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ISOLATION AND ANTIMICROBIAL ACTIVITY OF ISOLATED SOIL FUNGI FROM HOMALIN TOWNSHIP, SAGAING REGION

Hlaing Myint Thu[1], Zar Zar Yin[2], Ye Myint Aung[3]

Abstract

In the research work, soil samples were collected from six different places of Homalin Township during July 2017. Soil samples were collected from 0-15 cm depth after removing the surface soil for the isolation of fungi. After three days, soil fungi were isolated by the serial dilution method on six different media. A total of 41 fungal strains were isolated and the surface colour of isolated fungi HMT-1 to HMT-41 were white, black, green, pale green, greenish white, dark green, tan, yellow, yellowish green, plum, pale pink, gray and their reverse colour were cream, yellowish cream, greenish cream, buff, brown and purple. In the colony morphology, the isolated fungi were small, medium and large in size. The margin of isolated fungi were entire, undulate, filiform, lobate and the elevation of isolated fungi were raised, convex, flat, umbonate and the form of isolated fungi were circular, irregular, filamentous and rhizoid. Moreover, physicochemical properties of soil from different locations of Homalin Township were analyzed. Some physicochemical properties of soil samples were preliminaries determined and the collected soil samples were found to the rich in fungal strains due to acidic (4.67-5.80), moisture content (0.91 –7.23), organic matter (3.33- 7.98), temperature (25- 27 ˚C) and texture. Soil texture was examined by hydrometer method. Furthermore, all fungal strains were tested by seven test organisms for preliminary study of antimicrobial activity. Among them, twenty strains showed different levels of antimicrobial activity. Especially, HMT-33 showed the moderate antimicrobial activity against all test organisms. Therefore, strain of HMT-33 may be useful by the pharmaceutical industries for the production of antibiotics from local sources.

Keywords: soil fungi, filamentous, physicochemical properties, antimicrobial activity

Introduction

The soil serves as a reservoir for many microbial communities of plants and herbs which can be producing, CO_2 and nitrogen cycle. The microorganisms plays major role in soil ecosystem. Microbial composition and functioning changes the soil quality through decomposition of organic matter, recycling of nutrients and biological control (Stefanis *et al*., 2013). It is very hard to find substrata not isolated any microbes in nature. Therefore, any substrata collected in nature are useful materials for isolating microbes including fungi. Different materials have been reported as the substrata or the samples for fungi. The typical materials are soil, living and fallen leaves, leaf litters, dung, insects, fresh water, marine water and so on. Therefore, soil sample is the most effective and popular materials for especially isolating a number of fungi. The soil sample collected at various places such as paddy field, grain and vegetable fields, conifer or broadleaf wood forests, high mountain area, and etc. For the isolating different fungi, it is more effective to collected surface soil under leaf litter, because the fungal population is densely at the soil surface than the depth (Ando, 2004).

Antimicrobial agents play the most important role in the treatment of bacterial infections (Hacioglu and Dulger, 2011) and wide spread efforts have been carried out by many scientists in

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order to screen for novel antibiotic production microbes (Oskay *et al.*, 2004). The presence of fungi in soil has been well described showing that the environment contains an enormous biodiversity which can be screened for antibiotics production (Gunatilaka, 2006). Through their efforts, many antibiotics have been discovered successfully to combat pathogenic bacteria that cause diseases. Nevertheless, the emergence of new disease and reemergence of multiple-antibiotic resistance pathogens that render the effectiveness of existence clinically used antibiotics have spurred the needs for the discovery of new antibiotics (Roberts, 1998). Several mechanisms of actions of antibiotics have been discovered by scientists. These actions include the inhibition of cell wall, protein and nucleic acids synthesis (Lambert, 1977). However, soil ,which is a naturally occurring loose mixture of mineral and organic particles, still remains the most important target for most researchers in their efforts to discover novel antibiotics which have pharmaceutical values (Najad *et al.*, 2013). According to Dulmage and Rivas(1978), soil microorganisms have continually been screened for their useful biological active metabolites, such as antibiotics since long ago. Therefore, the aim of the research work was to produce antimicrobial compounds by isolated fungi from six different places soil from Homalin Township.

Materials and Methods

Method for Collection of Soil Sample

The soil samples were collected from six different places in various locations of Homalin Township, during July 2017 (Table 1). The soil samples were collected from different places (up to 15 cm depth) into sterilized polythene bags after removing the surface soil for the isolation of fungi and brought to the laboratory of Biological Resources and Biotechnology Development Center at Pathein University.

Physicochemical Analysis of Soil Samples

The collected soil samples were characterized for its physicochemical properties. Physicochemical parameters include organic carbon, organic nitrogen, pH, water content and temperature etc., microbial population density generally decreases with depth as a function of the availability of organic carbon and molecular oxygen, parameters which typically decrease with depth. Temperature and colour of the soil samples was recorded on the spot. The other physicochemical parameters of the soil samples were analyzed at Land Use, Department of Agriculture, Yangon in Myanmar

Isolation of Fungi from the Soil Samples

The soil micro fungi were isolated by serial dilution method (Dubey and Maheshwari, 2002) on different media such as Blaskeslee's Malt Extract Agar (BMEA Medium), Czapek-Dox Agar (CZA Medium), Malt Extract Agar (MEA Medium), Dichloram Rose Bengal–Chloramphenicol Agar (DRBC Medium), Glucose Ammonium Nitrate Agar (GAN Medium), Potato Glucose Agar (PGA Medium) .

Table 1 Collected Soil Samples from Six Different Places at Homalin Township

Soil sample No.	Place	Location
1	Nam Khan Village	N 24º 48' 32.572"
		E 95˚ 09' 23.267"
2	Tone Kham Village	N 24º 50' 05.750"
		E 95˚ 08' 33.089"
3	Naung Taw Village	N 24º 49' 39.211"
		E 95˚ 07' 07.066"
4	Naung Po Aung Village	N 24º 49' 05.805"
		E 95˚ 03' 52.663"
5	Hae Khamm Village	N 24º 51' 46.247"
		E 95˚ 02 12.275"
6	Naung-Pa-Kyit Quarter	N 24º 52' 26.395"
		E 94˚ 54' 43.868"

Serial Dilution Method

One gram of soil sample was introduced into a conical flask containing 99 mL of distilled water. The flask was than shaken for about 30 min in order to make the soil particles free from each other. This solution was then serial diluted from 10^{-3} to10^{-7} dilution in separate test tubes and 0.5 mL each of the above dilution was separately transferred into sterile petridishes under aseptic condition. The sterilized medium in conical flask was cooled down to about 45 ºC and separately poured into each of the petridish containing the respective soil dilutions. The inoculated plates were shaken clock-wise and anti-clock wise direction for about 5 min so as to make uniform distribution of the fungi inoculums. When the agar was solidified, the inoculated plates were inverted and incubated at 27-30 ºC for 3-7 days. Isolated pure fungi were preserved into slant culture (Atlas, 1993) containing BMEA medium for further experiments.

Agar Well Method

Isolated strains were tested by agar well method (Collins, 1965) for the preliminary antimicrobial activities. Cork borer was used to make the wells (8 mm in diameter) in the autoclaved basal antimicrobial test-medium. Wells impregnated with 3-6 days old culture fermented broth (20 µL/ well) were incubated at room temperature for 24-28 h. After 24- 28 h of incubation, the clear zones were measured. Therefore, the diameter of clear zones had been observed as potent activity as shown by respective strain. Clear zones surrounding the wells indicated the presence of antimicrobial activities which inhibit the growth of the test organisms selectively.

Test Organisms

The test organisms used for this experiment were *Agrobacterium tumefaciens* NITE 09678, *Bacillus pumilus*, *Bacillus subtilis* IFO 90571, *Candida albicans* NITE 09542, *Escherichia coli* AHU5436, *Pseudomonas fluorescens* IFO94307, *Staphylococcus aureus* AHU8465. The organisms were obtained from National Institute of Technology and Evaluation (NITE, Japan), and Pharmaceutical Research Department, Yangon, Myanmar.

Results and Discussion

Physicochemical analysis showed that pH of the soil is acidic and is rich with both macro and micro nutrients which is favorable for the growth of fungi. Fungal diversity of any soil depends on a large number of factors of the soil such as pH, organic content, and moisture (Rangaswami and Bagyaraj, 1998).

Eilers *et al.*, (2012) reported that soil microbial abundance and diversity are highest in the top 10 cm and decline with depth. Soil samples were collected from 0-15 cm depth after removing the surface soil for the isolation of fungi. Analysis of six different soils of Homalin Township revealed very optimum moisture and organic contents (Table 2). The colour of soil samples was red, brown and black with variation in pH (4.67–5.80). The temperature of soil environments of Homalin Township at the time of this investigation of July, 2017 (rainy season) revealed that the soil component of Homalin Township had temperature range between 25 °C and 27 °C with great variation in moisture content (0.91-7.23), percent organic carbon (1.93-4.63), organic nitrogen (1.66-3.99), percent humus (3.33-7.98). Total number of colonies obtained in Nam Khan is 14 with pH 4.67 (moisture 3.11%), Tone Kham is 11 strains with pH 5.03 (moisture, 0.91 %), Naung Taw is 11 with pH 5.49 (moisture 5.10 %), Naung Po Aung is one strains with pH 5.80 (moisture 1.91 %), Hae Khamm is 3 strains with pH 5.44 (moisture 7.23 %) and Naung Pa Kyit is 1 strain with pH 4.98 (moisture 4.32 %). This shows that low pH and optimum moisture content favour the growth of Fungi. Distribution of soil fungi depending upon the nature of the organic content, climatic conditions, surface vegetation and soil texture (Marchner *et al*., 2003). Direct relationship is observed between the soil texture and moisture content. Silt and clay soil holds the highest moisture content that is why there is increased population of fungi is observed. The frequency of mycoflora in different fields were found to be regulated by many factors like temperature, humidity, vegetation, organic and inorganic materials, soil type of texture. The fungi were moistly observed in month of June to September due to suitable temperature and humidity (Vinay *et al*., 2015). Brodie *et al.*, (2003) and Pfenning (2006) also reported that the forest areas sampled have higher plant diversity than the other systems of land use (agriculture, pasture and agroforestry). Besides, the results obtained from this study has shown to be similar with the research conducted by Tangjang and Arunachalam (2009) where they found out that there was greater amounts of bacterial and fungal populations in the top soil (0-10 cm) if compared to that of other depths. This might be due to the high organic contents found in the top soil where humus is abundantly presence, especially for the forest floor that is often covered by wilted leaves that tend to decompose.

Table 2 Physico- chemical Properties of the Soil Samples Collected from Six Different Places of Homalin Township

Sample No.	Place	Soil color	Texture	pH	T (˚C)	Moisture (%)	Organic carbon (%)	Organic nitrogen (%)	Humus (%)
1	Nam Khan	Red	CL	4.67	25	3.11	2.48	2.14	4.28
2	Tone Kham	Brown	SCL	5.03	26	0.91	1.93	1.66	3.33
3	Naung Taw	Black	SICL	5.49	26	5.10	2.70	2.33	4.66
4	Naung Po Aung	Red	SCL	5.80	27	1.91	1.94	1.67	3.34
5	Hae Khamm	Black	SIL	5.44	26	7.23	4.63	3.99	7.98
6	Naung Pa Kyit	Red	SICL	4.98	27	4.32	2.12	1.83	3.65

**CL-clay loam, SCL- sandy clay loam, SICL- silty clay loam, SIL- silty loam

In the present investigation, 41 strains fungal were isolated from six different soils samples of Homalin Township by using six different media including BMEA, CZA, MEA, DRBC, GAN and PGA medium and incubated for 3-7 days at room temperature (Table 3). HMT-1 colony diameter was 3.8-4.3 cm, HMT-2 (3.5-4.2 cm), HMT-6 (3.6-4.4 cm), HMT-7 (3.3-4cm), HMT-17 (4.2-4.2 cm), HMT-27 (4.33-6 cm), HMT-28 (3.8-4.5cm), HMT-29 (4.3-5.7 cm) after 5 days incubation on BMEA medium. So, these isolated fungi were large in size and HMT-3 colony diameter was (2.5-2.5 cm), HMT-4 (3.5-3.3 cm) HMT-8 (2.1-2.5 cm), HMT-9 (2.4-2.5 cm), HMT-11 (2-2 cm), HMT-15 (3.8-3.3 cm) HMT-16 (3.7-3.8 cm), HMT-18 (3.0-3.1 cm), HMT-19 (3.1-3.1 cm), HMT-24,30, 31, 33, 39, 40, 41 were (2.5-2.5 cm), HMT-25 (2.3-2.3 cm) after 5 days incubation on BMEA medium. Thus, these isolated fungi were medium in size and other strain colony diameters have less than 2 cm. Therefore, another strains colony diameter were small in size. The surface colour of HMT-1, 4, 6, 15, 20, 23, 26, 27, 28, 29, 34, 40 were white and the surface colour of other strains were greenish white, white cotton, dark green, tan, yellow, yellowish white, yellowish green, plum, black, pale pink, gray, greenish yellow, straw, pale green. The reverse colour of HMT-1, 2, 3, 4, 7, 10, 14, 17, 18, 10, 22, 23, 26, 29, 32, 38, 39 were cream and the other strains were brown, yellow, plum, green, yellowish cream, white cream, greenish cream, dark, buff cream, gray, yellow, purple respectively (Figures 1-5). All fugal strains were cultured, preserved, observed on BMEA medium, due to BMEA medium is best growth and isolated of fungi than other media. Ando (2004) reported that many fungi grow robustly on BMEA medium. After incubating fungi for 5 days, colony morphology of isolated fungi were photographed and measured. The form of colonies were circular, irregular, filamentous and rhizoid. The elevation of colonies were raised, convex, flat, umbonate, and the margin of colonies were entire, undulate, filiform, curled, and lobate.

Table 3 Isolation of Fungi by Using Six Different Media and Soil Samples

No. of Soil Samples	BMEA Medium	CZA Medium	MEA Medium	DRBC Medium	GAN Medium	PGA Medium	No.of isolated fungal strains
1	HMT-1, 2,3,4,5	HMT-6,7,8	HMT-9, 10,11,12,13	-	HMT-14	-	14
2	HMT-15, 16,17	-	HMT-18	HMT-19, 20	HMT-21, 22	HMT-23, 24,25	11
3	HMT-26, 27,28,29	-	-	HMT-30, 31	HMT-32, 33	HMT-34, 35,36	11
4	HMT-37	-	-	-	-	-	1
5	HMT-38, 39,40	-	-	-	-	-	3
6	HMT-41	-	-	-	-	-	1
	17	3	6	4	5	6	41

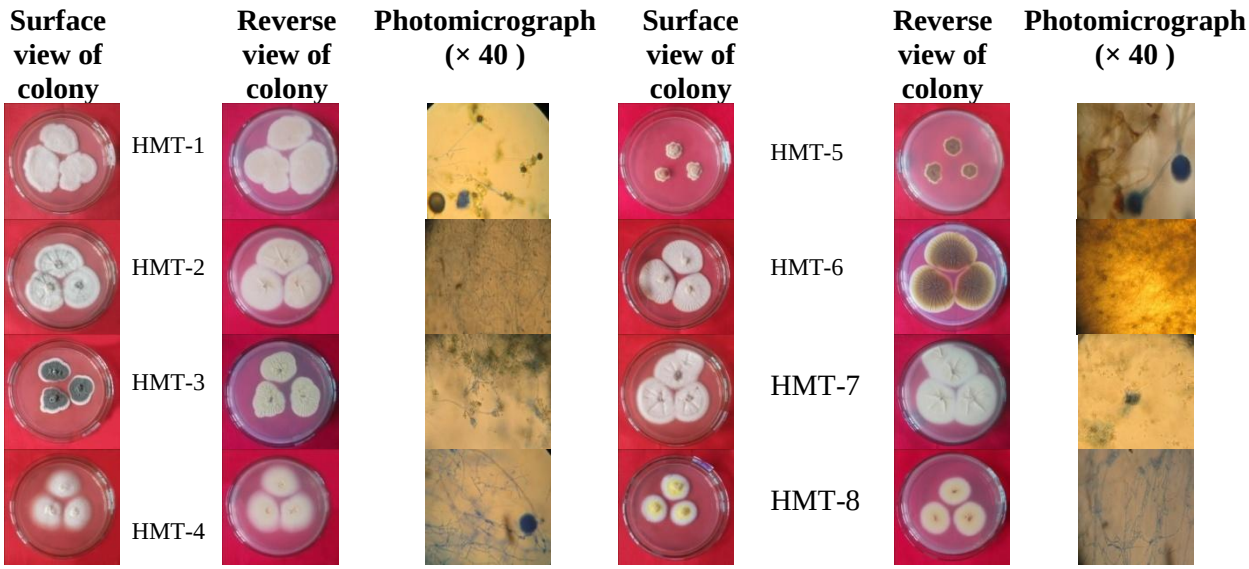

Figure 1 Morphology and photomicrograph characters of isolated fungi HMT-1, 2, 3, 4,5, 6,7,8

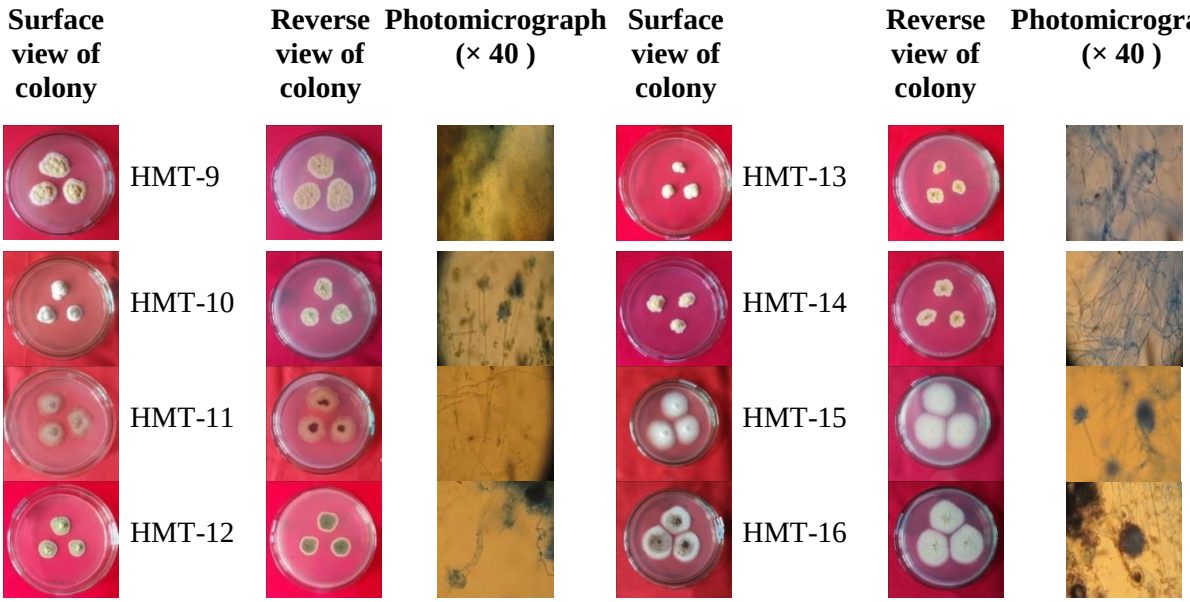

Figure 2 Morphology and photomicrograph characters of isolated fungi HMT-9, 10,11,12,13, 14, 15, 16

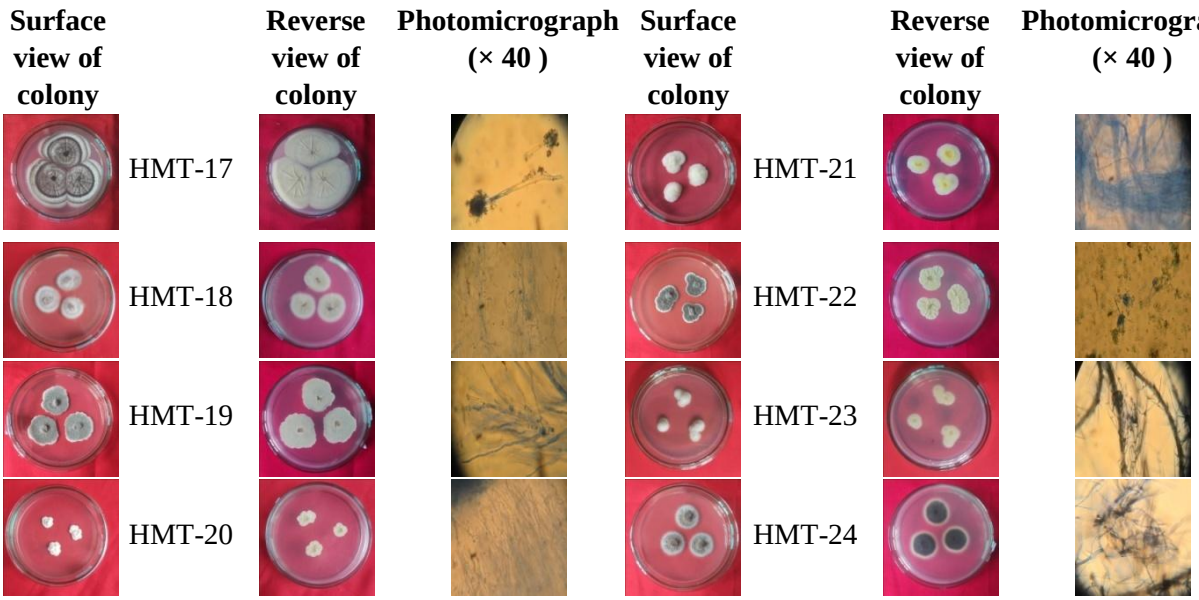

Figure 3 Morphology and photomicrograph characters of isolated fungi HMT-17, 18, 19, 20, 21, 22, 23, 24

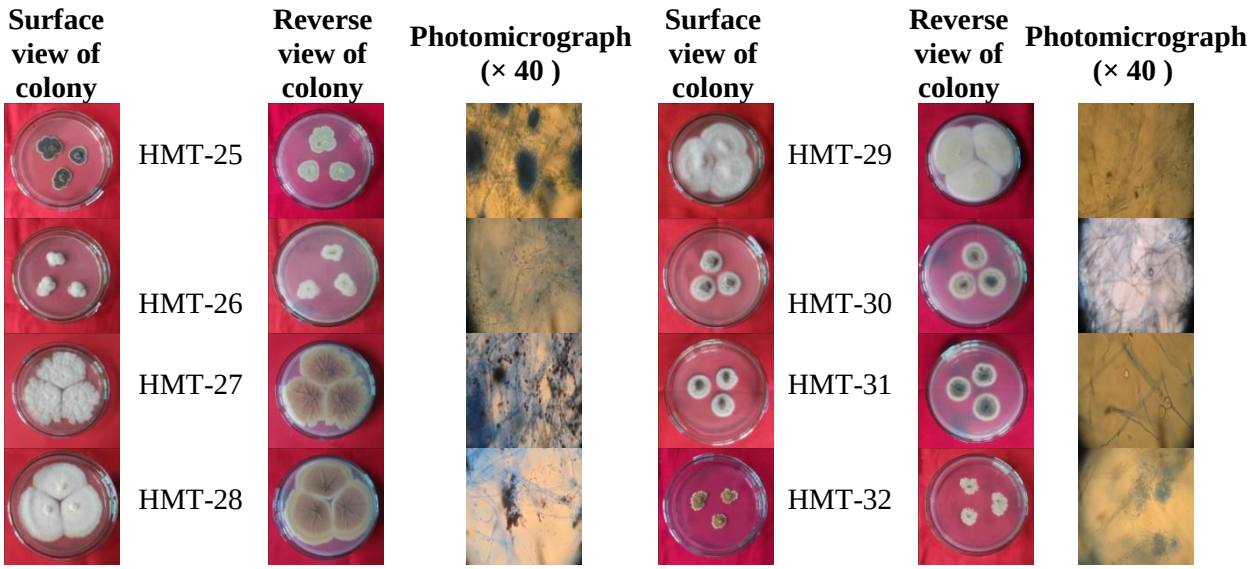

Figure 4 Morphology and photomicrograph characters of isolated fungi HMT-25, 26, 27, 28, 29, 30, 31, 32

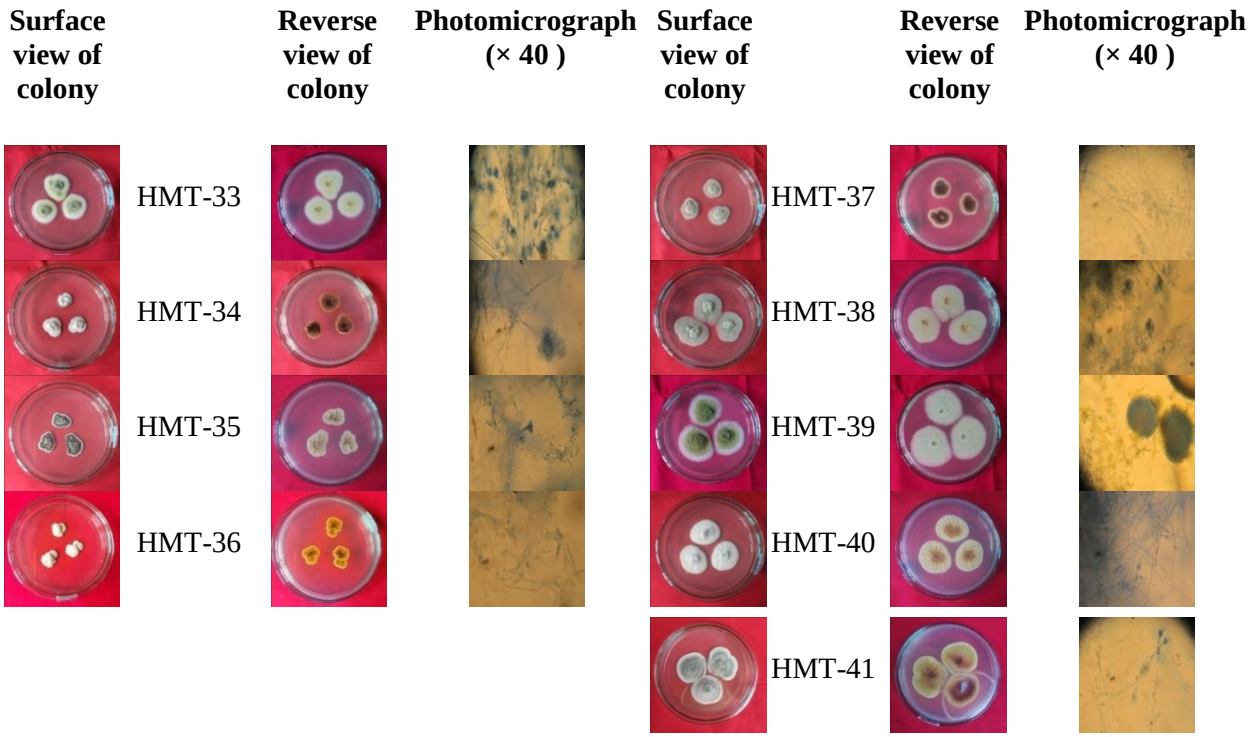

Figure 5 Morphology and photomicrograph characters of isolated fungi HMT-33, 34, 35, 36, 37, 38, 39, 40, 41

All fungal strains were tested by seven test organisms for preliminary study of antimicrobial activities. Among them, twenty strains showed different level of antimicrobial activities. HMT-38 exhibited the highest antibacterial activity (27.23 mm) against *Agrobacterium tumefaciens* at 5 days (Table 4 and Figure 6) and HMT-21 showed the high activity (23.26 mm) on *Bacillus pumilus* at 4 days (Table 5 and Figure 7). HMT-20 gave the moderate antimicrobial activity (24.54 mm, and 24.23 mm) against both *Bacillus subtilis* at 4 days(Table 6 and Figure 8) and *Candida albicans* at 5 days(Table 7 and Figure 9). And then, HMT-12 showed the antibacterial activity (24.67 mm) on *Esherichia coli* (Table 8 and Figure 10) and HMT-29

(21.69 mm) against *Pseudomonas fluorescens* (Table 9 and Figure 11) respectively. Especially, HMT-33 showed the moderated antimicrobial activity against all test organisms (Table 10 and Figure 12). Brooks (2001) reported that antibiotics are classified as broad-spectrum antibiotics when they have the ability to affect a wide range of gram- positive and gram-negative bacteria as well as fungi while antibiotic that only effective towards certain group of bacteria are known as narrow spectrum antibiotics. Therefore, HMT-33 strain was broad-spectrum antibiotics.

Table 4 Antibacterial Activity of the Isolated Fungal Strains against *A. tumefaciens*

No.	Isolated Fungi	Fermentation Period (days) and Inhibitory zone (mm)			
		3	4	5	6
1	HMT-1	+	24.84	27.21	20.99
2	HMT-5	-	22.56	26.45	19.75
3	HMT-7	+	22.38	22.37	21.32
4	HMT-29	15.55	13.57	23.04	-
5	HMT-33	+	13.93	20.75	17.16
6	HMT-37	+	+	26.80	+
7	HMT-38	+	+	27.23	-
8	HMT-39	-	-	25.17	+
9	HMT-40	+	+	26.80	+

(+) present (-) no activity Agar well =8mm

Figure 6 HMT-38 against *A. tumefaciens* at 5 days of fermentation period

Table 5 Antibacterial Activity of the Isolated Fungal Strains against *Bacillus pumilus*

No.	Isolated Fungi	Fermentation Period (days) and Inhibitory zone (mm)			
		3	4	5	6
1	HMT-1	-	+	-	-
2	HMT-5	-	+	-	-
3	HMT-12	12.99	-	-	18.36
4	HMT-18	+	+	+	-
5	HMT-20	+	21.61		-
6	HMT-21	+	23.26	--	-
7	HMT-29	-	15.87	--	-
8	HMT-30	+	15.60		-
9	HMT-33	19.61	19.74	19.67	15.24

(+) present (-) no activity Agar well =8mm

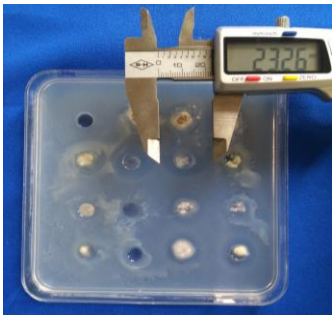

Figure 7 HMT-21 against *B.pumilus* at 4 days of fermentation period

Table 6 Antibacterial Activity of the Isolated Fungal Strains against *Bacillus subtilis*

No.	Isolated Fungi	Fermentation Period (days) and Inhibitory zone (mm)			
		3	4	5	6
1	HMT-12	+	-	19.20	20.16
2	HMT-16	-	22.65	+	-
3	HMT-18	-	+	17.00	-
4	HMT-20	-	24.54	21.61	-
9	HMT-21	-	23.29	-	-
6	HMT-27	-	+	13.43	13.43
7	HMT-29	-	19.57	18.63	18.63
8	HMT-30	-	16.92	-	-
9	HMT-33	18.61	19.06	17.35	17.54

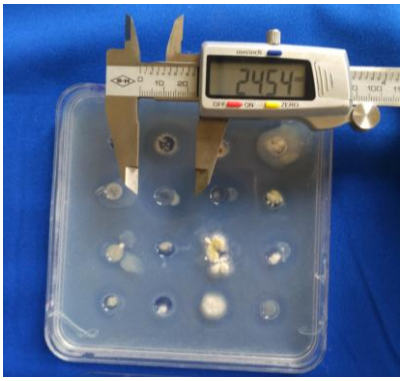

Figure 8 HMT-20 against *B.subtilis* at 4 days of fermentation period

(+)present (-) no activity

(+) present (-) no activity Agar well =8mm

Table 7 Antifungal Activity of the Isolated Fungal Strains against *Candida albicans*

No.	**Isolated Fungi**	**Fermentation Period (days) and Inhibitory zone (mm)**			
		3	**4**	**5**	**6**
1	HMT-12	12.99	-	18.24	17.98
2	HMT-16	-	-	18.65	-
3	HMT-17	-	+	14.85	-
4	HMT-18	-	+	17.98	-
9	HMT-20	-	+	24.23	-
6	HMT-27	-	+	13.42	-
7	HMT-28	-	+	+	-
8	HMT-29	-	+	15.45	-
9	HMT-33	18.59	22.97	18.47	17.54

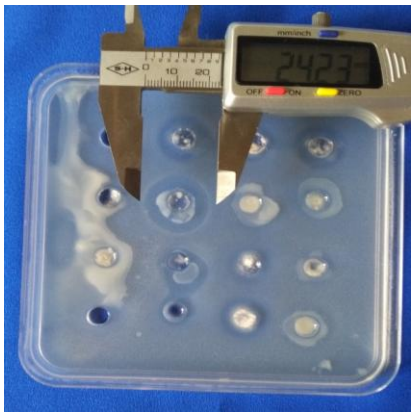

Figure 9 HMT-20 against *C. albicans* at 5 days of fermentation period

(+) present (-) no activity Agar well =8mm

Table 8 Antibacterial Activity of the Isolated Fungal Strains against *Escherichia coli*

No.	**Isolated Fungi**	**Fermentation Period (days) and Inhibitory zone (mm)**			
		3	**4**	**5**	**6**
1	HMT-1	-	24.27	-	-
2	HMT-5	-	24.27	-	-
3	HMT-12	13.58	24.67	-	17.63
4	HMT-16	-	17.52	22.12	-
9	HMT-17	-	17.14	21.40	-
6	HMT-18	-	15.82	19.17	-
7	HMT-20	-	19.30	+	-
8	HMT-21	-	16.86	-	-
9	HMT-33	19.48	20.75	18.60	16.44

Figure 10 HMT-12 against *E. coli* at 4 days of fermentation period

(+) present (-) no activity Agar well =8mm

Table 9 Antibacterial Activity of the Isolated Fungal Strains against *P. fluorescens*

No.	Isolated Fungi	Fermentation Period (days) and Inhibitory zone (mm)			
		3	4	5	6
1	HMT-16	-	-	+	-
2	HMT-17	-	-	+	-
3	HMT-18	-	-	+	-
4	HMT-20	-	-	-	-
9	HMT-21	-	-	-	-
6	HMT-27	-	-	-	-
7	HMT-28	-	-	-	-
8	HMT-29	-	-	20.52	21.69
9	HMT-33	18.06	18.79	20.13	18.70

(+) present (-) no activity Agar well =8mm

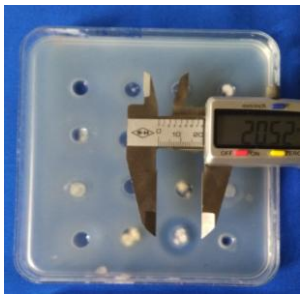

Figure 11 HMT-29 against *P. fluorescens* at 5 days of fermentation period

Table 10 Antibacterial Activity of the Isolated Fungal Strains against *S. aureus*

No.	Isolated Fungi	Fermentation Period (days) and Inhibitory zone (mm)			
		3	4	5	6
1	HMT-12	-	-	18.90	17.63
2	HMT-16	17.53	-	+	-
3	HMT-17	17.53	-	+	-
4	HMT-18	16.07	-	+	-
9	HMT-21	16.08	-	-	-
6	HMT-29	16.69	-	-	-
7	HMT-33	18.37	19.50	23.26	20.12
8	HMT-34	-	-	-	-
9	HMT-35	-	-	-	-

(+) present (-) no activity Agar well =8mm

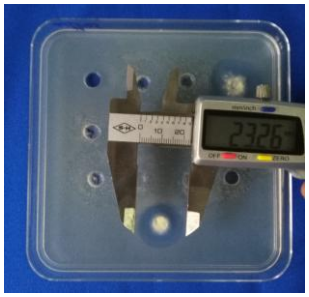

Figure 12 HMT-33 against *S. aureus* at 5 days of fermentation period

Conclusion

Results revealed that acidic pH and optimum moisture content and rich mineral content is most favourable condition for the growth of fungi. The soil which as large amount of organic matter due to acidic soil pH and slit and clay soil texture holds good amount of moisture content harboured a good quantitative isolated fungi in the soil for the purpose of recycling of dead organic matter thus making them available to the maintenance of global carbon cycle and ecological balance in the environment with dominant and sporulating genera. This research was done by the isolation of fungi occurring in the soil and their colony morphology and preliminary study of antimicrobial activity was performed by using seven test organisms. This investigation revealed that soil fungus HMT-33 isolated from soil of Homalin Township showed the moderated antimicrobial activity against (plant and animal pathogen) seven test organisms. It can be concluded that there is potential to discover useful antibiotic producing fungi in Homalin Township and produce some form of antimicrobial drug development programs.

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ISOLATION AND SCREENING OF SOIL FUNGI ISOLATED FROM SHIN MA TAUNG AND PONE TAUNG PONE NYAR AREAS

Moh Moh Htun[1] , Ye Myint Aung[2] and Zaw Lin Aung[3]

Abstract

Soil sample collection was carried out on the 28th June, 2018 from the Shin Ma Taung and on the 29th June, 2018 from Pone Taung Pone Nyar Areas. Totally, 23 fungi (13 from Shin Ma Taung and 10 from Pone Taung Pone Nyar) were isolated from 15 different soil samples by Chemical Treatment Dilution Method. The specimens were cultured on Low Carbon Agar (LCA) and Glucose Soluble starch Yeast extract (GSY) plates and incubated at room temperature. Fungus MMH-11 possessed the highest antibacterial activity among the fungi of Shin Ma Taung Area. Among the fungi of Pone Taung Pone Nyar Area, fungus MMH-23 possessed the highest antibacterial activity. Fungus MMH-23 was selected for further investigations because it exhibited more high antibacterial activity on Methicillin Resistance *Staphylococcus aureus* (MRSA). The growth phase of the selected fungus MMH-23 was between 36 h and 72 h. Age of inoculum (42 h seed culture), size of inoculum (20 %) and initial fermentation pH 6.0 were suitable for the production of bioactive compound against MRSA.

Keywords: antibacterial activity, Low Carbon Agar, Glucose Soluble starch Yeast extract, Methicillin Resistance *Staphylococcus aureus*, inoculum, bioactive compound

Introduction

Modern and global healthcare today face the problem of multi-resistant bacteria. Many bacterial species have developed resistance mechanisms against several classes of antibiotics in a relatively short period of time after the clinical introduction of antibiotics (Stefan *etal.*, 2011). Shin Ma Taung Area have severe weather and environmental conditions. In this area, low rainfall, high temperature and frequent drought (Kaung, 2013). Pone Taung-Pone Nyar region has been protected under the 1998 law. The fossils in Pone Taung Pone Nyar region are believed to be 4 million years older than the Egyptian counterpart which were thought to be the oldest before (Shwe, 2019). Therefore, soil samples from Shin Ma Taung and Pone Taung Pone Nyar Areas are very interesting to isolate filamentous fungi. In the present work, the production of antimicrobial substances from the isolated fungi was investigated with antimicrobial activity against both susceptible and resistant strains of bacteria.

Materials and Methods

Soil Sample Collection and Isolation of Soil Fungi

Sample collection was carried out in Shin Ma Taung Area, Yesagyo Township, Pakokku District, Magway Region and Pone Taung Pone Nyar Area, Saw Township, Gantgaw District, Magway Region. Isolation of soil fungi was carried out by Chemical Treatment Dilution Method

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(Hayakawa and Kobayashi, 2005). The specimens were cultured on Low Carbon Agar and Glucose Soluble starch Yeast extract plates and incubated at room temperature.

Preliminary Study for Antimicrobial Activities of Soil Fungi

The isolated fungi were grown for 7 days on Potato Dextrose Agar medium for sporulation. The isolated fungi were inoculated on seed medium and incubated for 3 days. Twenty milliliter of seed culture was transferred into the 30 mL fermentation medium and incubated for 9 days. Thirty microliter of fermented broth was put on paper disc and placed on assay plate containing test organisms. Seed medium, fermentation medium and assay medium were employed in the studies for antimicrobial activities. Test organisms utilized for antimicrobial activity were *Staphylococcus aureus,* Methycillin Resistance *Staphylococcus aureus, Bacillus subtilis,* Chloramphenicol Resistance *E. coli* (CREC), *Micrococcus luteus, Pseudomonas fluorescens, Salmonella typhi, Candida albicans* and *Agrobacterium tumefaciens*.

Study on Microbial Growth Kinetics of the Selected Soil Fungi MMH-23

The strain MMH-23 was inoculated into the Glucose Yeast extract NZ Amine type A medium at 25 ºC and incubated for 120 h. The culture sample (10 mL) was checked in 12 h interval for the growth. The sample (10 mL) was centrifuged at 2000 rpm for 30 mins and Packed Cell Volume % (PCV %) was calculated (Omura, 1985; Crueger and Crueger, 1989).

Study on the Effects of Ages and Sizes of Inoculum on the Fermentation

According to the results of microbial growth kinetics of MMH-23, (42, 48, 54, 60, 66 and 72 h) seed culture were utilized for the fermentation. Based on the results of the ages of inoculum of MMH-23, (5 %, 10 %, 15 %, 20 %, 25 % and 30 %) of 42 h seed cultures were utilized for the fermentation. Antibacterial activity was checked by paper disc diffusion assay method with 8 mm diameter paper discs.

Study on the Effects of Initial pH on the Fermentation Medium

Fermentation media for MMH-23 were adjusted to the pH 5.0, 5.5, 6.0, 6.5 and 7.0 respectively. Antibacterial activity was checked by paper disc diffusion assay.

Results and Discussion

Isolation of Fungi from Soil Samples and their Antimicrobial Activities

A total of 15 different soil samples were collected at Shin Ma Taung (9 soil samples) and Pone Taung Pone Nyar (6 soil samples) Areas. A total of 23 fungi (MMH-01 to MMH-23) were isolated from 15 different soil samples collected at Shin Ma Taung and Pone Taung Pone Nyar Areas. The study of antimicrobial activities of isolated fungi showed that MMH-03, MMH-05, MMH-11, MMH-20, and MMH-23 strains possessed the antibacterial activity on test organisms (Figures 1-6).

Among them, MMH-03 showed the antibacterial activity against Chloramphenicol Resistance *E. coli* (23.12 mm at 7 day fermentation). MMH-05 showed the antibacterial activity against *Staphylococcus aureus* (24.53 mm at 7 day fermentation). MMH-11 showed the antibacterial activity against *Salmonella typhi* (26.29 mm at 7 day fermentation). MMH-20 showed the antibacterial activity against *Agrobacterium tumefaciens* (23.35 mm at 7 day

fermentation). MMH-23 showed the antibacterial activities against MRSA (29.13 mm at 7 day fermentation), *Staphylococcus aureus* (17.30 mm at 7 day fermentation) and *Micrococcus luteus* (16.71 mm at 7 day fermentation). MMH-23 was selected for further investigations because it showed high antibacterial activity on Methicillin Resistance *Staphylococcus aureus* (Table 1).

Soil texture and soil pH of selected soil samples are shown in Table 2. Selected fungus MMH-23 was isolated from the soil sample PT-6 of Pone Taung Pone Nyar Area. Soil texture of PT-6 was sandy loan and soil pH was 6.60.

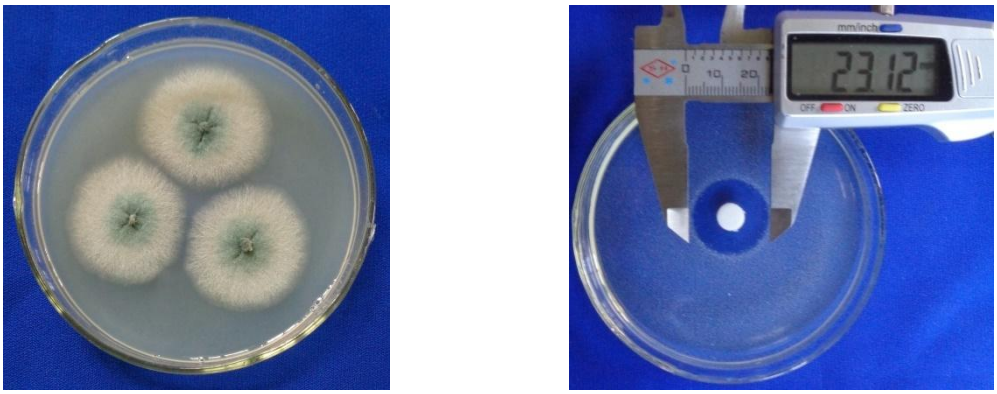


Figure 1 Morphology and activity of isolated fungus MMH-03
(7 day fermentation, Test Organism CREC)

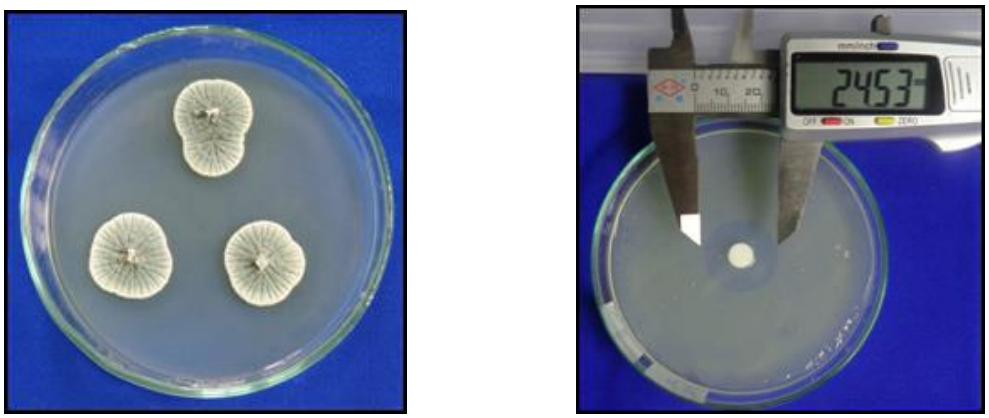


Figure 2 Morphology and activity of isolated fungus MMH-05
(7 day fermentation, Test Organism *Staphylococcus aureus*)

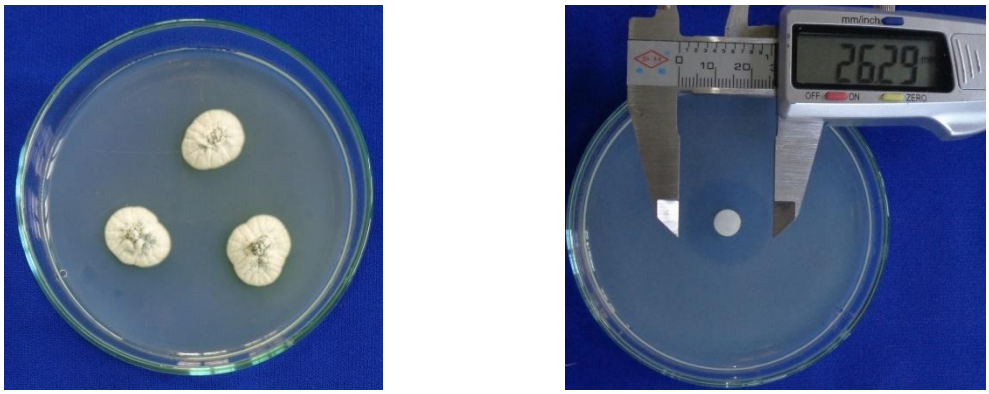


Figure 3 Morphology and activity of isolated fungus MMH-11
(7 day fermentation, Test Organism *Salmonella typhi*)

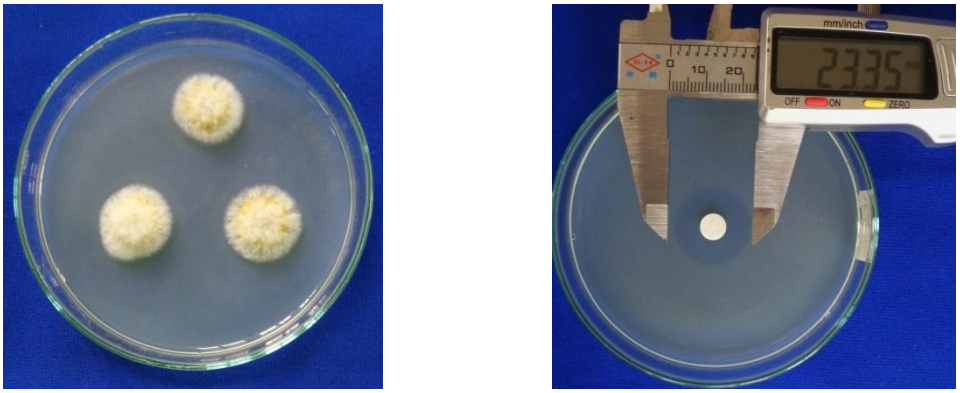


Figure 4 Morphology and activity of isolated fungus MMH-20
(7 day fermentation, Test Organism *Agrobacterium tumefaciens*)

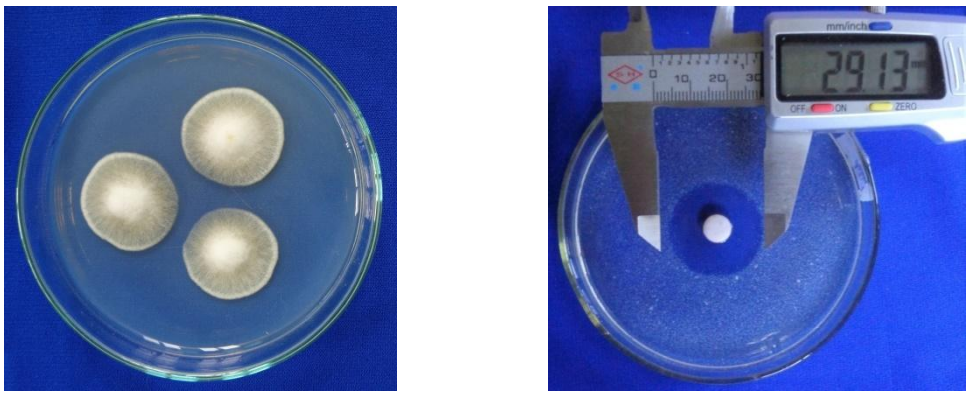
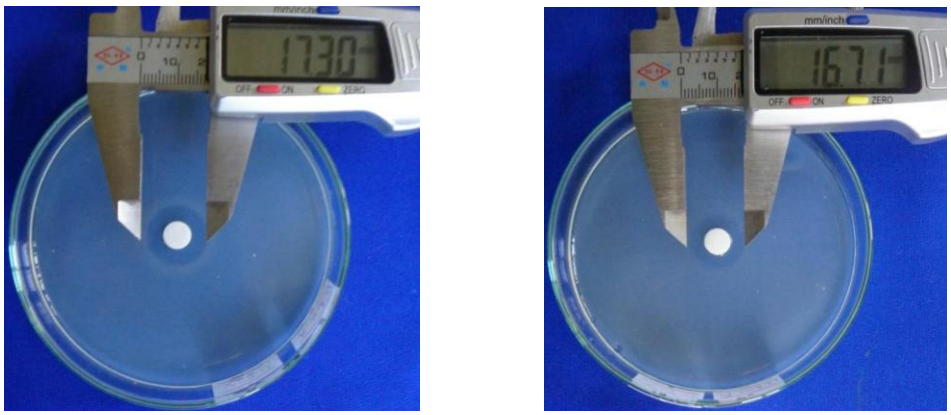


Figure 5 Morphology and activity of isolated fungus MMH-23
(7 day fermentation, Test Organism MRSA)



Staphylococcus aureus ***Micrococcus luteus***

Figure 6 Antibacterial activities of isolated fungus MMH-23
(8 day fermentation)

Table 1 Comparison of Antibacterial Activity Possessing Isolated Fungi

Soil No.	Isolated Fungus	Fermentation Period (Day)	Test Organism	Antibacterial Activity (mm)
SMT-2	MMH-03,GSY	7	Chloramphenicol Resistance *E. coli*	23.12
SMT-3	MMH-05,LCA	7	*Staphylococcus aureus*	24.53
SMT-7	MMH-11,GSY	7	*Salmonella typhi*	26.29
PT-4	MMH-20,GSY	7	*Agrobacterium tumefaciens*	23.35
		8	*Pseudomonas fluorescens*	15.56
		8	*Staphylococcus aureus*	17.30
PT-6	MMH-23,GSY	8	Methicillin Resistance *Staphylococcus aureus*	29.13
		8	*Micrococcus luteus*	16.71

Table 2 Soil Texture and Soil pH of Five Selected Soil Samples

Soil No.	Soil Texture	Soil pH
SMT-2	Sandy Loam	6.62 (Near neutral)
SMT-3	Sandy Clay Loam	9.24 (Extremely alkaline)
SMT-7	Sandy Loam	8.26 (Moderately alkaline)
PT-4	Sandy Loam	8.27 (Moderately alkaline)
PT-6	Sandy Loam	6.60 (Near neutral)

Microbial Growth Kinetics of MMH-23

In the growth kinetics study of fungus MMH-23, it was found that growth phase was between 36 h and 72 h .Thus, the ages of inoculum (42, 48, 54, 60, 66 and 72 h) were suitable for the optimization of fermentation (Figure 7).

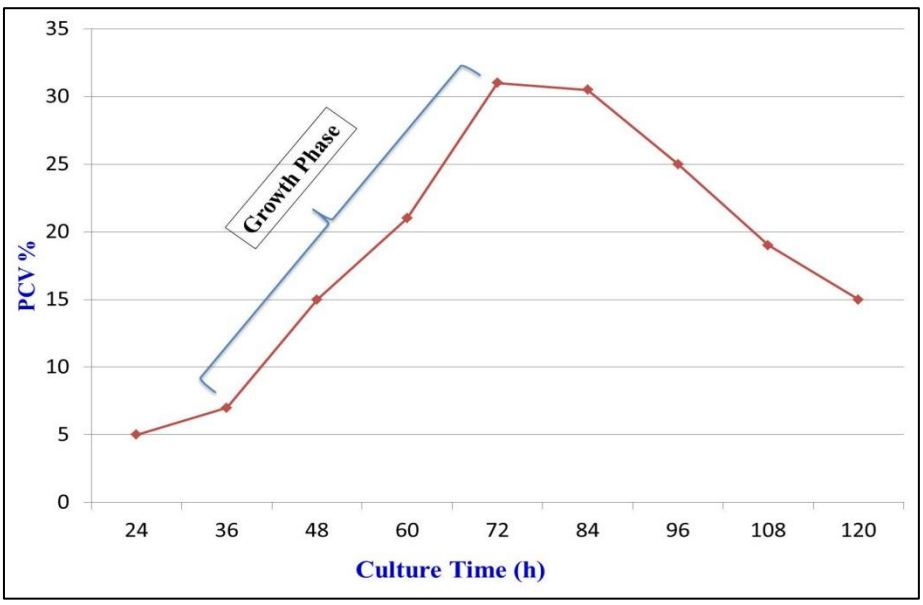


Figure 7 Microbial growth kinetics of the fungus MMH-23

Fermentation Optimization

In the study of ages of inoculum on the fermentation of MMH-23, 42 h seed culture showed the best activity on MRSA (Figure 8). Therefore, 42 h seed culture was selected for the fermentation. In the study of sizes of inoculum on the fermentation, 20 % size of culture showed the best activity on MRSA (Figure 9). Therefore, 20 % size of seed culture was selected for the fermentation. Fermentation medium adjusted to the pH 6.0 showed the highest antibacterial activity (Figure 10).

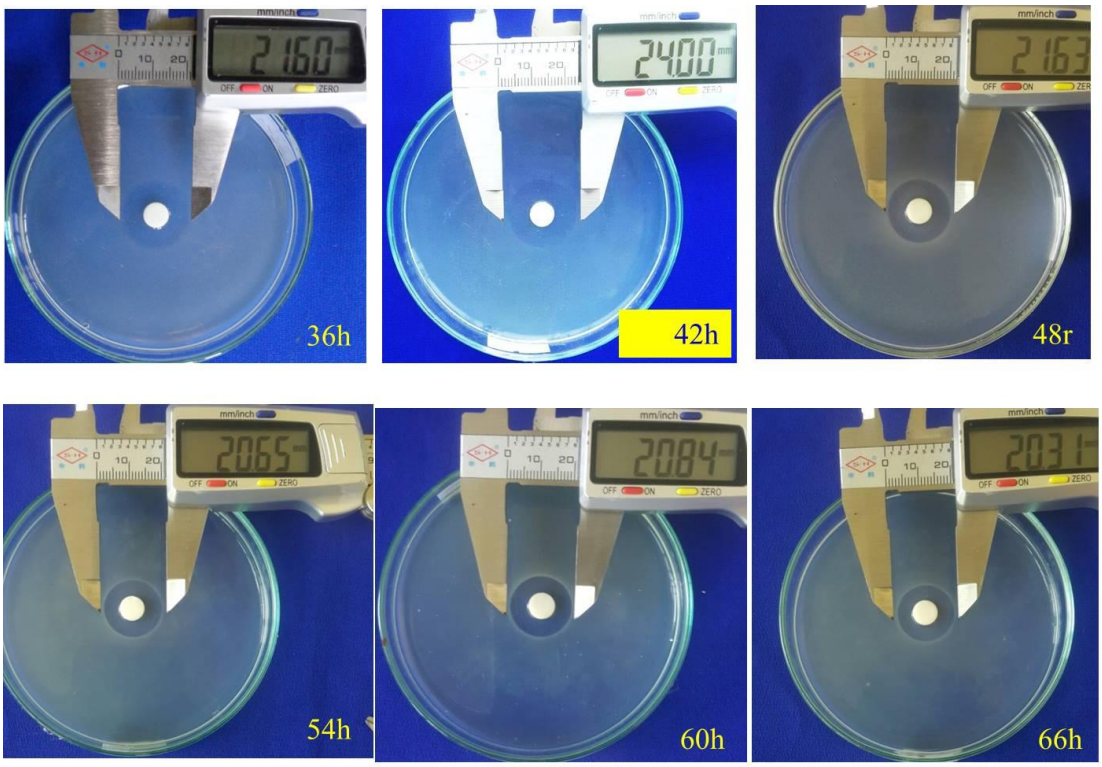


Figure 8 The effects of ages of culture on fermentation of MMH-23

Table 3 The Effects of Ages of Culture on the Fermentation

Ages of Culture (h)	Activity (mm, clear zone)
36	21.60
42	24.00
48	21.63
54	20.65
60	20.84
66	20.31

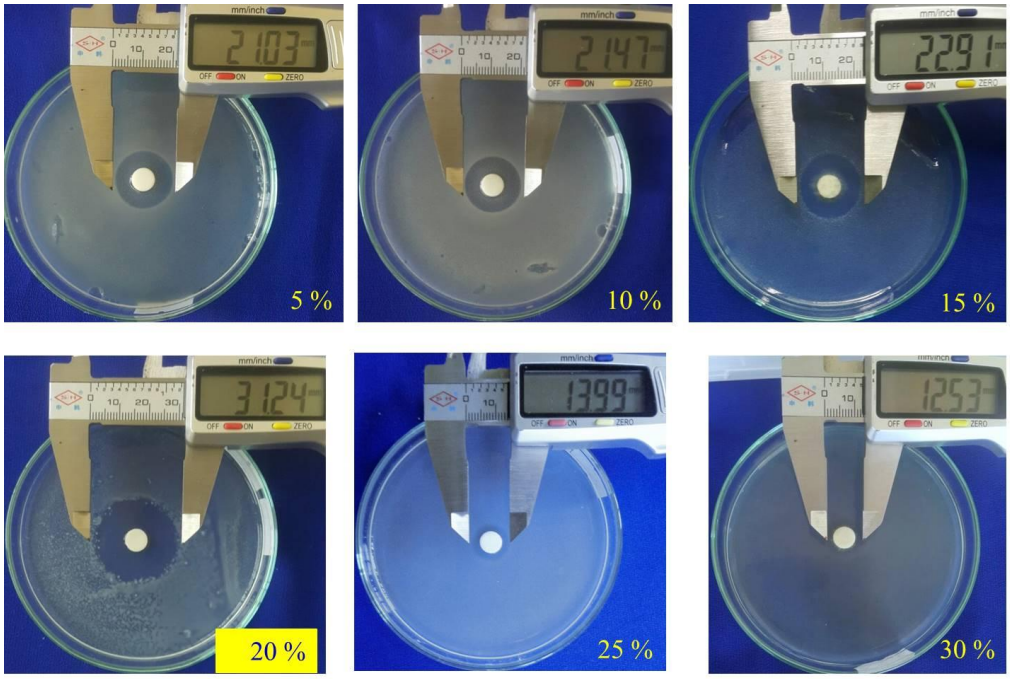


Figure 9 The effects of sizes of culture on fermentation of MMH-23

Table 4 The Effects of Sizes of Culture on the Fermentation

Sizes of Culture (%)	Activity (mm, clear zone)
5	21.03
10	21.47
15	22.91
20	31.24
25	13.99
30	12.53

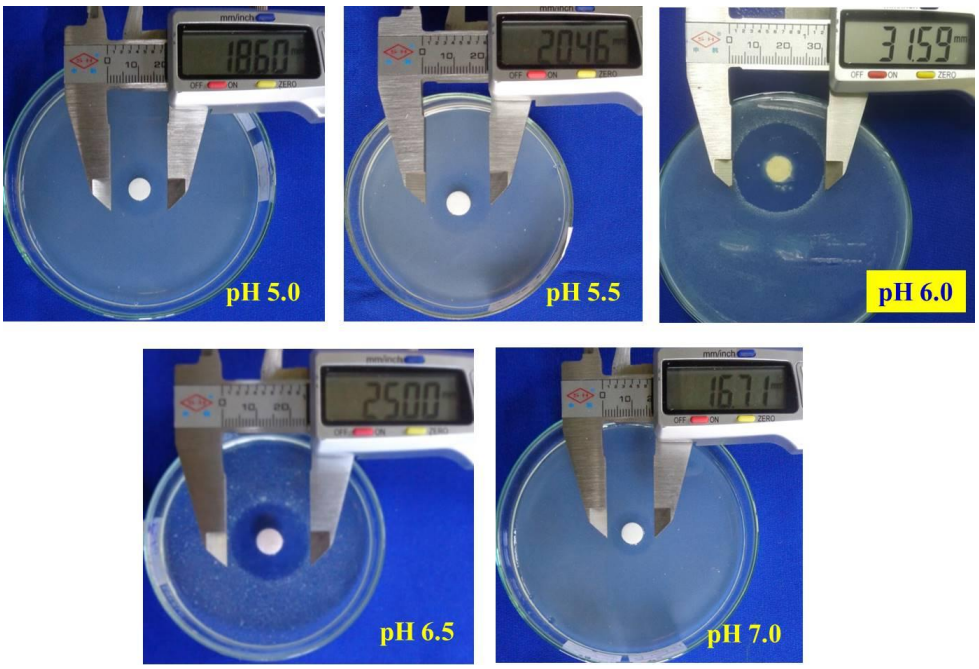


Figure 10 The effects of initial pH on fermentation of MMH-23

Table 5 The Effects of Initial pH on Fermentation of MMH-23

Initial pH	Activity (mm, clear zone)
5.0	18.60
5.5	20.46
6.0	**31.59**
6.5	25.00
7.0	16.71

Conclusion

In this research, sample collection was carried out from Shin Ma Taung and Pone Taung Pone Nyar Area, Magway Region. Soil samples are mostly sandy gravel and rocky in these areas. Soil fungi were isolated by Chemical Treatment Dilution Method.

MMH-03, MMH-05, MMH-11, MMH-20, and MMH-23 showed the antibacterial activity on test organisms. MMH-23 was selected for further investigations because it showed high antibacterial activity on MRSA. It was found that growth phase of MMH-23 from soil No. PT-6, Pone Taung Pone Nyar Area, was between 36 h and 72 h. In the study of the fermentation optimization of MMH-23, 42 h and 20 % size of seed culture showed the best activity on MRSA. It was found that fermentation medium adjusted to the pH 6.0 showed the highest antibacterial activity.

Drug-resistant pathogens are increasing. Therefore, the need for antimicrobial discovery and better treatments of these infections is becoming a rapidly growing concern. Further study is needed to isolate and to characterize the bioactive compounds responsible for antimicrobial activity.

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STUDY ON NUTRITIONAL QUALITY AND ANTIMICROBIAL, ANTIOXIDANT AND ANTI-PROLIFERATIVE ACTIVITIES OF *DIOSCOREA ALATA* L. (MYAUK U)

Mya Thandar Aung [1], Phyu Phyu Myint[2], Cherry Win[3], Yin Yin Myint[4]

Abstract

In this study, one of the *Dioscorea* species, *Dioscorea alata* L. (Myauk U) has been chosen to study nutritional values, chemical composition and some biological activities such as antimicrobial, antioxidant and anti-proliferative activities. The phytochemical constituents were screened by test tube method indicating the presence of alkaloids, α-amino acids, carbohydrates, glycosides, phenolic compounds, saponins, starch and terpenoids, however, cyanogenic glycosides, reducing sugars and tannins were absent. The nutritional values of the selected rhizomes were determined by AOAC methods. The antimicrobial activity of the different crude extracts such as pet-ether, ethyl acetate, ethanol and watery extracts was investigated against six microorganisms: *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus pumilus, Candida albicans and Escherichia coli* by agar well diffusion method. The total phenolic contents (TPC) of ethanol extract (469.1 ± 0.01 μg GAE/mg extract) and watery extract (441.1 ± 0.00 μg GAE/mg extract) were determined by Folin-Ciocalteau assay method. The antioxidant activity of ethanol and watery extracts was evaluated by 2, 2 - diphenyl -1- picryl – hydrazyl free radical scavenging assay. Both ethanol and watery extracts were found to possess antioxidant activity. However, both extracts possess weaker in antioxidant activity than standard ascorbic acid. The anti-proliferative activity or cytotoxicity of methanol extract of the rhizome of *D. alata* was evaluated by MTT assay using Hep G2 (human liver cancer cell).

Keywords: *Dioscorea alata* L., antimicrobial activity, antioxidant activity, anti-proliferative activity, MTT assay

Introduction

A number of wild crops remain unexplored in our country, Myanmar and among them some have excellent medicinal and nutritional properties. In this study, one of the *Dioscorea* species, also called yams, *Dioscorea alata* L. (Figure 1) has been chosen. Yam is a rich source of carbohydrate and also contributes to vitamins and minerals. It has many bioactive substances, such as phenolic compounds, alkaloids, steroidal saponins and proteins. *D. alata* has been used as a moderate laxative and vermifuge, hypertension (blood pressure), in skin diseases and for fever, gonorrhea, leprosy, tumors and inflamed hemorroids. *Dioscorea* species have been reported to have antifungal, antioxidant, anti-inflammatory, hypoglycemic and anticancer activity (Conlan *et al.*, 1998).

The tuber flesh is white or purplish and loose in texture. *Dioscorea* species are perennial through root system but are grown as annual crops, which serves as a staple food for millions of people in tropical and subtropical countries (Shewry, 2003). Its origin is Southeast Asia and is grown throughout the tropics and temperate regions of the world. It also grows in West India and West Africa (Acevedo-Rodriguez and Strong, 2012).

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Botanical Aspects of *Dioscorea alata* L.

Family	:	Dioscoreaceae
Genus	:	Dioscorea
Species	:	alata
Botanical Name	:	*Dioscorea alata* L.
English Name	:	Water yam, greater yam
Myanmar Name	:	Myauk U
Common Names	:	White yam, Water yam, Greater yam, Guyana arrowroot, winged yam, or simply yam
Part used	:	Rhizomes of *D. alata*

(a) Rhizome (b) Leaf (c) Plant

Figure 1 Rhizome, leaf and plant of Myauk U

Aim

The main aim of this research is to study on nutritional quality and to investigate antimicrobial, antioxidant and anti-proliferative activities of the crude extracts of *Dioscorea alata* L. (Myauk U).

Materials and Methods

Collection and Preparation of Plant Samples

The rhizome of *D. alata* was collected from Thapaung Township, Ayeyawady Region. The selected plant had been identified at Botany Department, University of Yangon. The collected sample was washed with water, sliced into small pieces and allowed to air - dried at the room temperature and then made into powder by using the grinding mill. The dried powdered sample was stored in the air-tight container for chemical and biological investigation.

Preliminary Phytochemical Investigation

Investigation of preliminary phytochemical screening was carried out on the dried powdered sample with a view to investigate the presence or absence of phytochemical constituents such as alkaloids, α-amino acids, carbohydrates, cyanogenic glycosides, organic acids, flavonoids, glycosides, phenolic compounds, reducing sugars, saponins, starch, steroids, tannins and terpenoids (M-Tin Wa, 1972), (Harbone, 1984).

Analysis of Nutritional Values

The analysis of the nutritional values on the dried powdered sample of *D. alata* was performed according to the procedures described by AOAC, 2000. The moisture content was determined by oven drying method, protein content by micro Kjeldahl distillation method, fat content by Soxhlet extraction method, fiber content by fiber cap method, ash content by ashing in furnace method and carbohydrates content by Phenol - sulphuric acid method (Agrawal *et al.*,2015).

Investigation of Antimicrobial Activity

Antimicrobial activities of various crude extracts such as PE, EtOAc, EtOH, and H_2O extracts were investigated by using agar well diffusion method (Cruickshank, 1960) at the Pharmaceutical Research Department (PRD), Ministry of Industry, Yangon. Fluconazole and tetracycline were taken as a positive control for antifungal and antibacterial tests. Bacterial cultures of *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus pumilus, Candida albicans* and *Escherichia coli* were used as the tested organisms for antimicrobial activities.

Determination of Total Phenolic Content

One of the antioxidative factors, total phenolic content (TPC) was measured by spectrometrically according to the Folin- Ciocalteu reagent method (Saxena, 2013).

Folin- Ciocalteau reagent (5 mL) was mixed with 0.5 mL of each sample in a test tube. After incubation for 5 min at room temperature, 4 mL of 10 % Na_2CO_3 was added, mixed and incubated for 30 min at room temperature. The absorbance was measured at 765 nm and then the TPC contents were calculated by the following equation,

$$C\ (GAE) = c \times \frac{V}{M}$$

where, c = concentration determined from standard curve (μg/mL),
V = volume used during the assay (mL),
M = mass of the extract during the assay (mg).

Results were expressed as microgram of gallic acid equivalents (GAE) per milligram of extract (GAE; μg/mg extract).

Investigation of Antioxidant Activity

One of the free radical scavenging assays, DPPH (2,2-diphenyl-1-1-picryl hydrazyl) free radical scavenging assay has been chosen to evaluate the free radical scavenging effectiveness of various flavonoids and polyphenols in crude extracts of *D. alata* (Marinova and Batchvarov, 2011).

Preparation of solutions

(i) Preparation of 0.002 % (w/v) DPPH solution

0.002 % DPPH solution was prepared by dissolving (2 mg) of DPPH powder in the 100 mL of ethanol.

(ii) Preparation of standard solutions (Ascorbic acid)

2 mg of ascorbic acid was dissolved with 100 mL of ethanol to obtain the stock solution. The standard solutions (20, 10, 5, 2.5, 1.25 and 0.625 μg/mL concentrations) were prepared from this stock solution by dilution with appropriate amount of EtOH.

(iii) Preparation of test sample solutions

2 mg of respective crude extract was prepared by dissolving 100 mL of ethanol and the stock solution was obtained. The sample solutions with the concentrations of (20, 10, 5, 2.5, 1.25, 0.625 μg/mL) were prepared from the stock solution by dilution with appropriate amount of EtOH.

(iv) Preparation of blank solution

Blank solution was prepared by mixing the sample solution (1.5 mL) with ethanol (1.5 mL).

Procedure

In this assay, the control solution was prepared by mixing 1.5 mL of 0.002 % DPPH solution and 1.5 mL of ethanol in the brown bottle. The sample solution was also prepared by mixing 1.5 mL of 0.002 % DPPH solution and 1.5 mL of test sample solution. These bottles were incubated at room temperature and were shaken on shaker for 30 min. After 30 min, the absorbance of different concentrations (0.625, 1.25, 2.5, 5, 10, 20 μg/mL) of the tested sample was measured at 517 nm using UV-1800 spectrophotometer. Absorbance measurements were used to calculate the percentage of radical scavenging activity (% RSA) by the following equation:

$$\% \text{ RSA} = [A_{DPPH} - (A_{Sample} - A_{blank})/ A_{DPPH}] \times 100$$

where,

% RSA = % radical scavenging activity of test sample
A_{DPPH} = absorbance of DPPH in EtOH solution
A_{Sample} = absorbance of sample + DPPH solution
A_{Blank} = absorbance of sample + EtOH solution

The antioxidant power (IC_{50}) is expressed as the test substances concentration (μg/mL) that results in a 50 % reduction of initial absorbance of DPPH solution and that allows to determine the concentration. IC_{50} (50 % inhibition concentration) values were calculated by linear regressive excel program.

Determination of Cytotoxicity or Anti-proliferative Activity by MTT Assay

The cytotoxicity or anti-proliferative activity of methanol extract of rhizomes of

D. alata was determined against human liver cancer cell line, Hep-G2 by 3- (4,5-dimethylthiazoyl - 2)- 2, 5- diphenyl tetrazolium bromide (MTT) assay at the College of Pharmacy and Natural Products Research Institute, Seoul National University, Seoul (Bahuguna *et al*., 2017).

Cytotoxic activity of crude extract was screened in 1 x 10^4 cells/well seeded in a 96 swell plate (30 mm) and incubated for 24 h for attachment and treated with MeOH extract at

concentration of 0 – 300 µg mL^{-1} and then kept for 24 h. Thereafter, 10 µL of MTT solution (5 mg mL^{-1}) were added to each well, and then incubated in darkness at 37 ºC for 4 h. The culture medium was discarded. The formazan crystals were solubilized by adding 100 µL DMSO per well and then mixed by gently shaking for 10 min. The amount of MTT – formazan is proportional to the number of living cells and the absorbance was measured in a microplate reader at 595 nm. The fractional absorbance was calculated by the following equation:

$$\% \text{ Cell survival} = \frac{\text{Mean absorbance in test well}}{\text{Mean absorbance in control wells}} \times 100$$

Results and Discussion

Phytochemical Constituents and Nutritional Values of *D.alata*

Phytochemical investigation was carried out to know the types of phytochemical constituents present in the rhizomes of *D. alata* by using test tube methods. According to these results, alkaloids, α-amino acid, carbohydrates, flavonoids, glycosides, phenolic compounds, saponins, starch, steroids, organic acids and terpenoids were found to be present but tannins, reducing sugars and cyanogenic glycosides were absent in the selected sample. Therefore, this study may provide the valuable scientific base for the use of herbs in the traditional medicine.

Nutritional values determined by standard AOAC methods were found to be protein (7.97 %), fat (1.98 %), fiber (22.61 %), ash (8.87 %), moisture (10.20 %) and carbohydrate (51.03 %). According to the observation, carbohydrate content has the highest composition than other nutrients. The corresponding energy value calculated is 253.82 kcal/100 g of the sample.

Antimicrobial Activity of *D. alata*

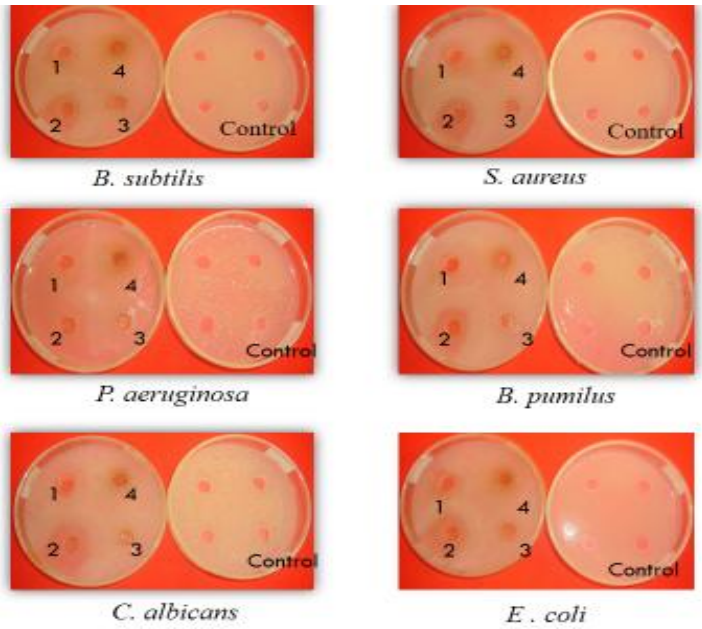
Screening of antimicrobial activity by agar well diffusion method, the measurable clear zone diameter, including the well diameter, shows the degree of antimicrobial activity. The larger the inhibition zone diameters, the higher the antimicrobial activity. The well diameter in the present study is 10 mm.

The results of antimicrobial activity are shown in Table 1 and Figures 2 and 3. It was found that all crude extracts possessed the antimicrobial activity against all tested microorganisms. Among them, the EtOAc extract exhibited the highest activity against all tested microorganisms, inhibition zone ranging from 20 - 24 mm. the PE extract and ethanol extract also showed antimicrobial activity on all tested microorganisms with inhibition zone diameter ranging from 18 - 23 mm and 12 – 13 mm, respectively. It was found that, the PE extract has the lowest antimicrobial activity against five strains except *Bacillus subtilis*. Therefore, the selected plant may be used in the treatment of diseases caused by microorganisms such as diarrhea, dysentery, food poisoning, boils, wound sepsis, respiratory tract infections and skin infections.

Table 1 Antimicrobial Activity of Four Crude Extracts of *D. alata* by Agar Well Diffusion Method

Microorganisms	Inhibition zone diameters of different crude extracts (mm)			
	PE	EtOAc	EtOH	H_2O
B. Subtilis	23 (+++)	20 (+++)	12 (+)	-
S. aureus	20 (+++)	22 (+++)	12 (+)	11 (+)
P. aeruginosa	12 (+)	24 (+++)	12 (+)	11 (+)
B. pumilus	22 (+++)	20 (+++)	12 (+)	11 (+)
C. albicans	23 (+++)	22 (+++)	12 (+)	11 (+)
E. coli	18 (++)	22 (+++)	13 (+)	11 (+)

Agar well - 10 mm
10 mm ~ 14 mm (+) 20 mm above (+++)
15 mm~19 mm (++) No activity (-)



1. PE extract 2. EtOAc extract 3. EtOH extract 4. H_2O extract

Figure 2 Effect of antimicrobial activity of four crude extracts of the rhizomes of *D. alata* on six microorganisms

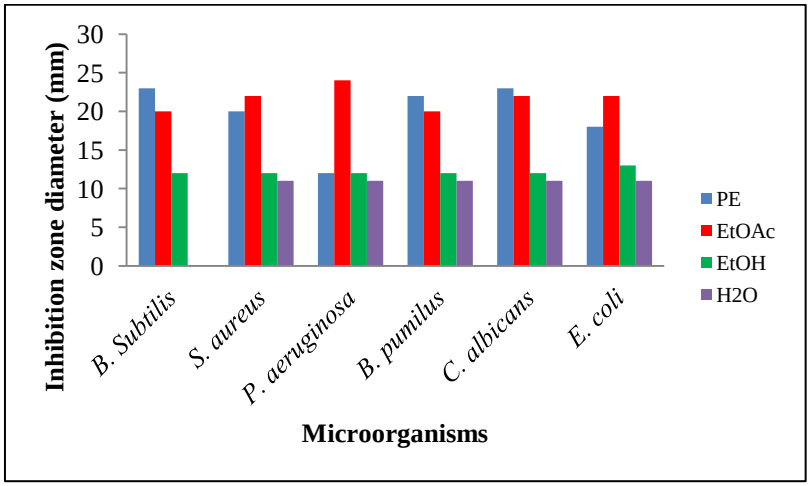


Figure 3 A bar graph of inhibition zone diameters of four crude extracts of the rhizomes of *D. alata* against six microorganisms

Total Phenolic Contents of *D. alata.*

In determination of the total phenolic contents by FCR method, phenols react with an oxidizing agent phosphomolybdate in F-C reagent under alkaline conditions and results in the formation of blue coloured complex which is measured at 765 nm. Gallic acid (3, 4, 5-trihydroxy-benzoic acid) was used to construct the standard calibration curve for total phenol estimation. Total phenolic content (TPC) was expressed as microgram of gallic acid equivalent (GAE) per milligram of crude extract (µg GAE/mg). The TPC content of ethanol extract (469.11 ± 0.01 µg GAE/mg) was found to be higher than that of watery extract (441.18 ± 0.00 µg GAE/mg). These correlations indicated that high total phenol contents in ethanol extract contributed to high in free radical scavenging activity of ethanol extract. The results are shown in Table 2 and Figure 4.

Table 2 Total Phenol Contents (TPC) of Ethanol and Watery Extracts of the rhizomes of *D. alata*

No.	Extracts	TPC (µg GAE/mg ± SD)
1	Ethanol extract	469.11 ± 0.01
2	Watery extract	441.18 ± 0.00

Antioxidant Activity of Crude Extracts of *D. alata*

In determining the antioxidant activity using DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay, a stable free radical from DPPH tends to capture hydrogen from the antioxidant. Due to its free radical, the ethanolic DPPH solution is violet and absorbance is measured at 517 nm. The colour changes upon neutralization of this free radical from violet to pale yellow by daylight. The decolouration of the initial colour is proportional to the test substances having antiradicalizing power.

It was found that as the concentration increased, the absorbance values decreased. The larger % RSA indicates the higher antioxidant activity. In contrast, the lower IC_{50} value indicates the more effective antioxidant activity. The antioxidant activity is expressed as % radical scavenging activity (% RSA) and 50 % inhibition concentration (IC_{50}) value was calculated by linear regressive excel program. The results of % RSA of two crude extracts of the rhizomes of *D. alata* and standard ascorbic acid are tabulated in Table 3 and Figure 5. Furthermore, their respective IC_{50} values are shown in Table 4.

According to the results, the ethanol extract (IC_{50} = 7.28 µg/mL) was found to be more potent than watery extract (7.32 µg/mL), in antioxidant power. Antioxidant potency of ethanol and water extracts were concluded to be weak by compared with the potency of standard ascorbic acid (IC_{50} = 1.73 µg/mL).

Table 3 Radical Scavenging Activity (%RSA) of Ethanol and Watery Crude Extracts of *D. alata* and Ascorbic acid

Sample	%RSA ± SD at Different Concentrations (µg/mL)					
	0.625	1.25	2.5	5	10	20
EtOH	13.46 ± 0.00	18.77 ± 0.01	26.93 ± 0.01	40.61 ± 0.03	61.12 ± 0.01	90.91 ± 0.00
H_2O	18.60 ± 0	22.44 ± 0.01	28.93 ± 0.00	41.92 ± 0	59.25 ± 0.00	65.64 ± 0.02
Ascorbic acid	26.80 ± 0.00	41.06 ± 0.00	61.27 ± 0.00	78.51 ± 0.00	88.93 ± 0.00	97.02 ± 0.00

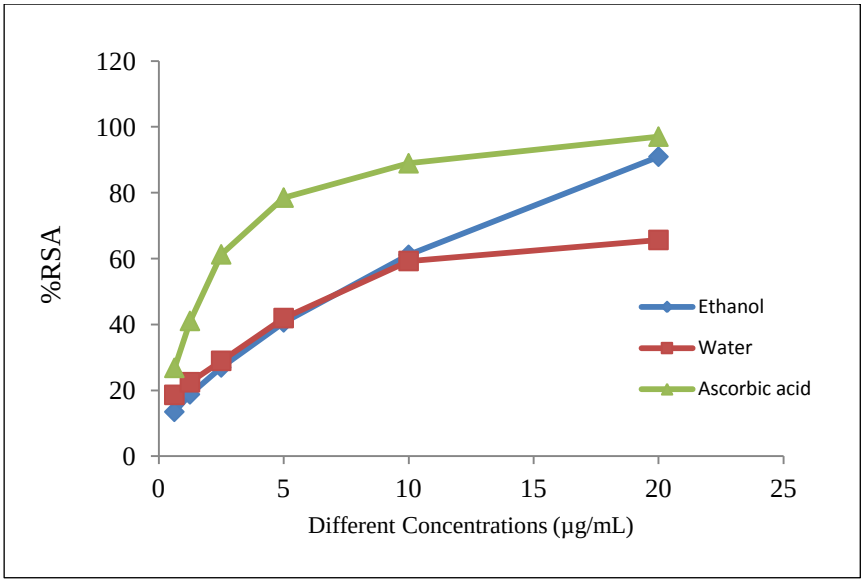


Figure 5 A plot of % RSA vs. concentrations of ethanol and watery extracts of the rhizomes of *D. alata* and standard ascorbic acid

Table 4 IC_{50} Values of Crude Extracts of the rhizomes of *D. alata* and Ascorbic acid

Samples	IC_{50} (µg/mL)
95 % ethanol extract	7.28
Watery extract	7.32
Ascorbic acid	1.73

Anti-proliferative Activity of *D. alata*

The anti-proliferative activity or cytotoxicity of methanol extract of the rhizome of *D. alata* was evaluated by MTT assay using Hep G2 (human liver cancer cell). The anti-proliferative activity or cytotoxicity of the crude extract is measured in terms of cells viability

and IC_{50} value (inhibitory concentration at which 50 % cells are died). Below IC_{50} range, the crude extract is toxic for mammalian cells. DMSO is used as a standard (Moo-Puc *et al*, 2009). The methanol extract of *D. alata* was found to possess the low anti-proliferative activity against Hep G2 (human liver cancer cell) having the IC_{50} value of 195.21 μg/mL, above this concentration the cell mortality rate is increased and below this, the cell survival rate is higher. The results are shown in Table 5.

Table 5 Anti-proliferative Activity of Crude Extract against Human Liver Cancer Cell Lines (Hep G2)

Sample	Cell viability ± SD at different concentrations (μg/mL)			IC_{50}(μg/mL)
	50	100	200	
MeOH - extract	0.8435 ± 0.0479	0.6808 ± 0.0549	0.4908 ± 0.0416	195.21

Conclusion

These observations provide the valuable suggestions for the study of phytochemical constituents concerned with the antimicrobial, antioxidant and anti-proliferative activities of the rhizome of *Dioscorea alata*. Due to the high content of carbohydrate, *D. alata* may be used as a functional food to supplement the fiber and carbohydrate. All extracts except petroleum ether extract, exhibited moderately antimicrobial activity on all tested microorganisms. According to the results, the high total phenol content in *D. alata* contributes to high in free radical scavenging activity. The methanol extract was found to possess low anti-proliferative activity against Hep G2 having the IC_{50} value of 195.21 μg/mL.

According to these observations, the traditional medicinal plant, *D. alata* may, therefore, be useful in the prevention of free radical reactions associated with the degenerative diseases like tumor or cancer and age related problems. In addition, the selected plant may be effectively used in the formulation for treatment of skin disease, wound infections, diarrhea, oxidative stress related diseases, anti-bacterial, anti-inflammatory, diabetic disease and other diseases infected by the microorganisms tested.

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SORPTION STUDY ON DYEING PROCESS OF NATURAL DYES EXTRACTED FROM *CASUARINA EQUISETIFOLIA* FORST. (KA-BWEE) BARK ON COTTON CLOTH

Zin Mar Oo[1], Mya Theingi[2], Nwe Nwe Aung[3]

Abstract

In this research work, the raw sample barks (Ka-bwee) were collected from Sittwe, Rakhine State. Physicochemical parameters of Ka-bwee raw bark powder such as moisture content, ash content, bulk density and pH were determined. Natural dyes were extracted from the bark of Ka-bwee by different solvents (water, ethanol and methanol).The prepared natural dyes were characterized by FT IR, EDXRF and UV-visible techniques. Relative abundances of elements in prepared natural dyes were analyzed by EDXRF which showed the chemical constituents of the elements. According to the FT IR, in Ka-bwee dyes extracted by different solvents, functional groups of O-H stretching, C=C, C-O (stretching) and benzene ring were observed. The phytochemical tests of dyes extracted with water were carried out. In phytochemical test, tannins, flavonoids, α-amino acids and others were observed in the dye solutions. Furthermore, antimicrobial activities of Ka-bwee dyes were investigated by agar disc diffusion method on six tested organisms. The wavelengths of maximum absorption (λ_{max}) of Ka-bwee dyes extracted with water, ethanol and methanol were observed at 510, 494 and 499 nm respectively. Sorption properties of 1000 ppm natural dye solutions dyeing on cotton were studied at different temperatures (40-90°C) by using UV-visible spectrophotometer. From the experiment, the optimum temperature of Ka-bwee dyes extracted with water, ethanol and methanol were at 80℃. The optimal conditions of dyeing on cotton cloth were observed at concentration of dye solution (1000 ppm), mordant alum dosage 0.1 g, contact time 60 min and pH 6. The dye solutions were prepared by natural dye powder with water. After dyeing on cotton cloth, the poor wash and light fastness of dyed fabrics improved with mordanting. The colour intensities of these dyed cotton were determined by Reflection Transmission Colour Densitometer. Depending on the type of mordant, such as alum, onion peel, teawaste and jengkol peel (natural mordant), colour fastness of the dyeing cotton cloth were studied.

Keywords: Natural dye, Ka-bwee bark, mordants, dyeing process

Introduction

Natural dyes produce very uncommon, soothing and soft shades as compared to synthetic dyes. Natural dyes obtained from plants are renewable and sustainable bio-resource products with minimum environmental impact and known since antiquity for their use, not only as ingredients in food and cosmetics but also in textile colouration. Natural dyes are eco-friendly, bio-degradable, less toxic and less algeric as compared to synthetic dyes (Nisar *et.al*., 2007). Natural dyes are those obtained from plants, animals and minerals. Most of the natural dyes are found to be non-carcinogenic in nature. Dyes are chemical substances used to impact colours to substrates such as cosmetics, foods, drugs, hairs, furs, textiles and polymers (Osabohien and Otutu, 2014).

Nowadays, extraction and application of natural dyes are becoming more popular owing to the growing awareness of environmental problems coupled with the toxicity associated with synthetic dyes (Rajeswari and Arivalagan, 2017).On the other hand, synthetic dyes, which are

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widely available at an economical price and produce a wide variety of colours, sometimes causes skin allergy and other harmfulness to human body, produces toxicity/chemical hazards during its synthesis, releases undesirable/hazardous/toxic chemicals etc. However, dyes commonly used in textile are seldom screened for use as antimicrobial agents for textile finishing (Singh *et al*., 2004).Dyeing of textiles using synthetic dyestuff is characterized by a high impact on the environment, the dyers as well as the end-users (Narayan, 2017). Natural dyes obtained from barks are renewable, traditionally extracted from animal and plant sources for use in coloring food substrate, leather, wood and natural fibres such as silk, cotton and flax from time immemorial.

Furthermore, natural dyes are known to exhibit better biodegradabilit, less toxicity, eco-friendly alternative to synthetic dyes and some dyes also medicinal properties. The major chemical constituents of Ka-bwee barks are elaggic acid, gallic acid, kaemferol and qurcetin and the barks contains astringent and antioxidant properties and also has significant anticancer and anthelminitic potential (Narayanswamy *et al*., 2013). In the present work, the dye from the barks of *casuarina equisetifolia* forst.were prepared and characterized by modern techniques. Physicochemical properties of Ka-bwee bark powder such as moisture content, ash content, bulk density and pH were studied. Functional groups, elements and maximum absorption wavelengths were studied on the prepared natural dyes by FT IR, EDXRF and UV-analysis, respectively. Furthermore, antimicrobial activities of extracted Ka-bwee dye were tested. The colour intensities of this dye on cotton cloth were determined by Reflection Transmission Colour Densitometer. Depending on the type of mordant, such as alum, onion peel, teawaste and jengkol peel (natural mordant), colour fastness of the dyeing on cotton cloth were studied.

Materials and Methods

Sample Collection

Ka-bwee bark is the sample used in this study for extraction of dye, which was collected from the Sittwe, Rakhine state. The part used for the dye extraction was only bark. And then, they were washed with distilled water and dried at room temperature, and made into fine powder.

Cotton cloth was purchased from Shwetaung Myoema Market, Bago Region.

Pretreatment of Cotton Cloth

The cotton cloth was soaked in mixture of 1 g / L of sodium carbonate and 2.5 g / L of detergent at 80 °C for 30 min and then washed with running tap water to remove the natural impurities and improve the texture of cotton cloth for dyeing.

Extraction of Tannin

The raw onion, teawaste and jengkol peel (10 g) each was extracted with distilled water (1 L) and then setup was kept for 60 min boiling. The extracted tannin was filtered and was used for mordanting.

Mordanting

Tannin extracted from onion, teawaste and jengkol peel has been used as bio-mordants to avoid toxicity caused by harmful chemical mordants. Extraction method has been standardized for maximum yield of tannin.

Mordanting by Using the Extracted Tannin

Bio-mordants (tannin extracted from onion, teawaste and jengkol peel) were applied on the cotton cloth with different concentrations (10 %, 20 %, 30 %) for 1 hr. The optimum concentration for each bio-mordant was selected and then dyeing method; pre-mordanting, simultaneous mordanting and post mordanting were carried out. The pre-mordanting of cotton cloth was done before dyeing. Dyeing and mordanting were carried out simultaneously. Post mordanting was also done after dyeing of cotton cloth.

Extraction of Dyes from Ka-bwee Barks with Different Solvents

The dried bark powder Ka-bwee (10 g) was extracted with 100 mL of each solvent (water, ethanol and methanol) in sonicator for 30 min and filtered. It was repeated for other two times. The filtrates were evaporated by distillation at various temperatures (100,78,65 ℃). And then, they were dried in oven and they were crushed in motar and pestle and sieved with 90 m aperture size. Finally, dye powders of water extract (4.2 g), ethanol extract (3.6 g) and methanol extract (3.2 g) were obtained.

Characterization of the Extracted Dyes

EDXRF spectroscopy

Elemental compositions in the extracted dye from Ka-bwee by using water was determined by EDXRF spectrometer (Shimadzu Co. Ltd., Japan).

FT IR spectroscopy

FT IR measurements were carried out to determine the functional group of natural dye extracted from Ka-bwee. All measurement were carried out in the range of 400-4000 cm^{-1} at a resolution of 4 cm^{-1}. The dye samples were measured by using Perkin Elmer GX system, FT IR spectrophotometer.

UV-visible spectroscopy

The dye extracts were analyzed in UV- visible spectrophotometer at the range of 400- 800 nm with a resolution of 1 nm, to determine the wavelength of maximum absorption (λ_{max}) of the dye pigments. The maximum absorption wavelengths (λ_{max}) of extracted (water, ethanol and methanol) were 510 nm, 494 nm and 499 nm, respectively.

Determination of the Antimicrobial Activities of the Extracted Dyes

The extracted dye solutions were tested with *Aspergillus flavus, Bacillus subtilis, Candida albicans, Pseudomonas fluorescen, Xanthomonas oryzae* and *Echerichia coli* species to investigate the nature of antimicrobial activities by agar disc difffusion Method.

Dyeing the Extracted Natural Dye with Cotton Cloth

The pretreated cotton cloth was dye using dyes extracted (water, ethanol and methanol) from Ka-bwee. The concentration of (1000 ppm) dye solution dyeing on cotton cloth were studied in terms of temperature, contact time, pH and alum dosage. The temperature was varied in 40-90 °C, contact time varied in the range of 10-80 min and pH was changed in the value of 3-9 and alum dosage used in 0.025-0.15 g by using UV-visible spectrophotometer. And then, the most suitable conditions for dyeing on cotton cloth was selected. The amount of adsorption at equilibrium q_t (mg/g) and % removal of Ka-bwee dyes were calculated by this equation:

$$q_t(\text{mg/g}) = \frac{\text{Co}-\text{Ce (mg/L)}}{\text{unit mass of } adsorbent\ (g)} \times \text{volume of solution (L)}$$

Where, q_t = adsorption capacity (mg/g), C_o= initial concentration (mg/L)

C_e = equilibrium concentration (mg/L), unit mass of adsorbent = 1 g

Effect of temperature

Bath adsorption experiments were conducted by 1 g of cotton cloth to 100 mL of dye solutions with water in a 250 mL beaker with a temperature control of 80 °C ± 5 °C. A 100 mL dye solution in a 250 mL beaker was put in water bath. Natural dye solution dyeing on cotton was allowed to reach the equilibrium for 60 min in a water bath at 40, 50, 60, 70, 80 and 90 °C. At 10 min intervals, the dye solution was taken from the beaker. The remaining dye concentration was determined by UV-visible spectrophotometer at λ_{max} 510 nm for watery extracted dye. Similarly, sorption properties of ethanol and methanol were also determined at 40, 50, 60, 70, 80 and 90 °C at 494 and 499 nm, respectively.

Effect of contact time

The effect of contact time on dyeing the cotton cloth with the extracted dye on cotton cloth was conducted by the same procedure for an equilibrium over a range of contact times (10 – 80 min) in 250 mL beaker with a temperature control of 80 °C. At 10 min intervals, the dye solution was taken from the beaker. The remaining concentration was determined spectrophotometerically at its corresponding λ_{max} (510, 494 and 499 nm).

Effect of pH

The effect of pH on dyeing the cotton cloth with the extracted dye was conducted by the same procedure for an equilibrium over a range of pH values (3,4,5,6,7,8 and 9) which were adjusted with HCl and NaOH. The extracted dyes of water, ethanol and methanol dyes were applied to dye the cotton cloth and allowed to equilibrate for 60 min in a water bath at 80 °C. At 10 min intervals, the dye solution was taken from the beaker. The remaining concentration was determined spectrophotometerically at its corresponding λ_{max} (510, 494 and 499 nm).

Effect of alum dosage

The effect of alum dosage on dyeing the cotton cloth with the extracted dye was conducted by the same procedure for an equilibrium over a range of alum dosage (0.025 – 0.15 g). At 10 min intervals, the dye solution was taken from the beaker. The remaining concentration was determined spectrophotometerically at its corresponding λ_{max} (510, 494 and 499 nm).

Results and Discussion

The physicochemical characteristics of Ka-bwee bark powder were determined. The results in Table 1 indicate 8.25 % w/w moisture content, 3.53 % w/w ash content, 0.80 g mL^{-1} bulk density, pH 6.70 in raw sample. The phytochemical results of the extracted dye from Ka-bwee are shown in Table 2. Alkaloids, flavonoids, glycosides, phenolic compounds, saponin, steroids and tannins were observed in Ka-bwee dye whereas carbohydrates and starch were not found in this extracted dye sample.

Table 1 Physicochemical Properties of Ka-bwee Dyes

No.	Characteristic	Content
1	Moisture Content (%)	8.25
2	Ash Content (%)	3.53
3	Bulk Density (g cm^{-3})	0.80
4	pH	6.70

Table 2 Phytochemical Results of Ka-bwee Dye

No.	Test	Extract	Test Reagents	Observations	Remark
1	Alkaloids	1 % HCl	Mayer's reagent	White ppt	+
2	α-Amino acid	H_2O	α-Ninhydrin reagent	Purple spot	+
3	Carbohydrates	H_2O	10 % Naphthol	Red ring	-
4	Flavonoids	EtOH	Mg turnings and conc;HCl	Pink	+
5	Glycosides	H_2O	10 % Leadacetate	White ppt	+
6	Phenolic compounds	EtOH	1 % $FeCl_3$	Deep blue	+
7	Saponins	H_2O	Distilled water	Frothing	+
8	Starch	H_2O	1 % Iodine solution	Deep blue	-
9	Steroids	PE	Acetic anhydride and conc:H_2SO_4	Blue or Blue green	+
10	Tannins	H_2O	1 % Gelatin	White ppt	+

EDXRF analysis

In this research, the natural dye was extracted from Ka-bwee by using water. The water extracted dye was characterized by EDXRF. From EDXRF analysis, the major constituent is calcium 46.38 % and potassium 23.26 % and silica 11.76 % were also observed.

Table 3 Relative Abundance of the Elements in the Watery Extracted Dye from Ka-bwee

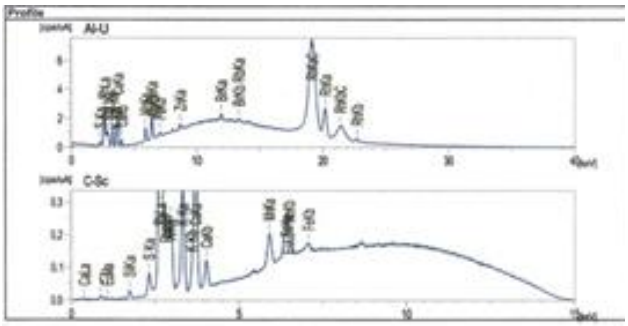
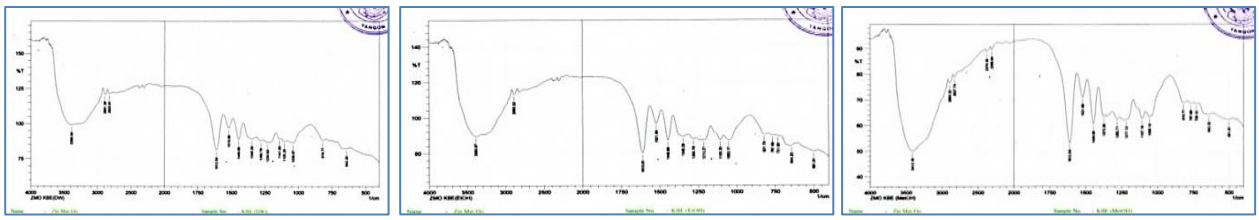


Figure 1 EDXRF spectra of the watery extracted dye from Ka-bwee

Element	Relative Abundance (%)
Ca	46.382
K	23.263
Si	11.763
S	6.846
Fe	5.425
Mn	4.603
Br	0.737
Zn	0.719

FT IR Analysis

Figure 2 shows the FT IR spectra of dyes extracted from Ka-bwee with water, ethanol and methanol. The characteristic absorption bands at 3300, 2891, 1446, 1105 and 642 cm^{-1} were observed. These peaks correspond to groups present in the sample and indicated to O-H stretching, C-H stretching, C-H bending, C-O stretching and benzene ring (quinone) which is the good correlation with that of literature. These bands confirmed the presence of alkaloids, tannins and flavonoids in natural dye (Table 4).



(a) (b) (c)

Figure 2 FT IR spectra of natural extracted dyes from Ka-bwee with (a) water, (b) ethanol and (c) methanol

Table 4 FT IR Assignments of the Extracted Dyes from Ka-bwee with Water, Ethanol and Methanol

Observed wavenumber (cm^{-1})			*Literature value(cm^{-1})	Possible Assignment
KBE-W	KBE-Et	KBE-Met		
3389.04	3390.97	3412.19	3950 - 3200	O-H stretching
2891.39	2893.32	2893.32	2980 - 2850	C-H stretching(CH_3) C-H stretching (CH_2)
1612.54	1610.61	1612.54	1700-1600	C=O stretching
1446.66	1446.66	1446.66	1465-1440	C-H bending
1105.25	1105.25	1055.10	1280-1030	C-O stretching
819.77	821.70	819.77	900-800	=CH bending
642.32	644.25	642.32	800-500	Benzene ring (quinone)

KBE-W =ka-bwee dye extracted with water
KBE-Et =ka-bwee dye extracted with ethanol
KBE-Met =ka-bwee dye extracted with methanol

* Silverstein *et al.*, 2003

UV- visible Analysis

Ultra violet spectra of natural dyes extracted from Ka-bwee dye are described in Figure 3. The wavelengthsof maximum absorption (λ_{max}) of extracted dyes were found to be 510 nm for water, 494 nm for ethanol and 499 nm for methanol extract.

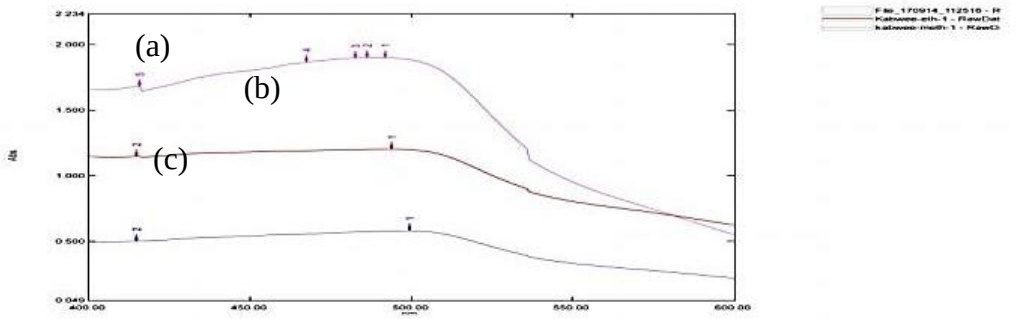


Figure 3 UV-visible spectra of Ka-bwee dyes extracted by (a) water, (b) ethanol and (c) methanol

Antimicrobial Activities of the Various Extracted Dyes from Ka-bwee

It was important to study the antimicrobial activity on dyes extracted from Ka-bwee because natural dyes showed inhibition effect against test bacterial in solution. The results are shown in Table 5 and Figure 4. Among these extracted dyes, watery extract did not show antimicrobial activity against test organisms. Petroleum ether extracted dye showed the highest activity while acetone, ethylacetate and methanol extract exhibited the lowest activity against six types of microorganisms. The antimicrobial activity might be due to ellagic acid and tannin components.

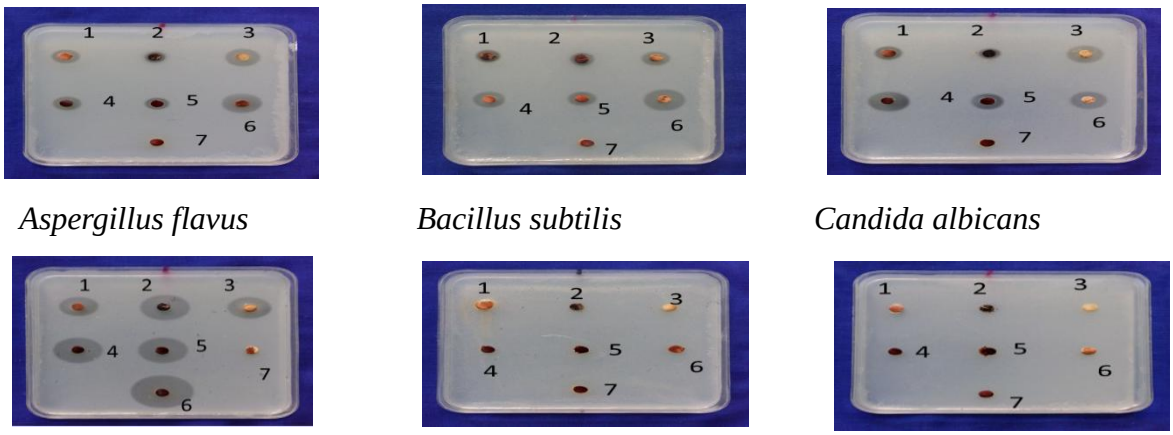


Figure 4 Antimicrobial activities of various solvent extracts of Ka-bwee dye(1.acetone, 2.chloroform, 3.ethylacetate, 4.ethnaol, 5.methanol, 6.pet.ether and 7. Water)

Table 5 Antimicrobial Activities of the Various Extracted Dyes from Ka-bwee

No.	Test Organisms	Acetone	$CHCl_3$	EtOAc	EtOH	MeOH	PE	H_2O
		Inhibition Zone Diameters (mm)						
1	*Aspergillus flavus*	+(12)	+(8)	+(14)	+(12)	+(10)	++(16)	-
2	*Bacillus subtilis*	+(12)	+(8)	+(14)	+(10)	+(12)	++(16)	-
3	*Candida albicans*	+(8)	+(10)	+(12)	++(14)	+(10)	+(12)	-
4	*Escherichia coli*	+(14)	++(18)	++(16)	++(18)	++(18)	+++(22)	-
5	*Pseudomonas fluorescens*	-	-	-	-	-	-	-
6	*Xanthomonas oryzae*	-	-	-	-	-	-	-

Agar Disc Diffusion Method -6 mm, 6 mm ~ 12mm (+), 15 mm ~ 19mm (++), 20 mm above (+++), No activity (-)

Sorption of the Extracted Natural Dye from Ka-bwee on Cotton Cloth

Effect of temperature

The adsorption properties of water, ethanol and methanol extracted dyes were studied at different temperatures (40-90 °C). In dyeing, the optimum temperature of extracted natural dyes on cotton was 80 °C.

Table 6 Adsorption Capacities of the Extracted Dyes at Different Temperature

Temperature (°C)	q_t(mg/g)		
	KBE-W	KBE-Et	KBE-Met
40	59.19	56.17	43.87
50	70.29	60.62	48.71
60	64.57	59.83	46.48
70	67.59	65.92	57.59
80	80.72	69.54	62.31
90	80.59	69.24	62.08

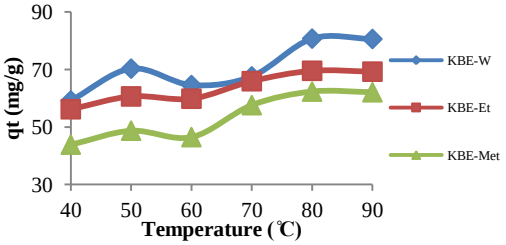


Figure 5 Effect of temperature on dyeing of Ka-bwee dye extracted by water, ethanol and methanol

Effect of contact time

Table 7 and Figure 6 show the amounts of extracted dyes applied on cotton cloth at different contact times (20, 30, 40, 50, 60, 70, 80 and 90 min) at 80 °C. It was found that the maximum sorption capacities were reached at 60 min.

Table 7 Adsorption Capacities of the Extracted Dyes at Different Contact Times

Time (min)	q_t(mg/g)		
	KBE-W	KBE-Et	KBE-Met
10	62.78	49.04	35.69
20	65.58	50.66	41.72
30	70.27	58.14	49.32
40	71.79	58.99	49.48
50	77.19	62.35	50.31
60	89.39	68.87	59.58

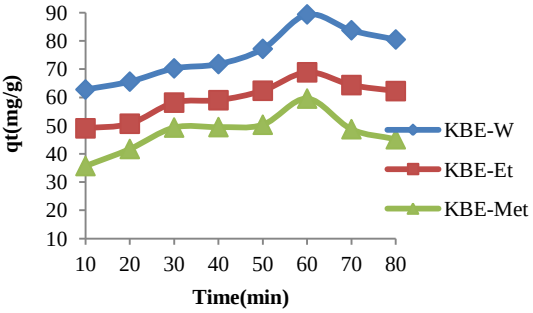


Figure 6 Effect of contact time on dyeing of Ka-bwee dye extracted by water ethanol and methanol

Effect of pH

The original pH of extracted dyes (water, ethanol and methanol) were 6.7, 6 and 7 respectively. The pH values of extracted natural dyes were adjusted with 1 % HCl and 1 % NaOH to reach the pH values of 3, 4, 5, 6, 7, 8 and 9. The optimum pH of extracted natural dyes was 6 (Table 8 and Figure 7).

Table 8 Adsorption Capacities of the Extracted at Different pH Values

pH	q_t(mg/g)		
	KBE-W	KBE-Et	KBE-Met
3	65.79	61.26	56.53
4	69.89	62.35	60.69
5	71.58	62.75	62.10
6	93.82	84.86	83.33
7	61.64	52.45	64.58
8	52.98	44.25	52.36

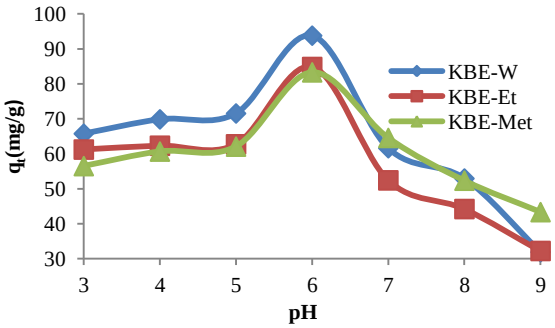


Figure 7 Effect of pH on dyeing Ka-bwee dye extracted by water, ethanol and methanol

Effect of mordant (alum) dosage

Table 9 and Figure 8 show the effective alum dosage of dyeing on cotton cloth. At the dye concentration 1000 ppm, contact time 60 min and temperature 80°C, the sorption properties of natural dye were studied at different alum dosages (0.0.25 to 0.15 g) by using UV-visible spectrophotometer. Among the different alum dosages, 0.1 g of alum dosage was the effective sorption capacity for dyeing process.

Table 9 Adsorption Capacities of the Extracted at Different Mordant (Alum) Dosage

Dosage (g)	q_t(mg/g)		
	KBE-W	KBE-Et	KBE-Met
0.025	73.12	61.58	56.12
0.050	76.40	65.63	57.14
0.075	72.77	61.45	62.74
0.100	80.72	75.52	73.78
0.125	74.34	67.66	69.34
0.150	65.04	60.45	62.31

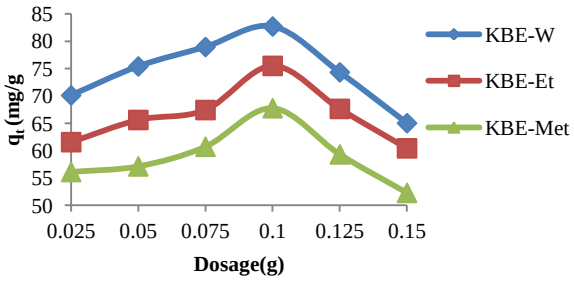


Figure 8 Effect of alum dosage on dyeing of Ka-bwee dye extracted by water, ethanol and methanol

Colour Fastness Properties of Ka-bwee Dye Extracted Dyes with Water Dyeing on Cotton Cloth

Colour density on the cotton cloth was increased significantly when a mordant was used. The colour fastness cotton cloth samples were prepared using pre-mordanting, simultaneous mordanting and post mordanting, 0.2 % v/v dye concentration and 60 min dyeing time because those conditions resulted in the highest colour strength for cotton cloth. The colour density for Ka-bwee dye extracted with water solution dyeing cotton cloth before and after colour fastness testing were compared in Tables 10,11,12 and 13 and Figures 9, 10, 11 and 12. For the dyeing on cotton cloth, the dyed cotton cloth without mordant was seen the lowest colour density. The dye (without) was seen the lowest colour intensity. Among mordants, natural mordant (jengkol peel) was used for the highest colour density whereas onion and teawaste were nearly equal colour density. It was also found that alum showed the good appearance and jengkol peel was good in colour fastness (Li *et al*., 2016).

Table 10 Colour Density of Cotton Cloth Dyed with Water Extract of Ka-bwee Dye

Type of mordanting	Colour Density/Mordants				
	Blank	Alum	Onion	Jengkol	Teawaste
Pre-mordanting	0.36	0.41	0.36	0.68	0.42
Simultaneous mordanting	0.35	0.40	0.38	0.75	0.45
Post mordanting	0.36	0.40	0.40	0.94	0.47

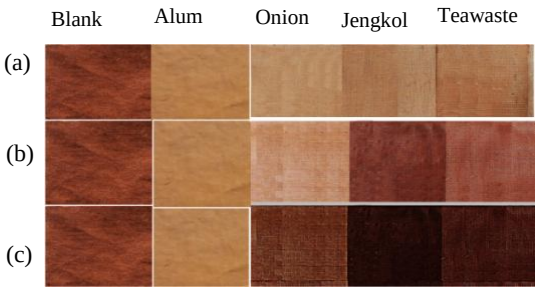


Figure 9 Colour of cotton cloth dyeing with water extract (a) pre-mordanting, (b) simultaneous mordanting and (c) post mordanting

Table 11 Variations in Colour Density of Cotton Cloth Dyed After Fastness Test (Pre-mordanting with Water Extract of Ka-bwee Dye)

	Colour Density / Mordants				
Mordant	**Blank**	**Alum**	**Onion**	**Jengkol**	**Teawaste**
Before	0.36	0.41	0.36	0.68	0.42
lighting	0.32	0.39	0.32	0.73	0.40
Washing	0.33	0.37	0.44	0.70	0.35

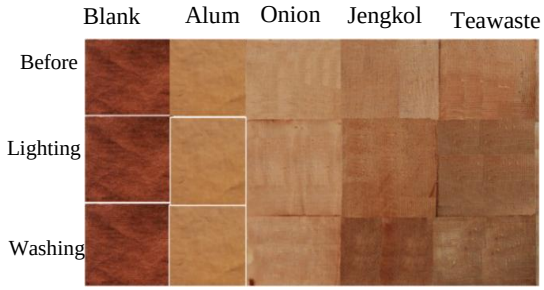


Figure 10 Colour of cotton cloth dyeing with water extract of pre-mordanting

Table 12 Variations in Colour Density of Cotton Cloth Dyed After Fastness Test (Simultaneous Mordanting with Water Extract of Ka-bwee Dye)

	Colour Density / Mordants				
Mordant	**Blank**	**Alum**	**Onion**	**Jengkol**	**Teawaste**
Before	0.35	0.40	0.38	0.75	0.45
lighting	0.34	0.38	0.33	0.76	0.42
Washing	0.32	0.37	0.45	0.73	0.37

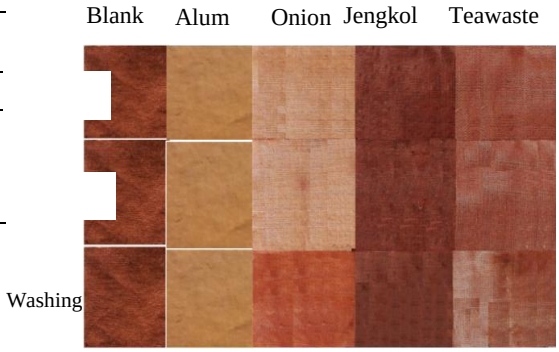


Figure 11 Colour of cotton cloth dyeing with water extract of simultaneous mordanting

Table 13 Variations in Colour Density of Cotton ClothDyed After Fastness Test (Post Mordanting with Water Extract of Ka-bwee Dye)

	Colour Density / Mordants				
Mordant	**Blank**	**Alum**	**Onion**	**Jengkol**	**Teawaste**
Before	0.35	0.38	0.40	0.94	0.47
Lighting	0.32	0.36	0.35	0.95	0.44
Washing	0.32	0.36	0.38	0.93	0.39

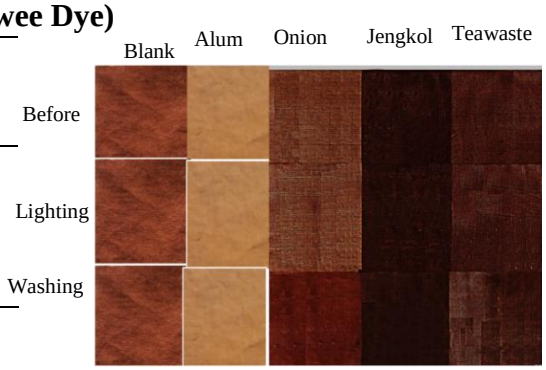


Figure 12 Colour of cotton cloth dyeing with water extract of post mordanting

Table 14 Difference before and after Wash Fastness (Post Mordanting) Properties of Five Dye Solutions on Cotton Cloth

Desorption values	**Blank**	**Alum**	**Onion**	**Jengkol**	**Teawaste**
	5.71	5.26	5	1.06	17.02

Conclusion

Natural dyes were extracted from Ka-bwee bark powder with different solvents (water, ethanol and methanol) by extraction method. The physicochemical properties of Ka-bwee raw bark were investigated. The physicochemical parameters of Ka-bwee raw bark, such as moisture content 8.25 %, ash content 3.53 %, bulk density 0.80 gcm^{-3}, pH 6.70 were observed. In phytochemical test, tannins, steroids, alkaloids, flavonoids, glycosides, phenolic compounds, saponins and α-amino acids were observed in the extracted dyes of water, ethanol and methanol. Whereas carbonhydrates and starch were not observed in this extracted dye. According to the FT IR analysis, Ka-bwee dyes extracted by different solvents, functional groups of OH, C-O, C=O(stretching),=CH bending and benzene ring were observed. From EDXRF analysis, the major constituent is calcium 46.38 % and potassium 23.26 % and silica 11.76 % were observed. Furthermore, antimicrobial activities of acetone, chloroform, ethyl acetate, ethanol, methanol, petroleum ether and water extracted dye from Ka-bwee were investigated by agar disc diffusion method. Among these extracted dyes, watery extract did not show antimicrobial activity in against test organisms. Among them, petroleum ether extracted dye showed the highest activity while acetone, ethylacetate and methanol extract exhibited the lowest activity against six types of microorganism. In this Ka-bwee dye, petroleum ether extracted dye inhibited antimicrobial activity in all tested organisms except *Pseudomonas fluorescens and Xanthomonas oryzae.* It was obvious that antimicrobial properties are closely related to the extracted solvent and dye structure, especially the presence of functional groups on it. Although dyes are tannin based, their antimicrobial activity differs greatly. The maximum absorption wavelengths (λ_{max}) of Ka-bwee dyes extracted by water, ethanol and methanol were 510, 494 and 499 nm, respectively. The factors effecting the dyeing on cotton cloth such as temperature, contact time, pH and alum dosage were studied. For dyeing on cotton cloth, the optimum temperature at 80 °C, alum dosage 0.1 g, contact time 60 min and pH 6 were observed. Among the various extracted natural dye, watery extracted dyes are the most suitable for dyeing on cotton cloth. The colour fastness of dyed samples by pre-mordanting, simultaneous mordanting and post mordanting were determined. In this, the light fastness of post mordanting method is better than the other two methods. For the dyeing on cotton cloth, the dyed cotton cloth without mordant was seen the lowest colour density. Among these mordants, natural mordant (jengkol peel) was observed the highest colour density whereas alum, onion peel and teawaste were nearly equal colour density. According to the desorption properties, natural mordant (jengkol peel) was good colour fastness among the five mordants. The dye plants produced fine colour on textile, food and drink. The present study showed that natural dye extracted from bark of plant was non-toxic and cost effective for the eco-system.

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PREPARATION AND CHARACTERIZATION OF SAWDUST-CEMENT PARTICLEBOARDS

Hnin Yu Wai*

Abstract

This work is mainly concerned with the preparation of particleboards from sawdust (SD). Cement (C) as used as binder. The physicochemical properties and characterization of sawdust and cement were also conducted. Six types of particleboards were prepared by mixing various proportions of sawdust (50 %, 40 %, 33.33 %, 28.57 %, 25 %, 22.22 %) and various proportions of cement (50 %, 60 %, 66.66 %, 71.43 %, 75 %, 77.78 %) and a chemical additive $CaCl_2$. The additive was based on 2 % by weight of cement used. Particleboards were prepared by cold compressing molding method. The prepared particleboards (SDC) were characterized according to physicochemical and physicomechanical parameters such as modulus of rupture, thickness, density, water absorption, swelling thickness and hardness. From the results, it was found that particleboard namely SDC 5 containing (25 % of sawdust, 75 % of cement and 1.5 % of $CaCl_2$) was a quality grade particleboard. It has 2058.34 psi modulus of rupture, 0.65 cm thickness, 1.2320 g cm^{-3} density, 13.05 % water absorption, 17.14 % swelling thickness and 98 D hardness. The SDC 5 particleboard, based on water absorption, modulus of rupture and hardness values, indicates that it was the best among all particleboards studied. The surface morphology of SDC 5 was studied by SEM and the thermal stability of particleboard was studied by TG-DTA.

Keywords: Particleboard, sawdust, cement, chemical additive, $CaCl_2$

Introduction

Wood as a raw material contributes significantly in improving a nation's economic base, industrialization and comfort of its teeming population. Globally the demand for wood and wood-based panel products has been on the increase (Youngs, 2002). Particleboard has become one of the most popular wood-based composite materials for integrating decoration because of its low density, good thermal insulation, sound absorption, and wonderful machining properties. The primary lignocellulosic material used in the particleboard industry is wood. The increased demands of raw materials in wood panel, pulp and paper manufacturing have led to worldwide shortages of forest resources (Fuwape, 1995).

The increasing population and growing concern about the environment have led to changes in forest management practices, resulting in a significant reduction in wood harvesting from national forests despite growing demands. In the meantime, a constantly increasing population has resulted in an escalating demand for wood in the forest products (Blankenhor, 1994). Cement-bonded particleboard (CBP) is one of the most important mineral bonded wood composites, a molded panel comprised of 10-70 wt % wood particles and 30-90 wt % Portland cement binder (Sorfa,1984). Unlike resin-bonded particleboards, CBP boasts excellent sound insulation, high resistance to water, termites and fungi, and excellent long-term weather durability in outdoor conditions. These advantages suggest its potential application as a replacement for traditional building materials and conventional wood composites in roofing, wall and flooring parts and noise absorbing partitions (Lee, 1984). CBP panel production provides attractive possibilities for using wood wastes and agricultural residues, which are otherwise environmentally problematic. In addition they have a low production cost (Karade, 2003).

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Aim

The aim of this research is to prepare the quality grade particleboard from renewable natural resource from sawdust, using cement as binder.

Materials and Methods

All necessary research facilities were provided by the Polymer Department, Department of Research and Innovation, (DRI). The apparatus consists of conventional Lab ware, glassware and modern equipment. Some of the instruments used in the experiments are E-Mettler balance (210 ± 0.1 mg) (LA – 310 S), Muffle furnace (Range 100-1100 ºC Gallenkamp, England), TG-DTA (Hi-TGA 2950, DTG-60 H Thermo gravimetric analyser), Mixer machine (Henschel Mischer, Germany), Hydraulic hot press (Apex Construction Ltd., Gravesend England), Electro-hydraulic tensile tester (Thwing-Albert Instrument Company Philadelphia, USA), Hardness tester (H.W Wallace and Co. Ltd., England), Vernier caliper (or) screw gauge, Specific gravity balance (Wallace Test Equipment), Sieves (US Series Equivalent, The Tyler Std. Screen Scale), Scanning Electron Microscope (No. JSM-5610, JEOL Ltd., Japan) and Thermal control status oven (H 053, 240 V, England).

Collection of Samples

In the experiments, sawdust was collected from Family Saw Mill, North Okkalapa Township and commercial cement from Chan Myae Aung Trading, Mingalar Taung Nyunt Township, Yangon Region, Myanmar.

Preparation of Sawdust

The collected sawdust was soaked in water for 24 h to reduce the amount of water-soluble sugars and tannins and then the cleaned sawdust was solar dried for five days. It was dried again at 115 °C for 1 h and cooled in desiccators. The dried samples were screened to pass through the sieve aperture of 25 mesh (0.5 mm). The sieved (25 mesh) material was then stored in an airtight plastic bag for further experiment.

Determination of Physicochemical Properties of Sawdust and Cement

The physicochemical properties (moisture content, ash content, solid content, bulk density and pH) of the samples were determined by conventional methods (Table 1).

Characterization of Sawdust and Cement

SEM analysis

The morphology of prepared sawdust and cement was studied by using Scanning Electron Microscope (JSM-5160, JEOL Ltd., Japan) for analysing micro and macro pores present on the surface of the samples. The scanning electron micrographs of sawdust and cement were obtained.

TG-DTA analysis

Thermal analysis of the samples was done by a DTA-60H (Hi-TGA 2950) thermal analyzer. The sample (*ca.* 5 mg) was required and measurements were made in the temperature range 0~600 ºC at 20.00 ºC/min and the nitrogen gas at 50.00 mL/min.

XRD analysis

The nature of the cement was determined by X-ray diffractometer.

Preparation of Sawdust-Cement Particleboards

In this research, all of the sawdust-cement particleboards were prepared by cold compressing molding method.

Effect of proportion of cement on the preparation of sawdust-cement particleboards

Each sawdust (50 %, 40 %, 33.33 %, 28.57 %, 25 %, 22.22 %) was dipped in 20 ^{o}C water (500 mL) for 3 h and mixed with (50 %, 60 %, 66.66 %, 71.43 %, 75 %, 77.78 %) of cement. The chemical additive $CaCl_2$ based on 2 % by weight of cement was then added by Henschel mixer for 10 min. The mixture was then laid in mold. Care must be taken to get uniform surface layer in cold press section. Later, this mat was carefully transferred to the hydraulic press machine. Excess water was squeezed out by compression at 2800 psi pressure for 24 h. The particleboards were kept at room temperature for 1-2 weeks and then the edges and both sides of the particleboards were trimmed and sanded (15.24 cm x 15.24 cm).

Determination of the Physicochemical and Physicomechanical Properties of Sawdust-Cement Particleboards

The physicochemical and physicomechanical properties (moisture content, modulus of rupture, thickness, water absorption, swelling thickness, density, and hardness) of the prepared sawdust-cement particleboards were determined by the conventional method and modern techniques.

Characterization of the Selected Particleboard (SDC 5)

SEM analysis

The morphology of prepared SDC 5 particleboard was studied by using Scanning Electron Microscope (JSM-5160, JEOL Ltd., Japan) for analysing micro and macro pores present on the surface of the samples. The scanning electron micrograph of SDC 5 particleboard was obtained.

TG-DTA analysis

Thermal analysis of the sample was determined by a DTA-60H (Hi-TGA 2950) thermal analyzer. The sample (*ca*. 5 mg) was required and measured in the temperature range 0~600 ºC at 20.00 ºC/min and nitrogen gas at 50.00 mL/min. The TG-DTA thermogram (Figure10) and the description data (Table 7) are given.

Results and Discussion

Table 1 shows that the physicochemical properties (moisture content, ash content, bulk density, and pH) of the sawdust and cement determined by the conventional method. The pH values of samples were determined by pH meter. The moisture content of sawdust and cement were determined by oven drying method at 115-120 ºC to obtain constant weight. It can be observed that moisture content of the sawdust is 20.11 %, ash content is 11.25 %, solid content is 79.89 % bulk density is 23.10 g mL^{-1} and pH is 6.32 (Table 1). Cement was used as a binder for making particleboards. It can be observed that moisture content of the cement is 0.94 %, ash content is 18.25 %, solid content is 99.06 %, bulk density is 80.47 g mL^{-1} and pH is 7.86.

Table 1 Physicochemical Properties of Sawdust and Cement

No.	Physicochemical Properties	Sawdust	Cement
1.	Moisture content (%)	20.11	0.94
2.	Ash content (%)	11.25	18.25
3.	Solid content (%)	79.89	99.06
4.	Bulk density (g mL^{-1})	23.10	80.47
5.	pH	6.32	7.86

Characterization of Sawdust and Cement

SEM analysis

Surface morphology of sawdust and cement was examined by SEM (Figures 1 and 2). It is obviously seen that many non-uniform cavities and pores are on the surface of the sawdust. It can be observed that the diameter of cement has ~20 μm from SEM image. In this image, amorphous nature of cement is not similar in size.

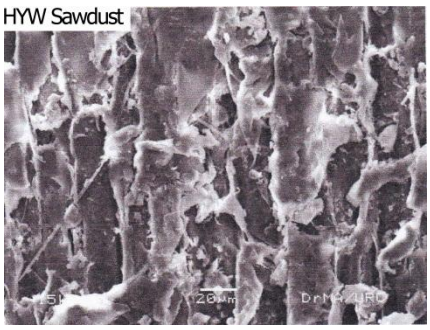

Figure 1 SEM photomicrograph of sawdust at 1.360 kx magnification

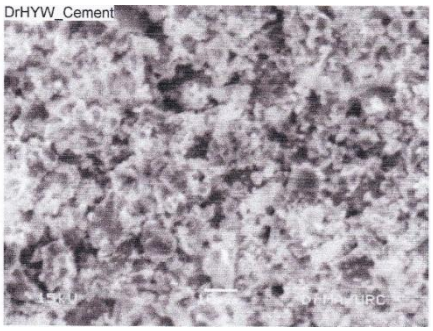

Figure 2 SEM photomicrograph of cement at 1.360 kx magnification

TG - DTA analysis

On the basis of the thermogram of sawdust (Figure 3), the initial region of the TG profile shows weight loss percent about 25 % at about 73.83 ºC. It may be dehydration process and at the temperature range of 39 ºC to 324 ºC. The major stage of the loss of about 50 % occurs from 324 ºC to 400 ºC. The exothermic peak was observed around 390 ºC. It may be attributed to decomposition of small segments from cellulose and lignin. The exothermic peak was found at 504 ºC with the weight loss percent about 20 %. It is due to the decomposition of cellulose backbones and formation on char and at the temperature range of 400 ºC to 600 ºC. Residual weight of sawdust was 0.146 mg (Table 2).

On the basis of the thermogram of cement (Figure 4) the weight loss % of about 9.44 % was found within the temperature range of 40 ºC to 140 ºC. This is due to the dehydration of moisture and absorbed water of cement, indicated by the endothermic peak at 122.04 ºC. The residual weight of cement was 19.023 mg (Table 3).

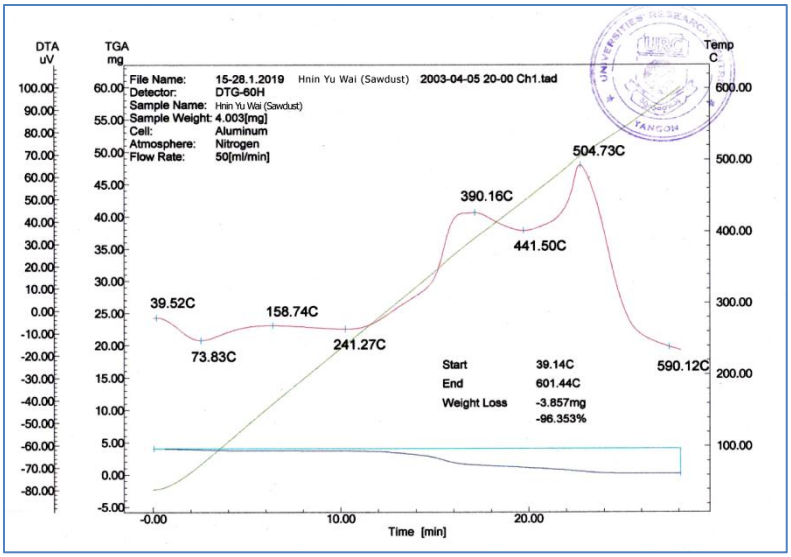

Figure 3 TG-DTA thermogram of sawdust

Table 2 Thermal Analysis Data of Sawdust

TG		DTA		
Break in Temperature (ºC)	**Weight loss (%)**	**Peak Temperature (ºC)**	**Nature of peak**	**Remarks**
39-324	25	73.83	endothermic	Dehydration due to surface water
324-400	50	390.16	exothermic	Decomposition of small segments from cellulose and lignin
400-600	20	504.73	exothermic	Decomposition of backbones of cellulose and burn into char, residual weight 0.146 mg

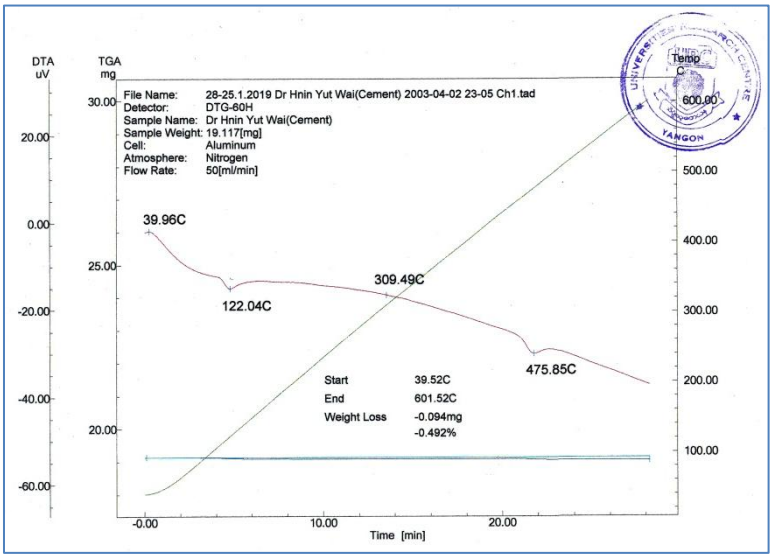

Figure 4 TG-DTA thermogram of cement

Table 3 Thermal Analysis Data of Cement

TG		DTA		Remarks
Break in Temperature (ºC)	**Weight loss (%)**	**Peak Temperature (ºC)**	**Nature of peak**	
40-140	0.48	122.04	endothermic	Dehydration due to moisture and absorbed water, residual weight of cement 19.023 mg

XRD analysis

In the X- ray diffraction profile of cement sample a big hump is observed at nearly 2 θ value of 30˚. The simplest and most widely used method for estimating the average crystallite size is from the full width at half maximum (FWHM) of a diffraction peak using the Scherrer equation, $d = K\lambda / \beta \cos\theta$, where d is the crystallite size, λ is the diffraction wavelength, β is the corrected FWHM, θ is the diffraction angle and K is a constant close to unity. The crystallite size of cement was calculated by this method (Table 4).

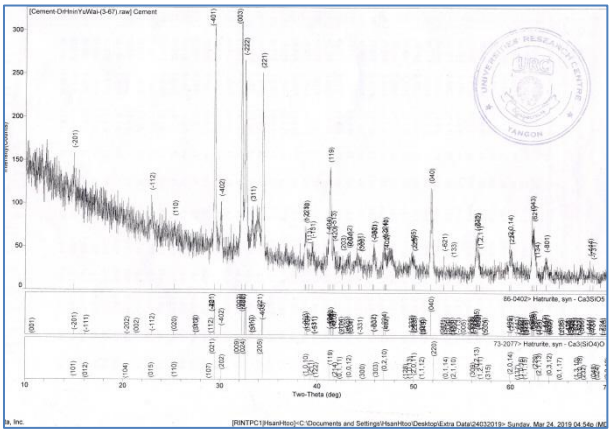

Figure 5 XRD diffractogram of cement

Table 4 XRD Data of Cement

No.	Miller Indice (h k l)	FWHM (degree)	2 Theta (degree)	Crystallite Size (nm)
1.	0 0 3	0.200	32.174	431.61
2.	2 2 1	0.209	34.344	415.36
3.	1 1 9	0.285	41.262	310.97
4.	0 4 3	0.276	62.185	350.96
Average Crystallite Size				377.225

On the Aspect of the Preparation of Sawdust-Cement Particleboards

For the preparation of particleboard, sawdust (SD) was mixed with cement (C) under the pressure 2800 psi. For all of the prepared particleboards, physicochemical and physicomechanical parameters such as thickness, swelling thickness, density, water absorption, modulus of rupture, and hardness were determined. Among these parameters, modulus of rupture is more specific than other for determining particleboards quality.

Effect of proportion of cement on the preparation of particleboards

SDC 1, SDC 2, SDC 3, SDC 4, SDC 5 and SDC 6 particleboards were prepared with various proportions of sawdust (50 %, 40 %, 33.33 %, 28.57 %, 25 %, 22.22 %), various proportions of cement (50 %, 60 %, 66.66 %, 71.43 %, 75 %, 77.78 %) and chemical additive $CaCl_2$ 2 % by weight based on the cement used. These particleboards were made at 2800 psi of pressure. The results of the physicochemical and physicomechanical properties including thickness, swelling thickness, water absorption hardness modulus of rupture (MOR) and density of SDC 1, SDC 2, SDC 3, SDC 4, SDC 5 and SDC 6 particleboards are presented in Table 6 and Figures 5, 6 and 7. It was found that the particleboard SDC 5 made with sawdust (25 %) and 75 % of cement has the highest modulus of rupture among them. Moreover, the water absorption tests of the particleboards are also found to be satisfactory. Water absorption test is a measure of the soaking characteristic of a test sample towards water when fully immersed in a quantity of water at room temperature for a certain time period (usually 24 h).The particleboards SDC 5 have the lowest water absorption percentage among them (Table 5). Therefore, particleboards SDC 5 was chosen to make the most suitable particleboard.

Table 5 Physicochemical and Physicomechanical Properties of Particleboards with Various Proportions of Cement (C)

Types of Particleboards	Cement (%)	Modulus of rupture (psi)	Thickness (cm)	Density (g cm^{-3})	*Water Absorption (%)	*Swelling Thickness (%)	Hardness Shore (D)
SDC1	50.00	820.25	0.60	0.5168	58.05	62.18	89
SDC 2	60.00	1030.01	0.63	0.7265	46.15	40.20	92
SDC3	66.66	1250.90	0.65	0.8060	35.42	38.19	94
SDC4	71.43	1648.48	0.65	0.9520	10.32	20.18	97
SDC5	75.00	2058.34	0.65	1.2320	13.05	17.14	98
SDC 6	77.78	1967.84	0.67	1.3460	18.70	30.28	96

*After 24h Applied pressure – 2800 psi Pressing time – 24 h

SDC1 = (50 %) sawdust with (50 %) Cement, SDC 4 = (28.57 %) sawdust with (66.66 %) Cement

SDC 2 = (40 %) sawdust with (60 %) Cement, SDC 5 = (25 %) sawdust with (75 %) Cement

SDC 3 = (33.33 %) sawdust with (140 g) Cement, SDC 6 = (22.22 %) sawdust with (77.78 %) Cement

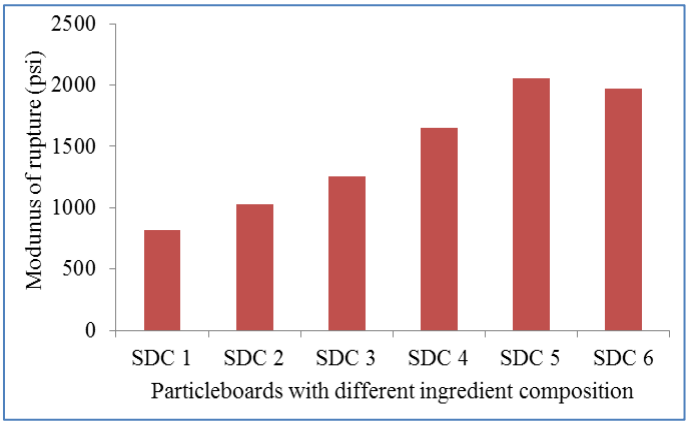

Figure 6 Modulus of rupture of SDC particleboards as a function of particleboards with different ingredient composition

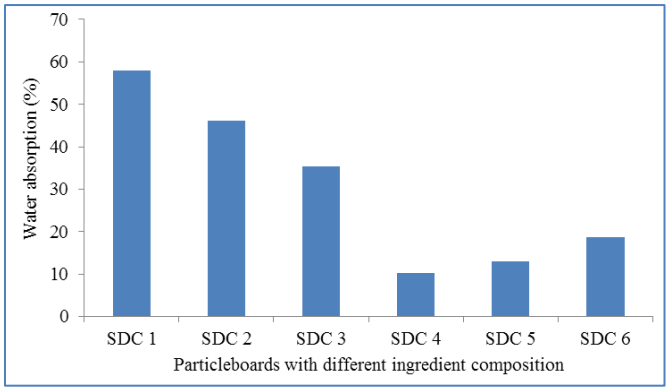

Figure 7 Water absorption of SDC particleboards as a function of particleboards with different ingredient composition

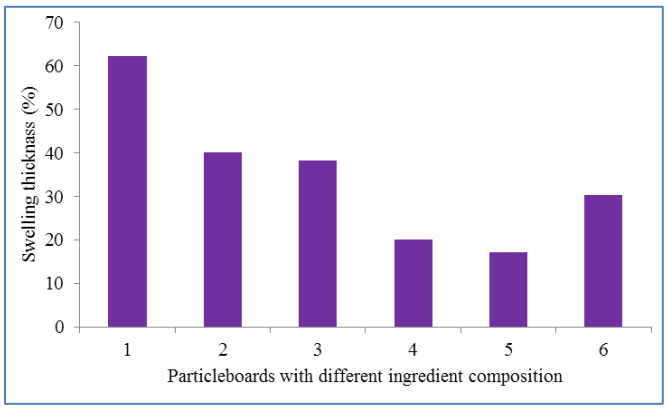

Figure 8 Swelling thickness of SDC particleboards as a function of particleboards with different ingredient composition

Characterization of SDC 5 Particleboard

SEM analysis

Scanning electron microscope (SEM) imaging technique has been used to identify the interfacial area between the wood particles and the cement matrix of the fracture surface of the selected particleboard (SDC 5). The SEM photomicrograph of SDC 5 particleboard shows that specimens containing calcium chloride exhibited cone-shaped, well-formed crystals in the cement (Figure 9).

Figure 9 SEM photomicrograph of SDC 5 particleboard

TG- DTA analysis

Thermal stability of particleboard (SDC 5), was investigated by TG-DTA analysis (Figure 10). On the basis of the thermogram of particleboard SDC 5, in first stage, the weight

loss % about 7.73 % that was found within the temperature range of 38 ºC to 120 ºC. This is due to the dehydration of surface giving an endothermic peak at 107.35 ºC. In the second stage, the weight loss % about 37.25 % was found within the temperature range of 120 ºC to 400 ºC. This is due to the decomposition of small segments from cellulose and lignin giving an exothermic peak at 365.91 ºC. In the third stage, the weight loss % about 14.76 % that was found within the temperature range of 400 ºC to 600 ºC. This is due to the decomposition of backbones of cellulose and burning into char giving an exothermic peak at 487.77 ºC. The residual weight of SDC 5 particleboard was 16.73 mg (Table 6).

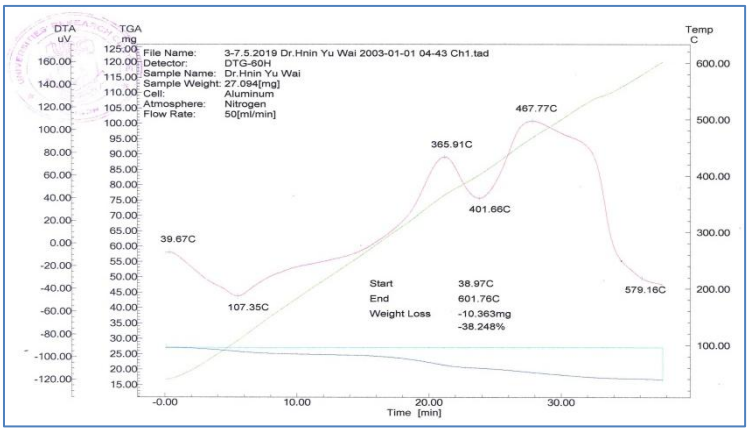

Figure 10 TG-DTA thermogram of SDC 5 particleboard

Table 6 Thermal Analysis Data of SDC 5 Particleboard

TG		DTA		Remarks
Break in Temperature (ºC)	**Weight loss (%)**	**Peak Temperature (ºC)**	**Nature of peak**	
38-120	7.73	107.35	endothermic	Dehydration due to surface water
120-400	37.25	365.91	exothermic	Decomposition of small segments from cellulose and lignin
400-600	14.76	487.77	exothermic	Decomposition of backbones of cellulose and burn into char, residual weight 16.73 mg

Some Possible Application of Prepared Particleboards

The sawdust-cement particleboard can be used in eaves, exterior wall, ceiling, partition wall, flooring and cladding. The photographs of SDC particleboards (15.24 cm x 15.24 cm) are presented in Figure 11.

Figure 11 Photographs of SDC particleboards

Conclusion

The sawdust (SD) was collected from the Family Saw Mill, North Okkalapa Township and waste cement (binder) from Chan Myae Aung Trading, Mingalar Taung Nyunt Township, Yangon Region. The collected sawdust was purified and sieved with 0.5 mm aperture size.

The particleboards were prepared by mixing different proportions of sawdust with different proportions of cement by cold pressing method. The physicochemical and physico-mechanical properties of the particleboards (SDC) were characterized. The optimum conditions of prepared particleboards were defined by the improvement of modulus of rupture (MOR) and least water absorption parameters. From the experimental results, (SDC 5) particleboard with 25 % of sawdust with 75 % of cement and 1.5 % of $CaCl_2$ was found to be a quality grade particleboard possessing 2058.34 psi of modulus of rupture, 0.65 cm of thickness, 1.2320 g cm^{-3} of density, 13.05 % of water absorption, 17.14 % of swelling thickness, and 98 Shore D of hardness, respectively. Sawdust particleboards prepared by simple hand lay-up process provide an opportunity of replacing materials with a higher strength, low cost alternative that is environmentally friendly. Particleboards are made of wastes by product from saw mill, which can change harm to benefit by developing the "green" particleboards from materials of natural degradation. Moreover, the use of sawdust-cement particleboard can reduce overall weight of the construction, since their density and weight are generally low. It is a very versatile material that can be used eaves, exterior wall, ceiling, partition wall, flooring, cladding even roofing provided that proper coating is applied and wire meshes imbedded to enhance the interlocking capacity especially for long life spans.

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PREPARATION AND CHARACTERIZATION OF BIODEGRADABLE CHITOSAN-CORNCOB-GLUTARALDEHYDE BIOCOMPOSITE FILM

Aye Myat Thu[1], Mya Kay Thi Aung[2], Khin Than Yee[3]

Abstract

This research work is concerned with the study on the properties of biodegradable composite film derived from chitosan, corncob and glutaraldehyde (used as cross-linking agent). Biodegradable composite films were prepared by using optimum composition of 0.2 g corncob and 1.5 % chitosan with different concentrations of cross-linking agent. The effect of cross-linking agent was studied over a range of concentrations from 6 % to 30 % (total solid weight). The comparative study of cross-linking agent and its concentrations incorporated into biodegradable films was investigated based on thickness, tensile strength, elongation at break and tear strength. From the results of physicomechanical parameters, the tensile strength of biodegradable films incorporated with glutaraldehyde was higher than that of chitosan-corncob biocomposite film. The physicochemical properties of biodegradable composite films such as degree of swelling was also determined. The degree of swelling increases with increasing immersion time. With increasing glutaraldehyde concentration, the ultimate degree of swelling after 4 days of immersion time of composite films was found to decrease. Comparative characterization of biodegradable composite films with corncob powder and glutaraldehyde were determined by SEM and TG-DTA analyses. From SEM analysis, the prepared chitosan film and chitosan-corncob biocomposite film had distinct phase structures whereas chitosan-corncob-glutaraldehyde biocomposite film showed homogeneous and smooth surface. In TG-DTA analysis, only exothermic peaks appeared in chitosan film but both endothermic and exothermic peaks were observed in chitosan-corncob and chitosan-corncob-glutaraldehyde biocomposite films. The biodegradable nature of prepared composite films was also studied according to the soil burial techniques.

Keywords: biodegradable composite film, cross-linking agent, degree of swelling, chitosan-corncob-glutaraldehyde biocomposite

Introduction

Chitosan is a biopolymer that is derived from chitin or by-products of seafood processing such as crab shell, lobster shell, and prawn shell waste (Mathew and Abraham 2008; Park *et al* ., 2004; Garcia *et al*., 2006). Among biopolymers, chitosan has been considered as one of the most promising materials for future applications on account of its excellent biocompatibility, biodegradability, anti-microbial activity, non-toxicity and its economic advantages (Rutnakornpituk *et al* ., 2006; Singh *et al* ., 2009; Dutta *et al.,* 2004). Generally, chitosan was used in producing plastic film due to its unique structure, multidimensional properties, and wide applications in biomedical and other industrial area (Ambri *et al*., 2013; Aranaz *et al*., 2009; Srinivasa *et al.,* 2007).

Corncob is a major part of the corn's waste, problematic waste is now being converted into useful industrial materials (Inglett, 1970). Powdered corncob is a naturally occurring composite material and thus does not have a specific molecular weight, molecular formula and structural formula. Corncobs contain a considerable reservoir of carbohydrates, most of which are of a polysaccharide nature. Corncobs are lignocellulosic materials composed of cellulose,

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hemicellulose and lignin. Corncobs contain approximately 39.1 % cellulose, 42.1 % hemicellulose, 9.1 % lignin, 1.7 % protein, and 1.2 % ash (Beall and Ingram, 1992).

Glutaraldehyde, a linear, 5-carbon dialdehyde, is a clear, colorless to pale straw-colored, pungent oily liquid that is soluble in all proportions in water and alcohol, as well as in organic solvents. Glutaraldehyde can react with several functional groups of proteins, such as amine, thiol, phenol, and imidazole (Habeeb, and Hiramoto, 1968) because the most reactive amino acid side chains are nucleophile. Glutaraldehyde is commonly used as a cross-linking agent for collagen-based biomaterials. Among cross-linking agents, glutaraldehyde is the most commonly used in chitosan (Uragami *et al*., 1994).

A composite is a structural material that consists of two or more combined constituents that are combined at a macroscopic level and are not soluble in each other. One constituent is called reinforcing phase and one in which it is embedded is called the matrix. Composites may be classified into three types which are green composites, hybrid composites, and textile composites. These composites can be easily disposed of or composed without harming the environment.

If the films consist of two organic materials that can be easily biodegradable, it is called biocomposite degradable films (Ting Liu, 2008). The production of biodegradable and edible films from carbohydrates and proteins adds value to low-cost raw materials and can play an important role in food preservation (Nasir *et al*., 2005; Peesan *et al*., 2003).

The aim of this research work was to study the preparation and characterization of the biodegradable chitosan-corncob-glutaraldehyde composite film using natural waste (corncob). Corncobs were used as filler in composites because it is naturally abundant in Myanmar, and low cost. Chitosan-corncob-glutaraldehyde biocomposite films were prepared by solvent evaporating method.

Materials and Methods

Sample Collection

The chemicals used in this research work were procured from British Drug House (BDH) Chemicals Ltd., England. Commercial chitosan was purchased from Shwe Poe Company, Hlaingtharyar Township, Yangon Region, Myanmar. Maize (*Zea mays*) sample was purchased from Thirimingalar Market, Kyeemyindaing Township, Yangon Region, Myanmar.

Preparation of Corncob Powder and Base Activated Corncob Powder

The collected raw corncobs were cut into pieces and washed with water (three times) to remove dust and impurities. Then they were directly dried in sunshine and dried in an oven at 80 °C for 24 h. The dried corncobs were obtained. They were made into powder form with blender. Corncob powders were sieved by a sieve (80 mesh size).

The 30 g of corncob powder was chemically treated with 300 mL NaOH solution having concentration of 0.1 M. The mixture was placed in an autoclave at 121 °C for 20 min to obtain homogeneous solution. Then, it was washed with distilled water until pH 7.0. After that it was dried in an oven at 120 °C for 24 h. The dried sample was ground in motar and pestle to obtain fine particle size. Finally, base activated corncob powders were sieved by a sieve (80 mesh size).

Preparation of Chitosan-Corncob Biocomposite Films

Chitosan solution (1.5 % w/v) was prepared by dissolving 1.5 g of chitosan in 100 mL of 2 % v/v acetic acid with often stirring and heating for 30 min at 100 °C to get a clear solution. After the chitosan was dissolved completely, the solution was filtered by a filter paper to remove any small lumps in the solution. Then 0.1 g to 0.5 g of base activated corncob powder was added to the above chitosan solution. The mixture was stirred and heated for 30 min at 100 °C to get a homogeneous solution. The mixture solutions were then made into films by pouring onto a melamine shallow plates.

The melamine plates containing the mixture solution were left for about three days at room temperature. Then they were heated at 50 °C in an oven for about 6 h. All of the prepared chitosan-corncob biocomposite films were kept under dry conditions before further use.

Preparation of Chitosan-Corncob-Glutaraldehyde Biocomposite Films

Chitosan solution (1.5 % w/v) was prepared by dissolving 1.5 g of chitosan in 100 mL of 2 % v/v acetic acid with often stirring and heating for 30 min at 100 °C to get a clear solution. After the chitosan was dissolved completely, the solution was filtered by a filter paper to remove any small lumps in the solution. Then 0.2 g of base activated corncob powder was added to the above chitosan solution. Various concentrations of glutaraldehyde (6, 12, 18, 24 and 30 % of the total solid weight) were added to the prepared chitosan-corncob solutions. The mixture solutions were stirred and heated for 30 min at 100 °C to get a homogeneous solution. These solutions were kept for sufficient time to remove any bubble formation and were cast onto a cleaned and dried melamine plate at room temperature.

The melamine plates containing the mixture solution were left for about three days at room temperature to obtain chitosan-corncob-glutaraldehyde biocomposite films. Then they were heated at 50 °C in an oven for about 6 h. All of the prepared chitosan-corncob-glutaraldehyde biocomposite films were kept under dry conditions before further use.

Characterization of the Prepared Samples

The physicochemical properties (pH, moisture, specific gravity and bulk density) of chitosan and base activated corncob powder were determined. The morphological structures of the prepared biocomposite films were characterized by SEM. Thermal properties of the biocomposite films were evaluated by TG-DTA.

The scanning electron microscopy (SEM) images of raw corncob powder, base activated corncob powder, chitosan film, chitosan-corncob biocomposite film, and chitosan-corncob-glutaraldehyde biocomposite film were obtained using JSM-5610 Model SEM, JEOL-Ltd., Japan.

Thermogravimetric analyses of samples were performed using TG-DTA instrument, (Hi-TGA 2950 model). The temperature range between 0 °C and 600 °C under nitrogen gas (at a rate of 50 mL/min).

Determination of Biodegradation

Soil burial test

The films were cut 1" × 1" dimension. The films were then accurately weighed and buried in soil at the depth of 5 cm. They were taken out from the soil at an interval of one week. Sample geometry on degradation was also recorded with photos and they are presented in Figure 10.

Results and Discussion

Sample Preparation

The corns were collected from local market. The seeds were removed from corns. The obtained corncobs were cut into small pieces and washed with water (three times). They were directly dried in sunshine and dried in an oven at 80 °C for 24 h. Then they were made into powder form with blender. Finally, the obtained corncob powder were sieved by a sieve (80 mesh).

Physicochemical Analysis of Chitosan and Base Activated Corncob Powder

Table 1 shows the physicochemical properties of chitosan and base activated corncob powder. From the physicochemical properties, the moisture content of chitosan is higher than the base activated corncob powder. However, the pH value of chitosan is lower than the base activated corncob powder.

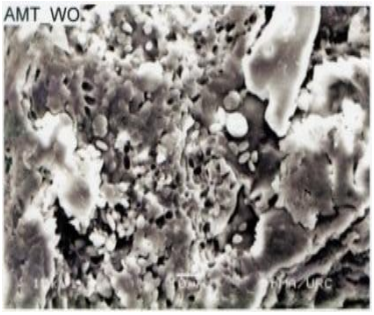
Table 1 Physicochemical Properties of Chitosan and Base Activated Corncob Powder

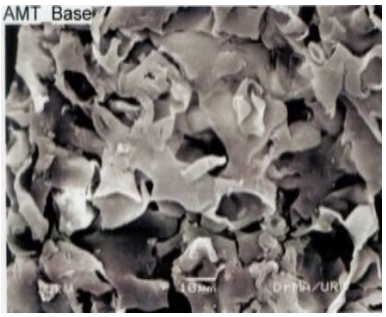
Physicochemical properties	Chitosan	Base Activated Corncob Powder
pH	4.10	7.49
Moisture (%)	13.050	10.1549
Specific gravity (gmL^{-1})	1.0205	-
Bulk density (gcm^{-3})	-	0.2609

Characterization of Corncob Powder

SEM analysis

The scanning electron micrographs of raw corncob powder (RCP) and base activated corncob powder (BACP) are shown in Figure 1. From SEM analysis, the base activated corncob powder (BACP) had more distinct features than the raw corncob powder (RCP).



(a)


(b)

Figure 1 Scanning electron micrographs of (a) raw corncob powder (RCP) and (b) base activated corncob powder (BACP)

Preparation of Different Types of Chitosan-Corncob Biocomposite Films

The various amount of base activated corncob powder (0.1, 0.2, 0.3, 0.4, and 0.5 g) were added to the chitosan solution (1.5 % w/v). The mixture solutions were stirred and heated for 30 min at 100 °C to get a homogeneous solution. These solutions were made into films by pouring onto a melamine shallow plates. These melamine plates containing the mixture solution were left for about three days at room temperature to obtain different types of chitosan-corncob biocomposite films. Then they were heated at 50 °C in an oven for about 6 h. All of the different types of chitosan-corncob biocomposite films were kept under dry conditions before further use.

Physicomechanical Properties of Chitosan-Corncob Biocomposite Films

The mechanical properties of chitosan-corncob biocomposite films are presented in Table 2 and Figure 2. The thicknesses of CC biocomposite films are approximately 0.20 to 0.34 mm. The composition of chitosan for preparing the biocomposite films was 1.5 % w/v and different weights of base activated corncob powder. In the concentration range of base activated corncob powder 0.1 g to 0.5 g, the tensile strength decreased slightly, but the maximum tensile strength was found to be 15.7 MPa for CC-2 film with CC content 0.2 g.

The more the elongation at break of a certain film, the higher the stability of the film. Therefore, based on the data of elongation at break, CC-2 film was the most suitable for the preparation of film. The elongation at break of corncob content 0.2 g was 5.2 %. The tear strength of chitosan-corncob CC biocomposite film increased significantly with progressively increase of corncob concentration up to 0.2 g and then it decreased in CC-3, CC-4 and CC-5 biocomposite films. The tear strength of CC-2 was 48.5 kN/m.

Table 2 Physicomechanical Properties of Chitosan Film and Chitosan-Corncob Biocomposite Films

Biocomposite Films	Weight of corncob powder (g)	Thickness (mm)	Tensile strength (MPa)	Elongation at Break (%)	Tear strength (kN/m)	Tensile strength/ elongation
C	-	0.17	18.8	6.0	51.5	3.13
CC-1	0.1	0.20	10.4	3.1	40.6	3.35
CC-2	0.2	0.21	15.7	5.2	48.5	3.02
CC-3	0.3	0.25	10.1	2.9	39.4	3.48
CC-4	0.4	0.31	6.28	2.3	35.7	2.51
CC-5	0.5	0.34	4.39	1.8	30.6	2.44

C = chitosan film with 1.5 % chitosan and 2 % acetic acid
CC-1 = composite film with 1.5 % chitosan + 2% acetic acid+ 0.1 g corncob
CC-2 = composite film with 1.5 % chitosan + 2% acetic acid+ 0.2 g corncob
CC-3 = composite film with 1.5 % chitosan + 2% acetic acid +0.3 g corncob
CC-4 = composite film with 1.5 % chitosan + 2% acetic acid +0.4 g corncob
CC-5 = composite film with 1,5 % chitosan + 2% acetic acid +0.5 g corncob

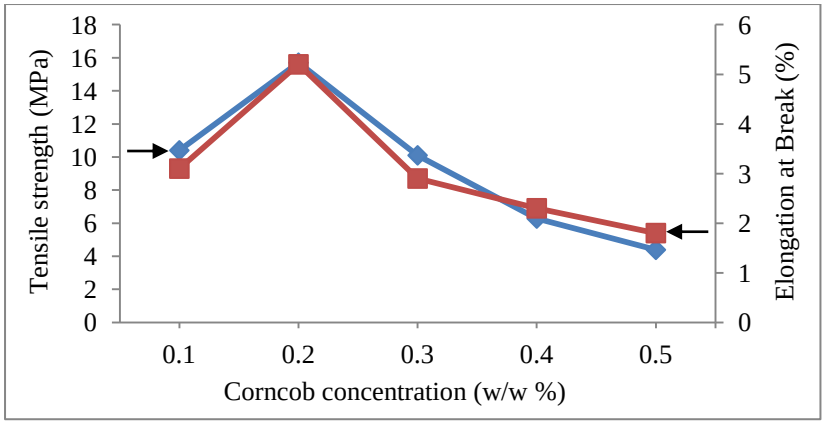


Figure 2 Tensile strength and elongation at break of chitosan-corncob biocomposite films as a function of different corncob concentrations

Preparation of Different Types of Chitosan-Corncob-Glutaraldehyde Biocomposite Films

Base activated corncob powder (0.2 g) was added to the chitosan solution(1.5 % w/v). The various concentrations of glutaraldehyde (6, 12, 18, 24, and 30 % of the total solid weight) were added to the above chitosan-corncob solutions. The mixture solutions were stirred and heated for 30 min at 100 °C to get a homogeneous solution. These solutions were kept for sufficient time to remove any bubble formation and were cast onto a cleaned and dried melamine plate at room temperature. The melamine plates containing the mixture solution were left for about three days at room temperature to obtain different types of chitosan-corncob-glutaraldehyde biocomposite films. Then they were heated at 50 °C in an oven for about 6 h. All of the different types of chitosan-corncob-glutaraldehyde biocomposite films were kept under dry conditions before further use.

Physicomechanical Properties of Chitosan-Corncob-Glutaraldehyde Biocomposite Films

The mechanical properties such as tensile strength, elongation at break and tear strength are important parameters which revealed the nature of the film. The mechanical properties of chitosan-corncob-glutaraldehyde biocomposite films are presented in Table 3 and Figure 3. The thicknesses of CCG biocomposite films are approximately 0.18 mm to 0.23 mm.

The tensile strength of prepared CCG biocomposite films decreased with increasing the amount of cross-linked glutaraldehyde beyond CCG-3. The value of tensile strength decreased from 19.5 MPa to 8.5 MPa when the amount of glutaraldehyde increased from 6 % to 30 %. The maximum tensile strength of 35.2 MPa was found at CCG-3 biocomposite film.

The elongation at break of CCG biocomposite films decreased from 5.4 % to 2.8 %. The percent elongation at break was found to be increased significantly at CCG-3 (ca 8.3 %) among the prepared CCG biocomposite films. The tensile strength and the percent elongation at break of CCG-3 biocomposite film were found to be higher than those of the other film. The tear strength of CCG-3 was found to be 59.8 kN/m.

Table 3 Physicomechanical Properties of Chitosan-Corncob-Glutaraldehyde Biocomposite Films

Biocomposite Films	Amount of glutaraldehyde (drops)	Thickness (mm)	Tensile strength (MPa)	Elongation at Break (%)	Tear strength (kN/m)	Tensile strength/ elongation
CCG-1	2	0.18	19.5	5.4	50.3	3.61
CCG-2	4	0.16	24.8	6.1	55.4	4.07
CCG-3	6	0.15	35.2	8.3	59.8	4.24
CCG-4	8	0.21	10.2	3.4	45.6	3.00
CCG-5	10	0.23	8.5	2.8	40.3	3.04

CCG-1 = chitosan-corncob biocomposite film with 6 % glutaraldehyde
CCG-2 = chitosan-corncob biocomposite film with 12 % glutaraldehyde
CCG-3 = chitosan-corncob biocomposite film with 18 % glutaraldehyde
CCG-4 = chitosan-corncob biocomposite film with 24 % glutaraldehyde
CCG-5 = chitosan-corncob biocomposite film with 30 % glutaraldehyde

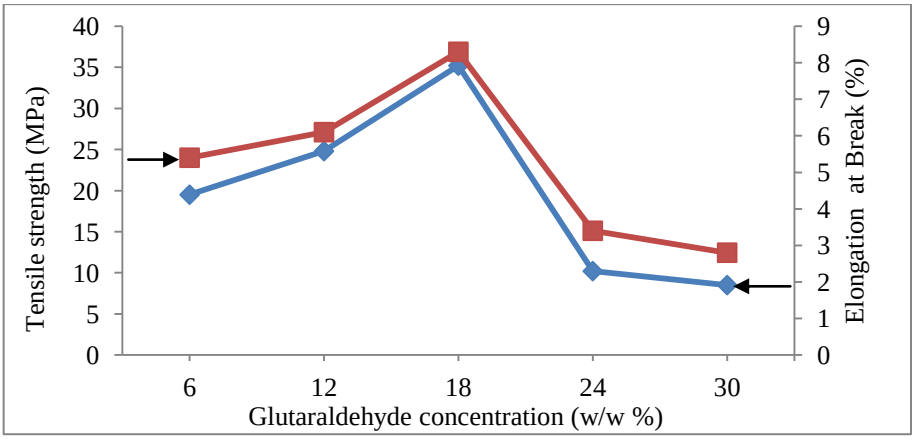


Figure 3 Tensile strength and elongation at break of chitosan-corncob-glutaraldehyde biocomposite films as a function of different glutaraldehyde concentrations

Degree of Swelling (%) of Chitosan-Corncob and Chitosan-Corncob-Glutaraldehyde Biocomposite Films

The degree of swelling of a series of chitosan-corncob (CC) biocomposite films as a function of immersion time in distilled water at room temperature are shown in Table 4 and Figure 4. According to the experimental data, the degree of swelling reached a mximum after 4 days. After 4 days of immersion time, the degree of swelling of CC biocomposite films decreased.

The degree of swelling of different chitosan-corncob-glutaraldehyde biocomposite (CCG) films with as a function of immersion time are also shown in Table 5 and Figure 5. For a given composition, mostly the degree of swelling increased with increasing immersion time. With increasing glutaraldehyde contents, the ultimate degree of swelling of CCG biocomposite films were found to decrease CCG-1 to CCG-5. Among them the degree of swelling of CCG-3 was found to be higher than that of others with respect to their immersion time. The CCG-3 biocomposite film had the most degree of swelling compared with the other CCG biocomposite films as a function of time and glutaraldehyde content. For CCG biocomposite film, the equilibrium degree of swelling was found to decrease from 85.77 to 62.33, as a function of

glutaraldehyde content. The degree of swelling was one of the essential parameters for determining the degradable biocomposite film. The values of degree of swelling of CCG biocomposite films were more than CC biocomposite films.

Table 4 Degree of Swelling (%) of Chitosan-Corncob (CC) Biocomposite Films Under Different Contact Time

No.	Biocomposite Films	Degree of Swelling (%)				
		1 h	2 h	3 h	4 h	4 days
1	CC-1	46.67	52.94	57.89	60.01	71.43
2	CC-2	50.01	68.33	71.54	94.29	105.25
3	CC-3	37.50	44.44	48.01	55.33	62.67
4	CC-4	30.52	39.15	45.37	53.65	59.43
5	CC-5	22.37	35.45	39.63	45.27	49.56

Table 5 Degree of Swelling (%) of Chitosan-Corncob-Glutaraldehyde (CCG) Biocomposite Films Under Different Contact Time

No.	Biocomposite Films	Degree of Swelling (%)				
		1 h	2 h	3 h	4 h	4 days
1	CCG-1	70.83	73.08	75.86	78.13	85.77
2	CCG-2	72.55	77.42	80.56	82.28	94.12
3	CCG-3	78.45	93.52	117.68	173.14	198.25
4	CCG-4	50.62	62.75	68.12	75.34	81.22
5	CCG-5	39.17	45.27	49.81	57.65	62.33

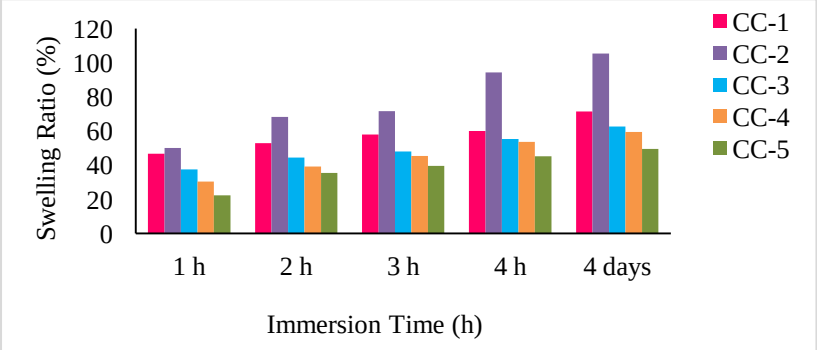


Figure 4 The effect of time on degree of swelling of chitosan-corncob (CC) biocomposite films

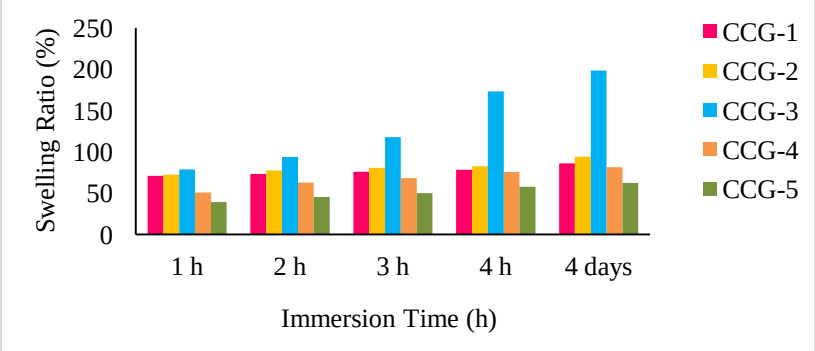


Figure 5 The effect of time on degree of swelling of chitosan-corncob-glutaraldehyde (CCG) biocomposite films

Characterization of Chitosan Film, Chitosan-Corncob Biocomposite Film and Chitosan-Corncob-Glutaraldehyde Biocomposite Film

SEM analysis

The scanning electron micrographs of chitosan (C) film, chitosan-corncob (CC-2) biocomposite film and chitosan-corncob-glutaraldehyde (CCG-3) biocomposite film are shown in Figures 6 (a), (b) and (c). The chitosan (C) film and chitosan-corncob (CC) biocomposite film have distinct phase structure. The chitosan-corncob-glutaraldehyde (CCG) biocomposite film had relatively smooth, homogeneous and continuous matrix without cracks with good structural integrity.

(a) (b) (c)

Figure 6 Surface morphologies of (a) C film (b) CC biocomposite film and (c) CCG biocomposite film

TG-DTA analysis

According to the TG-DTA thermogram profiles, the thermogram of chitosan (C) film is shown in Figure 7, having a weight loss in two stages. In the first stage, the temperatures range from 120 °C to 310 °C and the weight loss was 30.03 % due to the vaporization and elimination of volatile products. In the second stage, the weight loss started at 310 °C and that continued up to 600 °C where the weight loss was 19.42 % due to the degradation and decomposition of cellulose of chitosan polymer.

TG-DTA analysis also confirmed the chitosan-corncob (CC) biocomposite film in Figure 8. The first stage ranges between 39 °C and 120 °C with 11.31 % of weight loss due to the evaporation of absorbed water. The second stage of weight loss started at 120 °C and that continued up to 320 °C during which there was 36.36 % of weight loss due to the decomposition of cellulose and hemicellulose. The third stage in the loss in weight of about 30.68 % was actually observed from 320 °C to 600 °C due to the decomposition of lignin in corncob sample.

The nature regarding the thermogram profile of chitosan-corncob-glutaraldehyde (CCG) biocomposite film is presented in Figure 9. It indicated the two stages in weight loss. The percentage of weight loss was found to be 50.25 % at the temperature range between 120 °C and 320 °C. This weight loss was probably due to the vaporization and evaporation of volatile materials. The second stage in the loss in weight of about 33.52 % was observed within the temperature range of 320 °C up to 600 °C due to the decomposition of chitosan polymer.

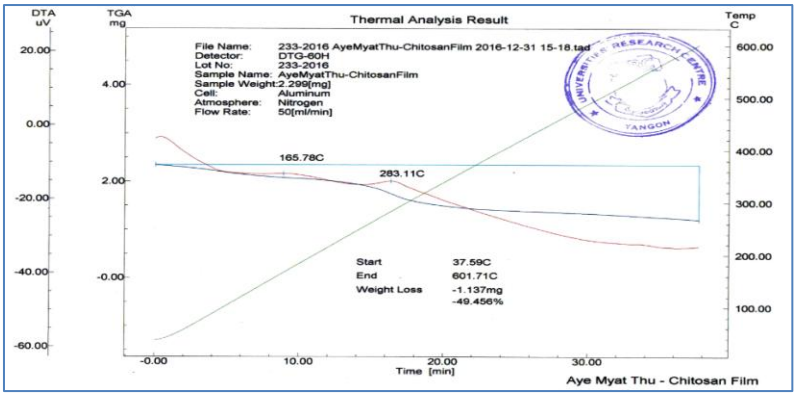


Figure 7 Thermal degradation analysis of pure chitosan film by TG-DTA analysis

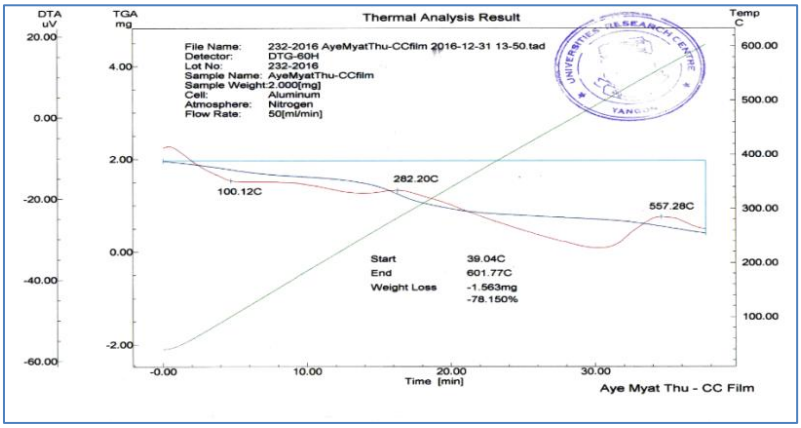


Figure 8 Thermal degradation analysis of chitosan-corncob biocomposite film by TG-DTA analysis

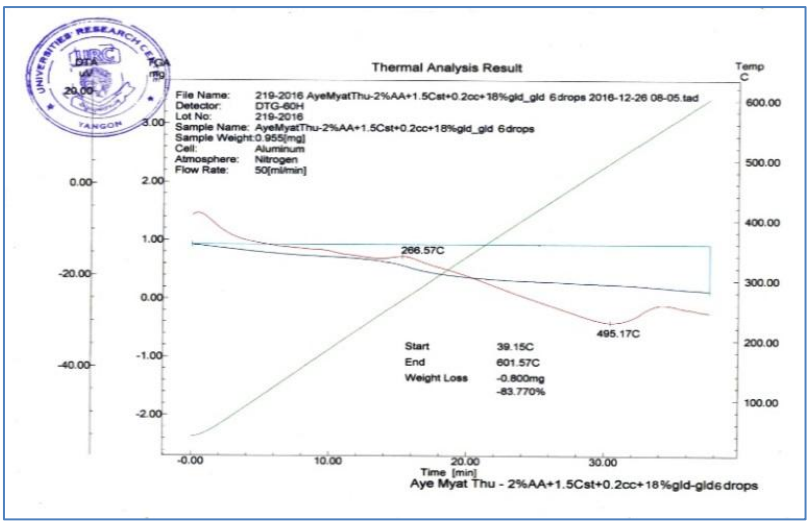


Figure 9 Thermal degradation analysis of chitosan-corncob-glutaraldehyde biocomposite film by TG-DTA analysis

Biodegradation of Biocomposite Films

In this work, biodegradation of chitosan (C) film, chitosan-corncob (CC) biocomposite film and chitosan-corncob-glutaraldehyde (CCG) biocomposite film were tested by soil burial method. Soil burial is a traditional way to test samples for degradation because of its similarity to actual condition of waste disposal. Uniformly sized samples were buried in the soil from

waste disposal. The physical parameters of the soil to be tested are presented in Table 6. From the physical parameters, the pH value of the tested soil is slightly greater than 7 and moisture content is 2.18.

Figure 10 shows biodegradation nature of C film, CC biocomposite film and CCG biocomposite film. These figures clearly show that significant deformation of C film, CC biocomposite film and CCG biocomposite film was found after two weeks although there was significant degradation of these films. However, quite deformation of C film, CC biocomposite film and CCG biocomposite film appeared after four weeks. Therefore, the prepared CC and CCG biocomposite film may be disappeared after short time duration by using burial method.

Table 6 Physical Parameters of the Tested Soil

Physical parameters	Soil
pH	7.64
Moisture (%)	2.18

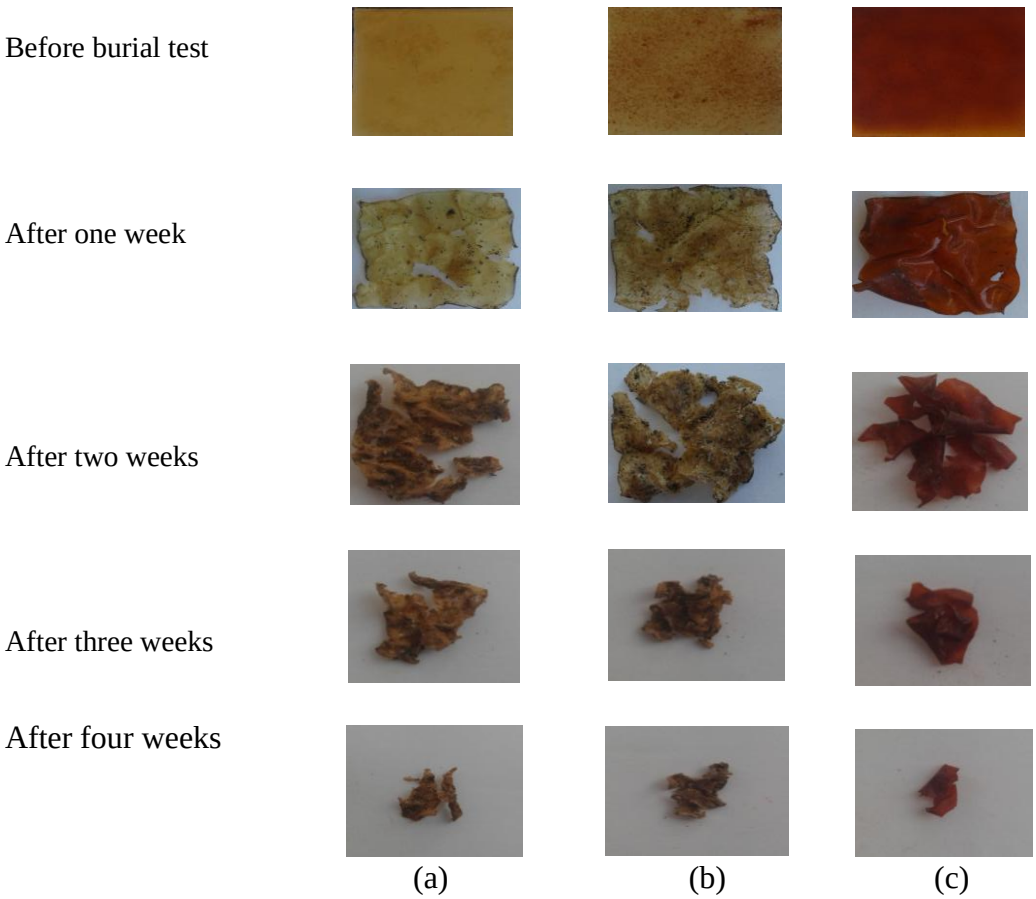


Figure 10 The physical appearances of (a) C film
(b) CC biocomposite film
(c) CCG biocomposite film

Conclusion

In this study, three types of films such as chitosan (C) film, chitosan-corncob (CC) biocomposite film and chitosan-corncob-glutaraldehyde (CCG) biocomposite film were prepared from the waste corncob with chitosan and various concentrations of cross-linking agent (glutaraldehyde) by solvent evaporating method. The effect of concentration of glutaraldehyde was studied over a range of concentrations from 6 % to 30 % (total solid weight). The optimum condition of CCG biocomposite film was achieved by using in the range of chitosan (1.5 %) and corncob (0.2 g) with glutaraldehyde (18 %). Based on the mechanical properties such as tensile strength (MPa), elongation at break (%) and tear strength (kN/m), the optimum condition was achieved by using in the range of 1.5 % chitosan and 0.2 g of corncob with different concentrations of glutaraldehyde. The CCG-3 biocomposite film provided the film with the highest mechanical strength (35.2 MPa), elongation at break (8.3 %), tear strength (59.8 kN/m) and film flexibility. The degree of swelling of biocomposite film had a lower degree of hydration, as measured by swelling ratio, which can be altered by varying weight percent of CC and CCG in the membrane matrix. From SEM analysis, the surface morphology of CC biocomposite film had distinct phase structure and CCG biocomposite film had homogeneous and smooth surface. From TG-DTA analysis, thermal stability of CC biocomposite film was found to be lower than that of CCG biocomposite film. According to the soil burial techniques, significant deformation and degradation of both CC biocomposite film and CCG biocomposite film occurred as the same as chitosan film. In addition, the prepared C film, CC biocomposite film and CCG biocomposite film can be disposed easily after using it, without environmental impact.

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ISOLATION OF SOME CHEMICAL CONSTITUENTS AND COMPARATIVE STUDY ON THE CURCUMIN CONTENT IN *CURCUMA LONGA* L. (NA-NWIN) COLLECTED FROM SOME REGIONS OF MYANMAR

Yu Nwe Moe[1], Khin Hnin Mon[2]

Abstract

In this study, *Curcuma longa* L. rhizomes were collected from six regions (Mawlamyine in Mon State, Kawkareik in Kayin State, Taungyi in Southern Shan State, Ann in Rakhine State, Aunglan in Magway Region and Pathein in Ayeyarwaddy Region) of Myanmar. Preliminary phytochemical investigation of *C. longa* revealed the presence of glycoside, carbohydrate, α-amino acid, flavonoid, terpenoid, steroid, saponin, tannin, phenolic compound. Alkaloid and reducing sugar were absent. The powdered samples were extracted with petroleum-ether (60-80 °C) and followed by 95 % ethanol. Percent curcumin and colour value were determined by UV–visible spectrophotometry. The percent curcumins and color values of *C. longa* were found to be 0.37 % and 62 in Mon State, 0.57 % and 96 in Southern Shan State, 2.27 % and 383 in Rakhine State, 2.94 % and 477 in Magway Region, 3.39 % and 570 in Ayeyarwaddy Region, and 3.8 % and 640 in Kayin State. Curcumin (R_f = 0.44, m.pt = 175 ºC) was isolated from ethanol extract of *C. longa* of Kayin State by column chromatography.

Keywords: *Curcuma longa* L., phytochemical, percent curcumin, colour value, curcumin

Introduction

Human diseases have been treated with plants for thousands of years still nowadays, many currently used medicines are derived from natural sources. *C. longa* requires warm and moist conditions. It can be cultivated in most areas of the tropics and subtropics (Ammon, 1991). It is an important herb and is widely used worldwide as medicine, condiment, dye and cosmetic (Jagan and Sakuriah, 2005). This plant is a perennial herb 60 – 100 cm high with a short stem and tufted leaves. Primary rhizomes are ovate, oblong, pyriform, denominated 'bulb' or round turmeric. The secondary rhizomes are more cylindrical and 4–7 cm long and 1– 1.5 cm wide, called 'fingers' (Eigner and Scholz, 1999). It has been used in traditional medicine as a remedy for various diseases including cough, diabeties and hepatic disorders. The content of rhizomes are very variable and depend on the site of cultivation, type of cultivar, moment of harvest method of processing and method of analysis (Ammon, 1991). *C. longa* is a group of phenolic compounds composed mainly of curcumin, demethoxycurcumin and bisdemethoxycurcumin (Paramasivam, 2008). Curcumin is the main chemical compound of *C. longa* and proven for its anti-inflammatory, anti-oxidant, anti-mutagenic, anti-diabetic, anti- bacterial, hepatoprotective, expectorant and anti-cancerous pharamacological activities. Curcumin has shown anti-proliferative effect in multiple cancers such as colon, skin, stomach, soft palate, tongue, sebaceous glands and breast (Gupta, 2010).

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C. longa (Na-nwin) (Figure 1) has been chosen for this research because it has various biological activities and bioactive chemical constituents. In this research work, screening of phytochemical constituents, comparison of percent curcumin and colour value and isolation of chemical constituents from crude extract of *C. longa* rhizome.

plant rhizome powder

Figure 1 The photograph of plant, rhizome and powder of *Curcuma longa* L.

Botanical Aspect of *Curcuma longa* L.

Family	Zingiberaceae
Genus	*Curcuma*
Species	*Curcuma longa*
Syn	*C. domestica* Valeton
Botanical name	*Curcuma longa* L.
Myanmar name	Na-nwin
English name	Turmeric (Chattopadhyay, 2004)

Materials and Methods

Collection of Sample

The rhizome of *C. longa* (Na-nwin) was collected from Aunglan in Magway Region, Pathein in Ayeyarwady Region, Taungyi in Shan State (South), Mawlamyine in Mon State, Kawkareik in Kayin State and Ann in Rakhine State.

Phytochemical Screening

Preliminary phytochemical investigation of *C. longa* tests such as alkaloids, glycosides, carbohydrates, α-amino acids, flavonoids, terpenoids and steroids, saponins, tannins, phenolic compounds and reducing sugars were carried out according to the appropriate reported methods (Harborne, 1993).

Determination of Percent Curcumin and Colour Value by UV-visible Spectroscopic Method

Preparation of authentic curcumin

25 mg of authentic curcumin was put in a 100 mL volumetric flask. It was dissolved and dilute with ethanol. Then 10 mL of above solution was taken in volumetric flask and again volume made up to 100 mL with ethanol. This authentic solution contains 2.5 mg (0.0025 g/L). Authentic curcumin solution was measured at 425 nm (Himesh, 2011).

Preparation of sample curcumin

0.1 g of dried powder sample was added with 25 mL ethanol and reflux for two and half hour. This solution was cooled and filtered the resulting filtrate was put in a 100 mL volumetric flask. It was dissolved and diluted with ethanol (100 mL). Then 10 mL of above solution was taken in a volumetric flask and again volume made up to 100 mL with ethanol. The absorbance was measured at 425 nm by using UV-1650 spectrophotometer (Himesh, 2011). The rhizome samples of six regions were separately prepared and then percent curcumin and colour value were determined. Percent curcumin and colour values were calculated by using following equation:

$$\text{Absorptivity of Curcumin} = A = \frac{a_1}{L \times C}$$

$$\% \text{ Curcumin sample} = \frac{a_1 \times 100}{L \times A \times m}$$

Where, a_1 = absorbance of authentic curcumin at 425 nm

a_2 = absorbance of extract at 425 nm

L = cell length in cm

C = concentration in gL^{-1}

m = mass in g of sample

colour value = $a_2 \times 1000$ (Himesh, 2011)

Isolation of Chemical Constituents from Crude extract of *Curcuma longa* L. (Rhizome)

Dried powdered rhizome sample of *C. longa* (100 g) was extracted with petroleum ether (250 mL), for 3 days at room temperature (3 times). The mixture was filtered with filter paper. The remaining residue was extracted with EtOH (200 mL) for 3 days at room temperature (3 times). This mixture was filtered with filter paper, and then, the resulting filtrate was evaporated in rotatory evaporator to volume reduce and then complete dryness. Resulting crude extract was weighed. Crude extract (0.21 g) was then subjected to column chromatography. The whole extract was dissolved in chloroform and throughly adsorbed on silica gel. The adsorbed material after being dried was transferred to column which was packed with 20 g of gel in chloroform. The column was eluted consecutively with chloroform: methanol (99:1, 600 mL). Collected each fraction was monitored by TLC using chloroform: methanol (99:1) as solvent system. The spot on TLC were visualized by spaying 5 % ferric chloride.

Determination of R_f Value of the Isolated Compound

R_f value of the isolated compound was determined by thin layer chromatogram using GF_{254}, (Merck) percolated silica gel on aluminum plate, as adsorbent and developed with suitable solvent systems. After the plate was dried, the R_f value of isolated compound was measured. Localization of spot was made by viewing directly under UV-254 nm and 365 nm lamp and or visualized by spraying with staining agent.

Determination of Melting Point

Isolated compound was individually introduced into capillary tubes. The tubes were placed on the Gallenkamp melting point apparatus and heating was started. The melting point was recorded when they started to melt.

Identification of the Isolated Compounds

The isolated compound was identified by FT IR and UV-visible spectroscopy.

Study on FT IR spectrum of the isolated compound

The infrared spectral of the isolated compound was recorded and examined whether the respective functional groups were presented or not by using FT IR spectrophotometer (Perkin Elmer Spectrum GX Fourier Transform in Infrared Spectrometer) at Department of Medical Research (DMR) in Yangon.

Study on the isolated compound by UV-Visible spectroscopy

For the identification of isolated compound, the ultraviolet observation spectrum of the isolated compound was recorded on Shimadzu UV-240, UV-visible spectrophotometer at Universities' Research Centre (URC).

Results and Discussion

Phytochemicals Present in *Curcuma longa* L.

Preliminary phytochemical analysis was performed in order to know different types of chemical constituent present in the plant samples of six regions. The types of chemical constituents were found to be identical in all regions. According to the results of preliminary phytochemical examination (Table 1), it indicated the presence of glycoside, carbohydrate, α-amino acid, flavonoid, terpenoid and steroid, phenolic compound, tannin, and saponins. However, alkaloid and reducing sugar were absent.

Table 1 Phytochemicals Present in *Curcuma longa* L.

No	Types of compounds	Extract	Test reagents	Observation	Remark
1	Alkaloid	1 % HCl	Mayer's reagent	no. ppt	-
			Dragendorff's reagent	no. ppt	-
			Wagner's reagent	no. ppt	-
2	Glycoside	H_2O	10 % lead acetate	ppt (white)	+
3	Carbohydrate	H_2O	10 % α-naphthol and conc: H_2SO_4	Redring	+
4	α-amino acid	H_2O	Ninhydrin reagent	Pink	+
5	Flavonoid	70 %EtOH	Mg turning and conc: HCl	Pink color	+
6	Terpenoids and steroid	PE	Acetic anhydride and conc: H_2SO_4	blue	+
7	Phenolic compound	H_2O	Ferric chloride (5 %)	blue	+
8	Tannin	H_2O	2 % NaCl and 1 % Gelatin	ppt (white)	+
9	Saponins	H_2O	Distilled water	frothing	+
10	Reducing Sugars	H_2SO_4 (dil)	NaOH (dil) and Benedict's solution	no ppt	-

(+) present, (-) absent, ppt = precipitate

Percent Curcumin and Colour Value of *C. longa* Rhizome

The percent curcumin and colour value of *C. longa* rhizome were determined by UV-visible spectrophotometry at 427 nm. The increasing order of percent curcumin contents and colour values from different regions are shown in Table 2.

From these data, the percent curcumin and colour value are the highest in *C. longa* rhizome from Kayin State and the lowest in that from Mon State, compared with other regions: Shan State (South), Rakhine State, Magway Region and Ayeyarwady Region.

Table 2 Quantitative Estimation of Curcumins and the Respective Colour Values in *Curcuma longa* L. (Na-nwin) from Some Regions of Myanmar

Sample	% Curcumin	Colour value
M	0.37	62
S	0.57	96
R	2.27	383
MG	2.84	477
A	3.39	570
K	3.80	640

M = the *C. longa* rhizome sample from Mon State
S = the *C. longa* rhizome sample from Shan Statez
R = the *C. longa* rhizome sample from Rakhine State
MG = the *C. longa* rhizome sample from Magway Region
A = the *C. longa* rhizome sample from Ayeyarwady Region
K = the *C. longa* rhizome sample from Kayin State

Identification of the Compound Isolated from the Crude extract

From the silica gel column chromatographic separation of crude (ethanol) extract of the rhizome of *C. longa*, a compound was isolated as orange needles in 0.6248 % of yield based of raw material. The R_f values of compound were found to be 0.44 and 0.34 ($CHCl_3$: MeOH, 99: 1 v/v and PE: EA, 1: 1 v/v) and identified with that of authentic curcumin and both of these compounds also gave the same behaviours on TLC. In addition, the melting point of isolated compound was observed to be 175 ºC and in literature curcumin is 177 °C (Merck Index, 2001). According to iodine vapour test and 5 % ferric chloride test, brown colour was observed, indicating that phenolic OH groups were present in curcumin. All of these physicochemical properties of compound leads to assign it as curcumin. After characterization, the isolated compound was generally classified and then was identified by FT IR and UV-visible spectroscopy.

Identification of the Isolated Compounds by FT IR and UV-Visible Spectroscopy

According to FT IR spectrum and spectral data, the broad bands at 3600 ~ 3317 cm^{-1} were due to –OH stretching vibration of phenolic hydroxyl groups. Aliphatic C–H stretching vibrations of CH_3 and CH_2 groups were found at 2924 and 2854 cm^{-1}, respectively, carbonyl stretching vibration of keto-enol system was observed at 1620 cm^{-1} and C=C stretching vibrations of aromatic ring system were found at 1597 cm^{-1}, 1504cm^{-1} and 1427 cm^{-1}, a sharp and strong signal at about 1273 cm^{-1} due to =CH in plane deformation. Two sharp and strong signal at about 1195 cm^{-1} due to C=O bending. Asymmetric and symmetric stretching vibrations of C–O–C group were found at 1026 cm^{-1}. Out of the plane bending vibration of trans olefinic C–H was assigned at 964 cm^{-1} and that of aromatic C–H were at 864 cm^{-1} and 810 cm^{-1}. Figure 3 represents FT IR spectrum of curcumin. The corresponding spectral data are summarized in Table 3. Ultraviolet spectrum of curcumin measured in EtOH solvent was illustrated in Figure 4. The wavelength of maximum absorption was observed at 427.86 nm, indicating the presence of conjugated double bond and found to be similar with the literature value (422 nm) (*Kasuge *et al*., 1985).

From the observation of physicochemical properties and spectroscopic evidences, the isolated compound was identified as curcumin.

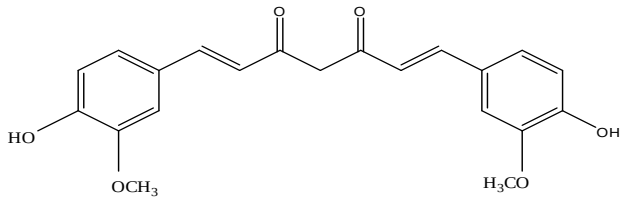

Figure 2 Structure of curcumin

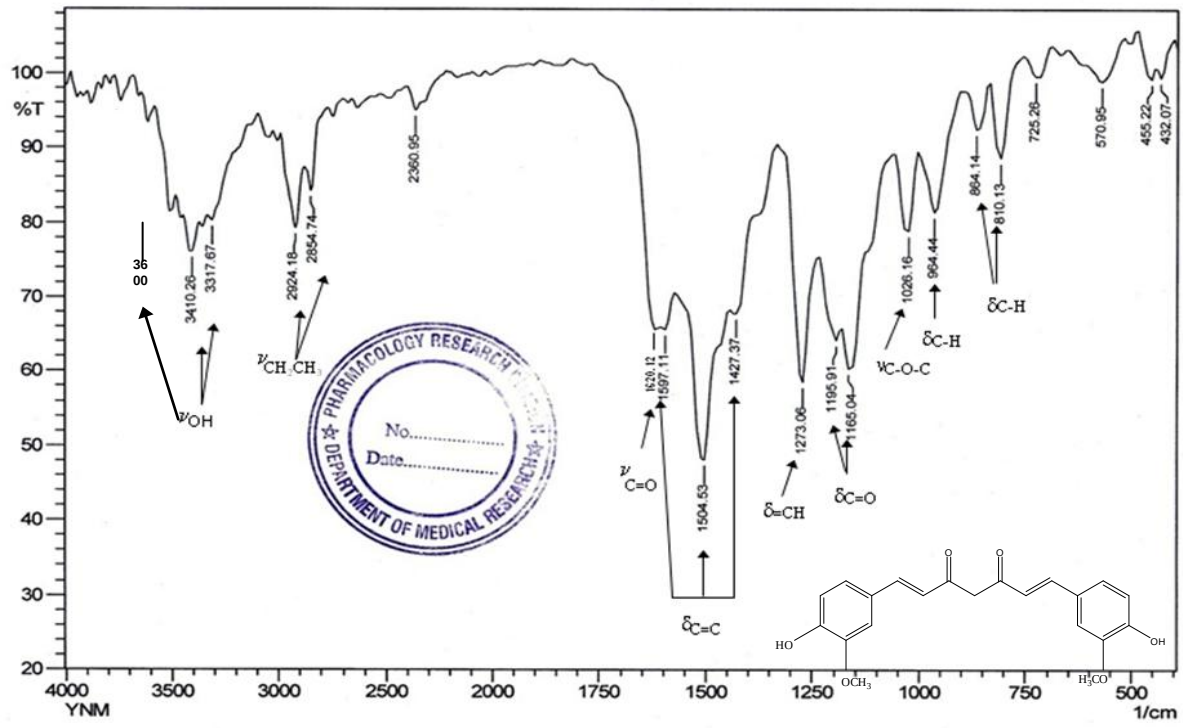

Figure 3 FT IR spectrum of the isolated compound

Table 3 FT IR Spectral Data of the Isolated Compound

Wave number (cm^{-1})		Assignment
Literature*	**Observed**	
3603	3600, 3317	OH stretching of phenolic –OH group
2943, 2827	2924, 2854	Aliphatic C–H stretching of CH_3 and CH_2 group
1628	1620	C=O stretching of keto-enol group
1603	1597	Aromatic C=C ring stretching
1509	1504	
1429	1427	
1283	1273	=CH in plane deformation
-	1195	C=O bending
1026	1026	Asymmetric and symmetric stretching of C–O–C group
962	964	C–H out of plane bending of trans olefinic group
856	864	Aromatic C–H out of plane bending
814	810	

(*Than Soe, 1984)

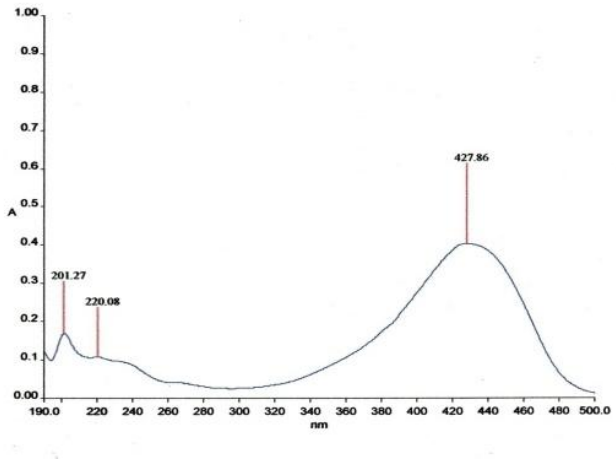

Figure 4 UV spectrum of the isolated compound (ethanol)

Conclusion

From the overall assessment of present work, the following inferences can be deduced.

In preliminary phytochemical investigation, glycoside, carbohydrate, α-amino acid, flavonoid, terpenoid and steroid, saponin, tannin, phenolic compound were present and alkaloid and however reducing sugars were not detected in the sample.

Percent curcumin and colours were determined by UV–visible spectrophotometry from powder samples. The percent curcumins and colour values were found to be 3.8 % and 640 in Kayin State > 3.39 % and 570 in Ayeyarwady Region > 2.94 % and 477 in Magway Region > 2.27 % and 383 in Rakhine State >0.57 % and 96 in Shan State > 0.37 % and 62 in Mon State from *C. longa* respectively.

One organic compound, curcumin (orange needles, 0.6248 %, R_f-0.44, mpt. 175 °C) was major chemical constituent from crude extract of the rhizome by using silica gel column chromatographic separation technique.

According to my research findings, curcumin content and color value were found to be highest in Kayin state and that of Mon State sample are the lowest. With these results, Na-nwin from Kayin State can be used as medicinal purpose, especially cancer diseases.

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ISOLATION OF SOIL FUNGI FROM MINHLA AREA, MAGWAY REGION AND SCREENING ON THE ANTIMICROBIAL ACTIVITY FROM SOIL FUNGI

Myint Thu[1], Ye Myint Aung[2], Zar Zar Yin[3]

Abstract

Three different soil samples were collected from Minhla Area, Magway Region during July 2018. A total of 15 fungal strains were isolated by serial dilution method from these soil samples. These strains were cultured on Blaskeslee's Malt Extract Agar (BMEA Medium), Czapek-Dox Agar (CZA Medium), Malt Extract Agar (MEA Medium), Dichloran Rose Bengal-Chloramphenicol Agar (DRBC Medium), Glucose Ammonium Nitrate Agar (GAN Medium) and Potato Dextrose Agar (PDA Medium). Pure colonies were preserved on slant culture containing PDA Medium. Among all of media, BMEA medium and PDA medium are better for isolation of soil fungi than other media. In the colony morphology, the isolated fungi were small, medium and large in size. The margin of isolated fungi were entire, undulate, and the elevation of isolated fungi were raised, convex, flat. In the form, isolated fungi were circular, irregular and filamentous. Moreover, physicochemical properties of soil from different locations of Minhla Area were analyzed. Furthermore, all fungal strains were tested by eight test organisms for the antimicrobial activity. Especially, the isolated fungi YY- 6, 11, 14 and 15 showed the antimicrobial activity against all test organisms. Among them, YY-6 showed the highest antimicrobial activity (32.82 mm and 31.47 mm) on *Candida albicans* and *Escherichia coli*, respectively.

Keywords: Soil fungi, serial dilution method, physicochemical properties, antimicrobial activity

Introduction

Soils are very complex, having numerous constituents performing different functions mainly due to the activity of soil organisms (Ullah *et al.,* 2017). The soil quality is determining by microbial composition and functioning changes during decomposition of organic matter, recycling of nutrients and biological control (Stefanis *et al*., 2013).

The microorganisms plays significant role in soil ecosystem. Fungi are very vital for the soil ecosystem since they play a key role in different essential processes including elemental release by mineralization and organic matter decomposition (Christensen, 1989). Moreover, the fungi are responsible for the decomposition of organic compounds and their activity contributes in the bio-deterioration and biodegradation of toxic substances in the soil (Rangaswami and Bagyaraj, 1998).

Fungi among other soil microorganisms are important in both of the formation and stabilization of soil aggregates. Fungi produce many antibiotics, having antibacterial and antifungal activity, which are widely used as drugs over the world especially the penicillin, cephalosporin and fusidic acid (Dobashi *et al.,* 1998). The recent decades are characterized by the novel discoveries of microorganisms capable of producing compounds, as a potential source of new antibiotics (Ullah *et al.,* 2017). Due to the importance of fungi, this research aimed to obtain the fungal species that could be isolated from soil. Fungi are not only beautiful but play a

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significant role in the daily life of human beings besides their utilization in industry, agriculture, medicine, food industry, textiles, bioremediation, natural cycling, as biofertilizers and many other ways. Fungal biotechnology has become an integral part of the human welfare (Karthikeyan *et al.*, 2014). The aim of the present work is to analyze physicochemical properties of soil from Minhla Area, to study the cultural characteristics of isolated soil fungi on six different culture media and to determine the preliminary antimicrobial activity of isolated fungi.

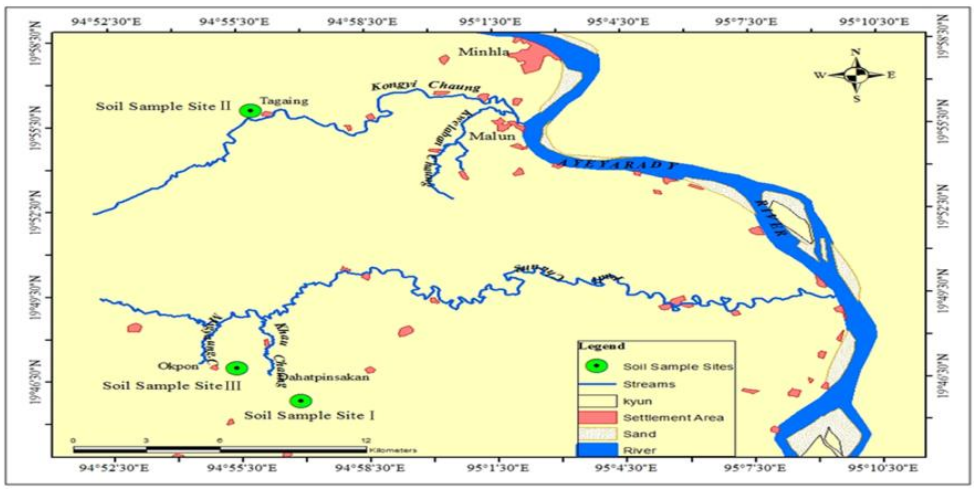
Materials and Methods

Collection of Soil Samples

The soil samples that collected from three different places (Table 1 and Figure 1) were utilized for the isolation of microorganisms especially fungi. The soil type as its some physicochemical properties was analyzed by Department of Agriculture (Land Use), Insein Township, Yangon Region.

Table 1 Collected Soil Samples from Three Different Places of Minhla Area

Sample No.	Place	Soil type	pH	Location
1	Dahatpin Village	Clay Loam	7.23	N 19º 46' 57.588" E 094˚ 55' 23.721"
2	Tagaing Village	Sandy Clay Loam	8.38	N 19º 56' 03.911" E 094˚ 55' 49.429"
3	Dahatpin Village	Clay	7.24	N 19º 45' 46.891" E 094˚ 56' 52.518"



Source: Department of Geography, Pathein University

Figure 1 Map of soil collection (Minhla Area)

Isolation of Fungi from Soil Sample by Serial Dilution Method

Soil samples (1 g each) were introduced into a conical flask containing 99 mL of distilled water. The flask was then shaken for about 30 min in order to make the soil particles free from each other. This solution was then serial diluted from 10^{-3} to10^{-7} dilution in separate test tubes and 0.5 mL each of the above dilution was separately transferred into sterile petridishes under aseptic condition. The sterilized culture medium in conical flask was cooled down to about 45ºC and separately poured into each of the petridish containing the respective soil dilutions. The inoculated plates were moved clock-wise and anticlock-wise direction as to make uniform distribution of the fungi inoculums. When the agar was solidified, the inoculated plates were inverted and incubated at room temperature for 3-6 days. Isolated pure fungi were preserved into slant culture containing PDA medium for further experimentations (Dubey and Maheshwari, 2002).

Inoculation Method

The working benches in the laboratory were thoroughly swapped methylated sprit soaked in cotton wool, and also a burning blue flame was allowed to sterile the surrounding air before the inoculation proper. The conical flasks were corked tightly with cotton wool and the petri were fully autoclaved (Ania *et al.,* 2011).

Lacto Phenol Cotton Blue Teased Mount (LPCB-TM)

Staining of the Soil Fungi

Fungal morphology were studied macroscopically by observing colony features (Colour and Texture) and microscopically by staining with lacto phenol cotton blue and observed under microscope for the conidia, conidiophores and arrangement of spores. Firstly, one drop of Lacto phenol Cotton Blue was placed on the slide. By using a sterile needle, a tiny piece of the colony was transferred into the lacto phenol Cotton Blue on the slide and fungal colony was carefully teased into very tiny pieces preparation using sterile needles. The prepared slide was covered with a cover slip. Finally, fungal spores were observed under microscope using 10×and 40× objective lens. Lacto phenol Cotton Blue is a stain used for making semipermanent microscopic preparation of fungi. Phenol kills any organism. Lactic acid preserves fungal structures, and acts as a clearing agent. Cotton blue stains the chitin and cellulose of the fungal cell wall intensely blue. Glycerol prevents drying (Larone, 1995).

Screening of Effective Soil Fungi by Agar Well Diffusion Method

The isolated fungi were grown on PDA medium at room temperature for 5 days. Isolated strains were tested by agar well diffusion method for the preliminary antimicrobial activities. Cork borer was used to make the wells (8 mm in diameter) in the autoclaved basal antimicrobial test-medium. Wells impregnated with 3-6 days old culture fermented broth (20 µL) were incubated at room temperature for 24-28 h. After 24-28 h of incubation, the clear zones were measured. Therefore, the diameter of clear zones had been observed as potent activity as shown by respective strain. Clear zones surrounding the wells indicated the presence of antimicrobial activities which inhibit the growth of the test organisms selectively. In this study eight pathogenic microorganisms (Table 2) were utilized for antimicrobial activity (Collins, 1965).

Table 2 Eight Kinds of Test Organisms Used for Antimicrobial Activity (NITE, PRD and UY)

Test No.	Test Organisms	Diseases
1	*Escherichia coli* AHU5436	Diarrhoea, abdominal pain
2	*Bacillus subtilis* IFO 90571	Fever
3	*Bacillus pumilus*IFO 90571	Wound and burn infection
4	*Candida albicans* NITE 09542	Candidasis, skin disease
5	*Pseudomonas fluorescens* IFO94307	Septicemia
6	*Staphylococcus aureus* AHU8465	Boil and food poisoning
7	*Agrobacterium tumefaciens* NITE 09678	Crown gall disease
8	*Malassezia furfur* UY	Dandruff, Seborrhoeic dermatitis

NITE = National Institute of Technology Evaluation
PRD = Pharmaceutical Research Development
UY = University of Yangon

Results and Discussion

Physicochemical Properties of Soil Samples

In present study, soil samples were collected from 0-15 cm depth after removing the surface soil for the isolation of fungi. The results obtained from this study has shown to be similar with the research conducted by Tangjang *et al*., 2009 where they found out that there was greater amounts of bacterial and fungal populations in the top soil (0-10 cm) if compared to that of other depths. This might be due to the higher organics contents found in the top soil where humus is abundantly presence, especially for the forest floor that is often covered by wilted leaves that tend to decompose.

Physicochemical analysis of three different soils from Minhla area revealed moisture and organic contents. The colour of soil samples was pale red and red with variation in pH (7.23-8.38). Most soil microorganisms and plants prefer a near-neutral pH range of 6 to 7 because the availability of most soil nutrients is best in this pH range (Atlas and Bartha, 1998). Neutral to alkaline soils provided favorable conditions for the growth of fungi (Rashmi, 2005). The temperature of soil environments of Minhla area at the time of this investigation (rainy season) revealed that the soil environment of this Township had temperature range between 30˚C to 35˚C. Moita *et al*., 2005 reported that temperature between 27 to 34˚C and pH 6.0 favoured cell growth. The soil moisture measured at each Dahatpin village and Tagaing village were (3.53-6.16%), organic carbon (0.66 - 1.06 %), organic nitrogen (0.11- 0.15 %), humus (1.14-1.83 %) respectively. Soil moisture is essential for soil microorganisms, without some water; there is no microbial activity (Sylvia *et al.*, 2005).

The soil physical factors as moisture content, texture and structure, which affect soil aeration, have a profound influence on fungal activity (Griffin, 1963). Organic matter in an ecosystem is frequently the limiting factor for growth of heterotrophic microorganism, nitrogen is required for protein formation, in addition to being required for protein synthesis, ammonium or nitrite is oxidized by some microorganisms for generating energy (Atlas and Bartha, 1998).These results are shown in Table 3.

Isolation of Fungi

In the investigation of fungi, 15 fungi were obtained and seven fungi were isolated from soil sample 1, one fungus from soil sample 2, and seven fungi from soil sample 3. The isolated fungi were designated as YY-1 to YY-15**.** These results are shown in Table 3.

Table 3 Some Physicochemical Properties of the Isolated Soil Fungi

Sample No.	Place	Texture	pH	T(˚C)	Moisture (%)	Organic carbon (%)	Organic Nitrogen (%)	Humus (%)	Isolated Fungi
1	Dahatpin Village	CL	7.23	30	4.98	1.06	0.15	1.83	YY-1,2,3,4,5, 6,7
2	Tagaing Village	SCL	8.38	35	3.53	0.92	0.11	1.59	YY-8
3	Dahatpin Village	C	7.24	30	6.16	0.66	0.11	1.14	YY-9,10, 11,12,13,14,15

*__CL__-clay loam **SCL**- sandy clay loam **C**- clay

Colony Morphology of the Isolated Soil Fungi

In the present study, 15 fungal strains were isolated from three different samples collected from Minhla area. The fungi have been isolated by using soil serial dilution method and six culture media. The surface colour of isolated fungi YY-1 to YY-15 were white, gray, pale yellow, pale green, yellow, pale red, black, pinky white, and their reverse colour were cream, pale yellow, pale brown, white, gray, red, deep yellow and yellow. In the colony morphology, the isolated fungi were small, medium and large in size. The margin of isolated fungi were entire, undulate, and the elevation of isolated fungi were raised, convex, flat and the form of isolated fungi were circular, irregular and filamentous. Tin Tin Hla (2017) reported that a total of 35 fungi were isolated from seven different soil samples and their colony color were red, green, gray, white, yellow, brown, pink and black. In this study, six culture media were used and isolated fungi were better growth on BMEA medium and PDA medium than other culture media. Ando *et al.* (2004) reported that many fungi grow robustly on BMEA medium. Agrios, (1988) described that PDA is general medium most widely used in the isolation of fungi, having a complete nutritional basis and this is probably the reason why colony development was faster with respect to other media. Earlier work reported maximum growth of fungi, potato Dextrose was most favourable (Maheshwari *et al*., 2000). These results are shown in Table 4.

Table 4 Colony Morphology of the Isolated Soil fungi

Strain No.	Surface color	Reverse color	Form	Elevation	Margin	Size of colony
YY-1	Pale yellow	Pale yellow	Circular	Flat	Undulate	Small
YY-2	White	Cream	Circular	Raised	Entire	Large
YY-3	White	Pale yellow	Circular	Flat	Entire	Large
YY-4	White	Cream	Circular	Raised	Entire	Large
YY-5	Gray in the center White in the edge	Cream	Filamentous	Convex	Entire	Large
YY-6	White	Pale yellow	Irregular	Flat	Undulate	Medium
YY-7	White	Pale yellow	Circular	Flat	Entire	Large

Strain No.	Surface color	Reverse color	Form	Elevation	Margin	Size of colony
YY-8	White	Pale yellow	Circular	Flat	Entire	Large
YY-9	Pale green	Yellow	Circular	Convex	Entire	Large
YY-10	White	White	Circular	Flat	Entire	Large
YY-11	Green in the center White in the edge	Cream	Circular	Raise	Entire	Large
YY-12	White	White	Circular	Flat	Entire	Large
YY-13	Pale green	Yellow	Circular	Flat	Entire	Medium
YY-14	Yellow	Pale yellow	Circular	Raised	Entire	Medium
YY-15	Gray in the center White in the edge	Cream	Circular	Raised	Entire	Large

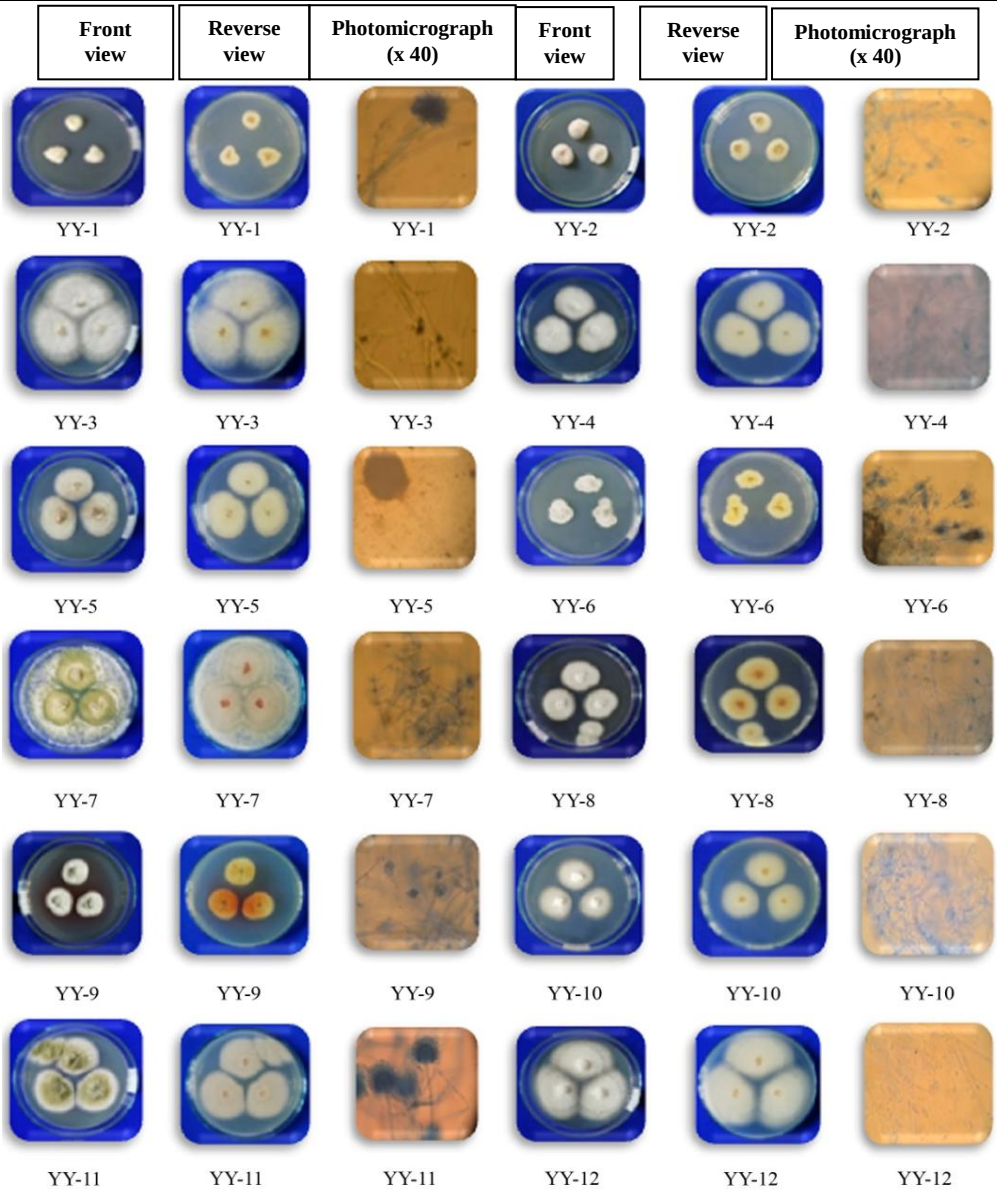


Figure 2 Morphologies of the isolated soil fungi on BMEA medium

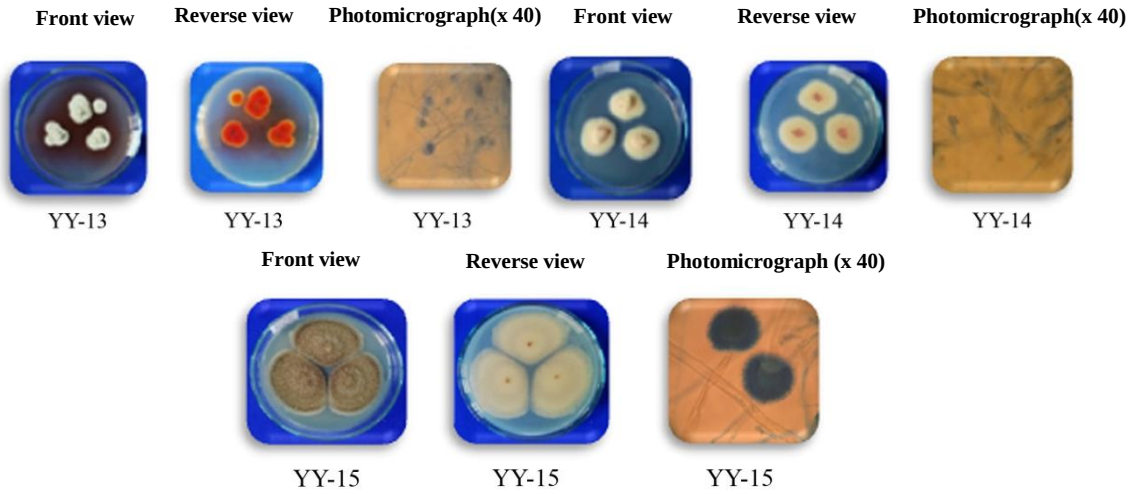


Figure 3 Morphologies of the isolated soil fungi on BMEA medium

Antimicrobial Activities of the Isolated Fungal Strains

All fungal strains were tested by eight test organisms for preliminary study of antimicrobial activities. Among them, four strains showed different level of antimicrobial activity. Especially, YY-6 and 11 showed the antimicrobial activity against all test organisms.

Among them, YY-6 showed the highest antimicrobial activity (32.82 mm and 31.47 mm) on *C. albicans* and *E. coli* respectively. Moreover, YY-6 showed the highest antifungal activity (31.90 mm) on *M. furfur*. According to Dulmage and Rivas 1978, soil microorganisms have continually been screened for their useful biological active metabolites, such as antibiotic since long ago. Brooks, 2001reported that antibiotics are classified as broad-spectrum antibiotics when they have the ability to affect a wide range of gram-positive and gram-negative bacteria while antibiotic that only effective towards certain group of bacteria are known as narrow spectrum antibiotics. Therefore, YY-6 strain was broad-spectrum antibiotics. These results are shown in Tables 5-12 and Figures 4-11.

Table 5 Antibacterial Activities of the Isolated Fungal Strains against *E. coli*

Isolated Fungi	Fermentation period (Days) and Inhibitory Zone (mm)							
	3 -days	4-days	5-days	6-days	7-days	8-days	9-days	10-days
YY-1	-	-	-	-	-	-	-	-
YY-2	-	-	-	+	+	12.81	20.28	-
YY-3	-	-	-	12.35	13.46	18.82	12.14	12.35
YY-4	-	-	-	+	+	+	14.21	-
YY-5	-	13.23	14.33	14.48	12.38	12.37	11.70	+
YY-6	19.73	24.05	27.04	30.99	31.47	31.03	27.08	24.21
YY-7	-	-	+	+	+	12.55	15.28	15.82
YY-8	-	-	+	+	+	+	+	-
YY-9	-	-	-	+	+	+	+	-
YY-10	-	-	-	+	+	+	+	-
YY-11	13.54	15.71	16.24	16.40	20.58	20.04	19.30	16.97
YY-12	-	-	-	11.99	12.84	12.22	+	-
YY-13	11.60	16.26	15.14	15.11	13.30	11.91	10.62	-
YY-14	17.24	16.56	15.57	15.55	15.29	15.19	12.48	12.34
YY-15	13.34	15.02	15.16	16.53	15.46	15.30	14.05	13.32

(+) present (-) No activity Agar well =8mm

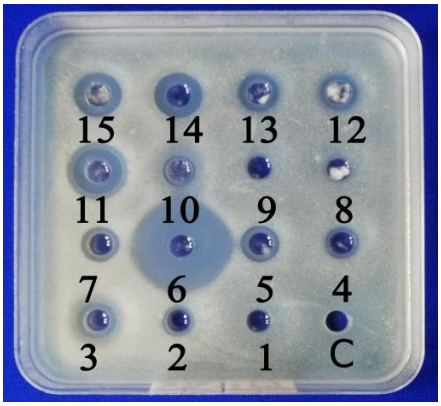


Figure 4 Antibacterial activity of the isolated fungal strains against *E. coli*

Table 6 Antibacterial Activity of the Isolated Fungal Strains against *B. subtilis*

Isolated Fungi	Fermentation period (Days) and Inhibitory Zone (mm)							
	3 - days	4- days	5- days	6- days	7- days	8- days	9- days	10- days
YY-1	-	-	+	13.55	+	+	-	-
YY-2	-	-	12.62	16.91	15.93	15.81	15.73	-
YY-3	-	-	+	14.54	15.29	15.21	13.52	-
YY-4	-	12.16	12.28	15.17	17.60	13.40	12.98	-
YY-5	-	-	+	14.04	12.50	12.24	+	-
YY-6	-	-	+	13.70	14.42	+	+	-
YY-7	-	-	+	+	+	+	-	-
YY-8	-	-	+	13.51	+	+	+	-
YY-9	-	-	+	13.53	+	+	+	-
YY-10	-	-	+	13.91	+	+	+	-
YY-11	-	-	+	17.26	13.81	+	+	-
YY-12	-	-	+	16.55	17.44	15.62	15.20	-
YY-13	-	-	+	12.95	13.99	13.29	+	-
YY-14	-	11.27	12.02	13.45	14.33	13.70	+	-
YY-15	-	12.25	12.70	13.71	15.24	14.46	+	+

(+) present (-) No activity Agar well =8mm

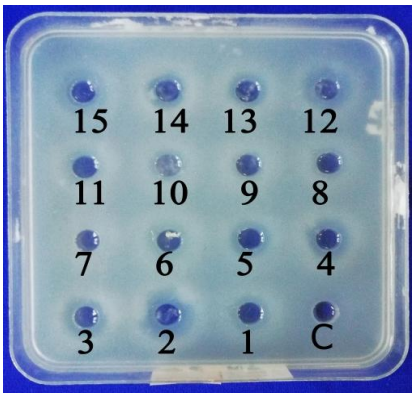


Figure 5 Antibacterial activity of the isolated fungal strains against *B. subtilis*

Table 7 Antibacterial Activity of the Isolated Fungal Strains against *B. pumilus*

Isolated Fungi	Fermentation period (Days) and Inhibitory Zone (mm)							
	3 -days	**4-days**	**5-days**	**6-days**	**7-days**	**8-days**	**9-days**	**10-days**
YY-1	-	-	-	-	-	-	-	-
YY-2	-	-	+	10.68	13.50	11.96	11.53	-
YY-3	17.56	12.38	+	+	-	-	-	-
YY-4	-	12.22	11.84	+	+	-	-	-
YY-5	-	-	12.48	13.94	12.81	10.95	-	-
YY-6	+	25.69	29.60	29.71	30.25	29.69	25.40	22.06
YY-7	14.63	14.01	11.56	+	-	-	-	-
YY-8	-	-	-	-	-	-	-	-
YY-9	-	-	-	-	-	-	-	-
YY-10	-	-	-	-	+	+	-	-
YY-11	-	-	15.18	18.49	18.80	19.60	17.82	14.44
YY-12	-	-	-	12.38	13.43	12.58	+	+
YY-13	-	-	-	11.20	11.82	12.16	14.13	10.58
YY-14	11.35	12.10	12.77	16.03	14.92	13.45	13.18	12.83
YY-15	11.87	13.53	15.44	16.75	16.81	14.51	12.88	12.21

(+) present (-) No activity Agar well =8mm

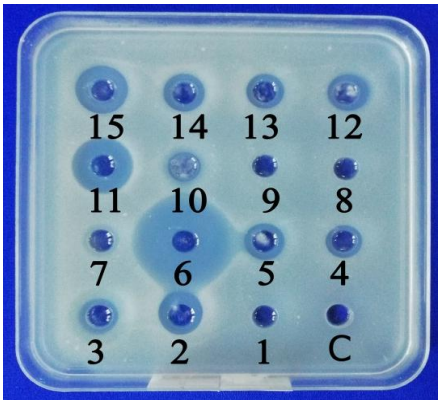


Figure 6 Antibacterial activity of the isolated fungal strains against *B. pumilus*

Table 8 Antifungal Activity of the Isolated Fungal Strain against *C. albicans*

Isolated Fungi	Fermentation period (Days) and Inhibitory Zone (mm)							
	3 -days	4-days	5-days	6-days	7-days	8-days	9-days	10-days
YY-1	-	-	-	-	-	-	-	-
YY-2	-	-	-	11.04	11.73	12.86	11.86	-
YY-3	-	16.15	20.99	18.76	15.94	15.58	14.31	-
YY-4	-	+	11.65	11.89	11.87	11.75	+	-
YY-5	-	+	13.10	13.72	14.02	13.37	12.19	12.01
YY-6	-	20.79	22.49	29.40	30.03	32.82	28.75	21.64
YY-7	-	-	-	-	-	-	-	-
YY-8	-	-	-	-	-	-	-	-
YY-9	-	-	-	-	-	-	-	-
YY-10	-	-	-	13.92	10.96	-	-	-
YY-11	-	18.19	19.36	20.57	16.73	16.23	15.72	+
YY-12	-	-	-	12.05	13.25	12.74	+	-
YY-13	-	11.59	12.16	14.39	12.92	12.65	12.35	-
YY-14	-	+	15.53	16.22	17.39	14.81	14.45	13.72
YY-15	-	+	16.70	17.23	16.96	15.39	14.37	14.11

(+) present (-) No activity Agar well =8mm

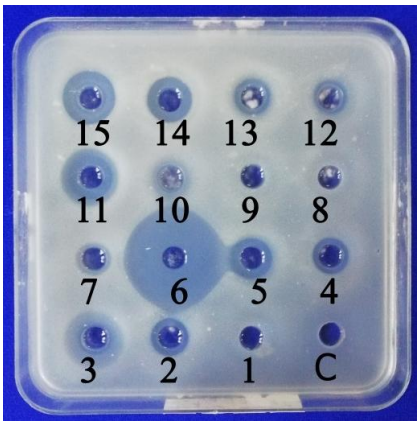


Figure 7 Antifungal activity of the isolated fungal strains against *C. albicans*

Table 9 Antibacterial Activity of the Isolated Fungal Strains against *P. fluorescens*

Isolated Fungi	Fermentation period (Days) and Inhibitory Zone (mm)							
	3 -days	4-days	5-days	6-days	7-days	8-days	9-days	10-days
YY-1	-	-	-	-	-	-	-	-
YY-2	-	-	13.30	12.02	11.83	11.79	+	+
YY-3	-	-	-	11.33	19.12	13.23	+	+
YY-4	-	11.75	12.58	11.93	11.89	11.24	+	-
YY-5	-	-	-	-	11.34	12.93	11.82	+
YY-6	-	+	+	21.97	24.41	27.39	28.33	27.25
YY-7	-	-	-	-	-	10.25	13.04	15.36
YY-8	-	-	-	-	-	-	-	-
YY-9	-	-	-	-	-	-	-	-
YY-10	-	-	-	-	+	13.51	+	-
YY-11	-	-	+	15.50	20.90	21.07	19.40	19.05
YY-12	-	-	-	+	+	12.09	+	-
YY-13	-	-	-	+	+	12.04	13.28	+
YY-14	-	11.77	13.29	15.59	14.38	13.43	12.33	+
YY-15	-	+	14.39	14.58	16.02	14.61	14.85	+

(+) present (-) No activity Agar well =8mm

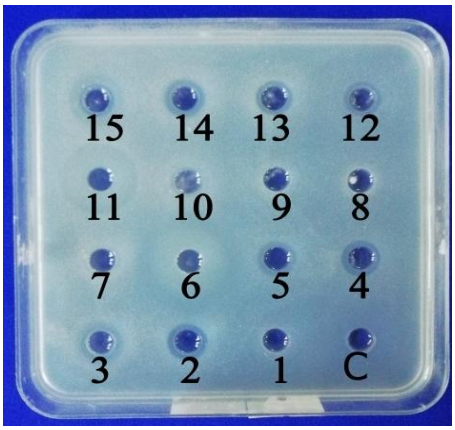


Figure 8 Antibacterial activity of the isolated fungal strains against *P. fluorescens*

Table 10 Antibacterial Activity of the Isolated Fungal Ftrains against *S. aureus*

Isolated Fungi	Fermentation period (Days) and Inhibitory Zone (mm)							
	3 -days	4-days	5-days	6-days	7-days	8-days	9-days	10-days
YY-1	-	-	-	-	-	-	-	-
YY-2	-	-	+	12.19	11.26	+	+	-
YY-3	-	-	-	+	+	17.87	13.30	-
YY-4	-	-	-	+	+	+	+	-
YY-5	-	-	-	13.29	11.48	+	+	-
YY-6	-	-	+	21.69	27.14	28.24	26.02	21.94
YY-7	-	-	-	-	-	-	13.28	15.53
YY-8	-	-	-	-	-	-	-	-
YY-9	-	-	-	-	-	-	-	-
YY-10	-	-	-	-	+	+	+	-
YY-11	-	-	-	17.38	18.04	19.71	19.92	20.05
YY-12	-	-	-	+	+	+	-	-
YY-13	-	-	-	+	+	11.18	12.25	+
YY-14	-	11.47	12.32	13.93	14.14	13.61	13.28	+
YY-15	-	-	+	16.76	16.10	15.63	15.51	13.12

(+) present (-) No activity Agar well =8mm

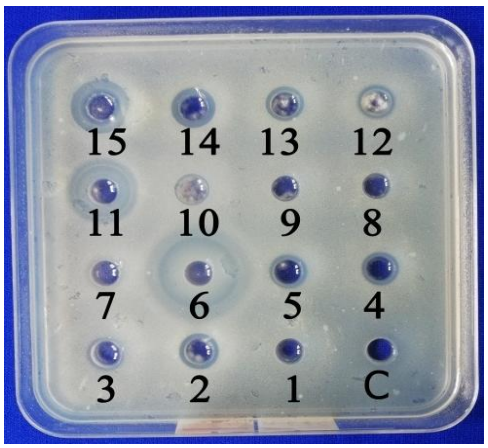


Figure 9 Antibacterial activity of the isolated fungal strains against *S. aureus*

Table 11 Antibacterial Activity of the Isolated Fungal Strains against *A. tumefaciens*

Isolated Fungi	Fermentation period (Days) and Inhibitory Zone (mm)							
	3 -days	4-days	5-days	6-days	7-days	8-days	9-days	10-days
YY-1	-	-	-	-	-	-	-	+
YY-2	-	-	-	-	+	+	16.22	16.65
YY-3	-	-	-	-	+	+	15.46	+
YY-4	-	-	-	-	11.36	14.77	+	+
YY-5	-	-	-	-	-	-	-	+
YY-6	-	-	-	+	+	+	+	+
YY-7	-	-	-	-	+	-	-	+
YY-8	-	-	-	-	+	-	-	+
YY-9	-	-	-	-	+	-	-	+
YY-10	-	-	-	-	+	-	-	+
YY-11	-	-	+	+	+	+	-	-
YY-12	-	+	+	+	+	+	15.66	+
YY-13	-	-	+	+	+	+	13.95	+
YY-14	-	-	+	+	+	12.26	13.10	+
YY-15	+	+	+	+	+	+	-	-

(+) present (-) No activity Agar well =8mm

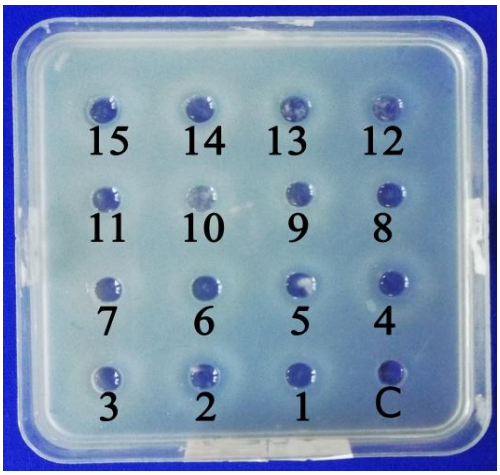


Figure 10 Antibacterial activity of the isolated fungal strains against *A. tumefaciens*

Table 12 Antifungal Activity of the Isolated Fungal Strains against *M. furfur*

Isolated Fungi	Fermentation period (Days) and Inhibitory Zone (mm)							
	3 -days	4-days	5-days	6-days	7-days	8-days	9-days	10-days
YY-1	-	-	-	-	-	-	-	-
YY-2	-	-	-	12.64	13.48	13.09	+	
YY-3	-	-	16.18	18.09	18.87	13.78	+	-
YY-4	-	-	-	12.57	13.74	11.37	+	-
YY-5	-	-	-	14.96	12.54	12.32	11.32	+
YY-6	-	-	18.90	29.71	31.40	31.90	28.70	23.63
YY-7	-	-	-	-	-	-	12.22	14.67
YY-8	-	-	-	-	-	-	-	+
YY-9	-	-	-	-	-	-	-	+
YY-10	-	-	-	+	+	+	+	-
YY-11	-	-	-	15.44	18.45	18.52	16.82	15.73
YY-12	-	-	-	11.83	12.84	12.69	+	-
YY-13	-	-	-	12.52	12.54	13.76	13.47	11.11
YY-14	-	-	-	13.88	13.89	14.18	14.27	15.52
YY-15	-	+	18.16	16.93	15.99	15.86	14.60	13.64

(+) present (-) No activity Agar well =8mm

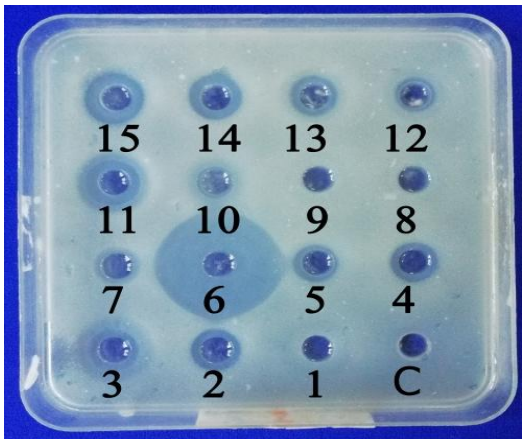


Figure 11 Antifungal activity of the isolated fungal strains against *M. furfur*

Conclusion

The present study is mainly involved in the isolation and antimicrobial activity of soil fungi based on the cultural, morphological and antagonistic activity. The results of this study indicated that 15 fungi were isolated from soil samples. Out of these isolates, 8 fungal strains were white colour and other were pale yellow, pale green and gray colour while their reverse colour were cream, white, yellow and pale yellow. Antimicrobial activities of all fungal strains were observed on eight test organisms and all fungi had the activity. Among them, isolated fungi (YY-11, 14 and 15) showed the antimicrobial activity on *E.coli, B. pumilus, C. albicans* and *M. furfur* while YY-2 showed the antibacterial activity on *B. subtilis.* Especially, YY-6 showed the highest antimicrobial activity on six test organisms. Therefore, it is expected that the current attempt will be useful for the identification and the best fermentation conditions of selected soil fungi and extraction of secondary metabolites.

Acknowledgements

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REMOVAL OF HEAVY METALS AND BIOFERTILIZATION ACTIVITY OF BIOCHAR

Su Su Aung[1], Myat Hla Wai[2], Myo Myo Myat[3]

Abstract

This research work deals with the removal of heavy metals and biofertilization activity of contaminated soils by using biochar (biomass): two organic residues rice husks from husker and bamboo leaves. These contaminated soil samples were collected from the Pagaye mining site S_c(I), Pagaye village S_c(II) and Battery service area from Weigyun S_c(III), Dawei Township. The three contaminated soil samples and biochar (rice husks and bamboo leaves) were treated under a close atmosphere using plastic housing. Soils were incubated at 27 °C for three months. Physiochemical properties (moisture, pH, texture, organic carbon, humus) of contaminated soils and treated soils were determined and major nutrients contents (total N, available P and available K), exchangeable cations (K^+, Ca^{2+} and Mg^{2+}) were studied by using instrumental and analytical methods. Generally, it was found that moisture, pH, organic carbon and humus were improved in treated soils and nutrients and exchangeable cations: K^+ and Ca^{2+} were also improved. Elemental compositions and heavy metals (Pb and Cd) in these soils were also determined with different interval of times by EDXRF and AAS methods. Both biochars removed the toxic metals Pb and Cd in all the contaminated soil samples. In addition, the comparison of the soil fertility on the contaminated and treated soils was carried out within one month. The soil fertility was also improved in the treated soil samples.

Keywords: Contaminated soils, biochar, treated soils, heavy metals, soil nutrients

Introduction

Myanmar is the agricultural country. Globally 30.4 million ha of agricultural land are currently managed organically. Organic farming is basically a simple idea, beginning with soil, compost, natural cycles that need to return garbage, sludge and wastes back to land, the hazards that pesticides, artificial fertilizers and toxic heavy metal from industrial waste cause to the environment and personal health benefits that result from eating quality nutrition food (Bhawalker, 1989). From the stand point of crop yields and quality, nutrient supply both from organic and inorganic sources are important. As such it is essential to substitute the inorganic fertilizers with nutrients from organic sources (Roy *et al* ., 2006). A number of diverse organic sources are available for use in agriculture. These source can be reduced the mining of soil nutrients and improve overall soil productivity. Recycling of wastes and their conservation into easily transportable and usable forms. Improper solid waste disposal (farm wastes) poses a major threat to the environment and high risks to human health. Most of these wastes are biodegradable and can be converted into valuable resources the reduces their otherwise negative impacts. To convert solid wastes into a valuable resources bio-fertilizer and its subsequent utilization as a source of plant nutrients, soil health and productivity (Birkeland, 1999) .

Soil is made up of minerals (rock, sand, clay and silt), air, water and organic material (dead plants and animals). Soil provides a substrate for plants (roots anchor in

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soil), a source of food for plants. Soil is a natural body consisting of layers (soil horizons) of mineral constituents of variable thicknesses. A layer of natural materials on the earth's surface contains both organic and inorganic materials and capable of supporting plant life. The material covers the earth's surface in a thin layer. It may be covered by water, or it may be exposed to the atmosphere. Soil contains four main components; inorganic material, organic matter, water and air(Biswas and Kherjee ,1994).

The post green revolution era witnessed a multiple nutrient deficiency because of higher crop harvest in the intensively cultivated areas where use of organic manure had declined while chemically pure fertilizers like Urea, Di-ammonium phosphate, and other allied items became the major source of plant nutrients (Blakemore *et al*, 1987). As a result of continued use of chemical fertilizer soil became poorer due to the deficiency of microbial contents of the soil. Agriculture devoid of organic manures crop residues has resulted in reduction of physicochemical and biological properties of the soil (Zin May Paing , 2014) .

Heavy metals are chemical elements with a specific gravity that is at least 5 times the specific gravity of water. Heavy metals become toxic when they are not metabolized by the body and accumulate in the soft tissues. Heavy metals may enter the human body through food, water, air, or absorption through the skin when they come in contact with humans in agriculture and in manufacturing, pharmaceutical, industrial, or residential settings. Industrial exposure accounts for a common route of exposure for adults (Johns,1998). Ingestion is the most common route of exposure in children. Children may develop toxic levels from the normal hand-to-mouth activity of small children who come in contact with contaminated soil or by actually eating objects that are not food dirt or paint chips (FAO, 2008). Generally the research aimed to (a) develop and disseminate technology on solid waste composting for the production of composted soil (b) remove the toxic heavy metals from contaminated soil and (c) determine the efficacy with which composted soil under anaerobic conditions generates major nutrients for vegetable production and it effect on some physical properties.

Materials and Methods

Materials

All chemicals and reagents used in this research were analytical grade and purchased from the British Drug House (BDH) Chemical Ltd, England. All other chemicals used were of analytical reagent grade. In all investigations, the recommended standard methods and techniques involving both conventional and modern methods were provided.

General

Two kinds of organic residues (rice husk and bamboo leaves) were used as biochar to remove toxic heavy metals from contaminated soils and to determine biofertilization activities.

Sample Collection

The contaminated soil samples were collected from Pagaye mining site S_c(I), Pagaye village S_c(II) and Battery service area from (Weigyun) S_c(III), Dawei Township. Tanintharyi Region in the month of July, 2016 (Figure 1). Soils which contained the greatest amount of organic matter from the upper position near to the surface about (15 cm) was dug in zigzag manner according to the according to the soil sampling method. The two plant residues such as rice husks (**R**) from husker, Launglone Township and bamboo leaves **(B)** were collected from Launglone township area, Dawei District. The kind of collected bamboo leaves was Wabo.

Sample Treatment

The contaminated soil samples and two plant residues (biochar) such as rice husks (R) and bamboo Wabo leaves (B) were placed in six polyethylene pots. In the pot, the first layer was contaminated soil with thickness 2 cm and biochar from rice husks (R) was placed in the second layer 2 cm thickness and then covered with contaminated soil 2 cm thickness. Another plant residue: bamboo leaves and another contaminated soil sample were placed in each pot as the above procedures and labeled the pots were labeled as S_c(I), S_c(II) and S_c(III) for contaminated soil samples, S_c(I)+R, S_c(II)+R, S_c(III)+R, S_c(I)+B, S_c(II)+B and S_c(III)+B for treated soil samples, respectively (Figure 2). The pots were covered with plastic sheet and placed in plastic housing to maintain ambient temperature and pressure. The soil samples were moisturized with water and temperature was measured every day. The experiments were conducted in August 2016 to November 2016. The incubation experiment; two types of biochar such as rice husks and bamboo leaves were applied to contaminated soil (three replicates per treatment) at 20 kg^{-1}, was conducted in August 2016 to November 2016 under a close atmosphere using plastic housing; 6 plastic pots were using as a soil treated pile and covered with plastic sheets. Soils were incubated at 27 ˚C for three months and rewetted to 70 % of water holding capacity.

Figure 1 Soil sampling sites of contaminated soil samples

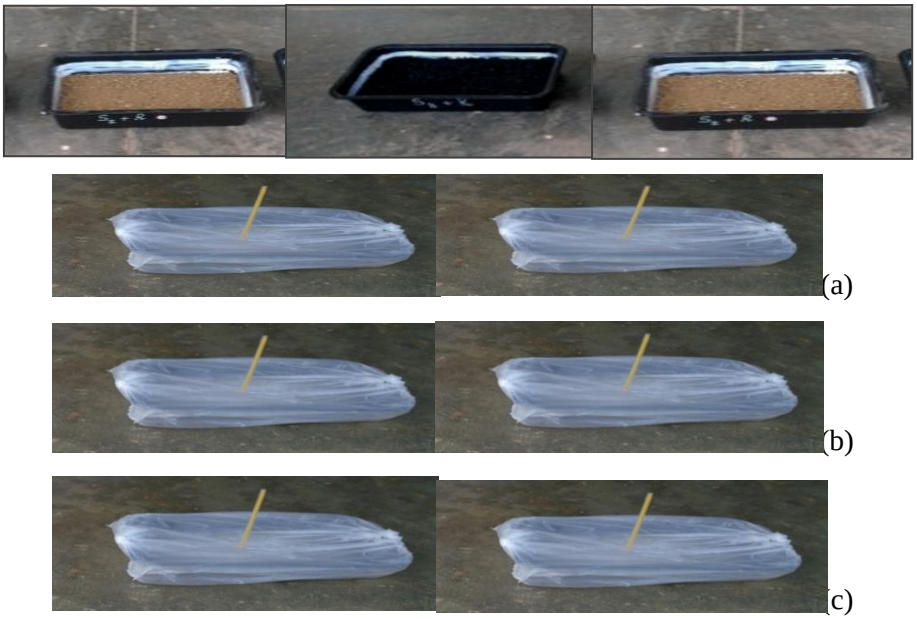

Figure 2 (a) Treated soil sample S_c(I) with rice husks (R) and bamboo leaves (B)
(b) Treated soil sample S_c(II) with rice husks (R) and bamboo leaves (B)
(c) Treated soil sample S_c(III) with rice husks (R) and bamboo leaves (B)

Soil Fertility by Cultivation of Plant (Lettuce)

The contaminated soil sample S_c(III) and treated soil samples with (S_c(III)+B and S_c(III)+R) were placed in provided containers and then inserted the lettuce seeds in each container 0.25 to 1 inch (0.6 to 2.5 cm) depth. The seed were covered with 0.5 inch (1.3 cm) of soil. The seeds were kept moist and then the soil fertility were also determined by growing plants within one month.

Results and Discussion

Physicochemical Properties of Contaminated Soil Samples

The type of contaminated soil samples for S_c(I) and S_c(II) were loam type but S_c(III) was sandy loam (Table 1). Moisture is constantly being taken by plants together with nutrients and lost by transpiration. The moisture percents of soil samples indicated that contaminated soil from near battery service area of S_c(III) was highest than the other (Table 2). The pH range for soils were 3.68 to 6.22. The pH showed that all the contaminated soil S_c(I), (II) and (III) were in acidic condition and S_c(III) was most acidic than the others. The organic carbon and humus percent of contaminated soil samples from battery service area S_c(III) was found to have the highest percent than the others. The major nutrients such as total nitrogen, phosphorus and potassium in three contaminated soil samples, total nitrogen were determined found to be in the range of 0.11 to 0.17 %, available phosphorus for 6.05 to 178.49 ppm and potassium were found to be in the range of 4.96 to 9.91 mg/100g (Table 3). And exchangeable cations : K^+ was highest amounts found in S_c(I), $Mg^{2+}$and Ca^{2+} were highest amounts found in S_c(II) (Table 4).

Table 1 Texture of Three Different Contaminated Soil Samples

Sr.No	Sample	Texture (%)			Total (%)	Soil type
		Sand	Silt	Clay		
1	S_c(I)	42.00	41.40	15.15	95.55	Loam
2	S_c(II)	40.70	40.70	17.50	98.90	Loam
3	S_c(III)	60.85	21.70	15.70	98.25	Sandy Loam

S_c(I) = Original sampling contaminated soil sample from Pagaye mining site
S_c(II) = Original sampling contaminated soil sample from Pagaye village
S_c(III) = Original sampling contaminated soil sample from Battery service area

Table 2 Moisture, pH, Organic Carbon and Humus Values of Three Different Contaminated Soil Samples

Sr. No.	Sample	Moisture (%)	pH	Organic Carbon (%)	Humus (%)
1	S_c(I)	3.07	6.27	0.40	0.69
2	S_c(II)	5.33	5.10	1.32	2.29
3	S_c(III)	3.26	3.68	1.68	2.89

Table 3 N, P and K Contents of Soil Samples

Sr. No.	Sample	Nitrogen (%)	Phosphorous (ppm)	Potassium(mg/100g)
1	S_c(I)	0.11	6.05	9.91
2	S_c(II)	0.17	23.95	7.60
3	S_c(III)	0.15	179.49	4.96

Table 4 Exchangeable K^+, Ca^2, Mg^{2+} Contents of Three Different Contaminated Soil Samples

Sr.No.	Sample	Potassium (meq/100g)	Calcium(meq/100g)	Magnesium (meq/100g)
1	S_c(I)	0.21	4.13	0.69
2	S_c(II)	0.16	4.93	2.11
3	S_c(III)	0.11	3.45	0.69

Physicochemical Properties of the Treated Soil Samples

Generally, it was found that moisture, pH, organic carbon and humus were improved in treated soils and nutrients (Table 5) and exchangeable cations: K^+ and Ca^{2+} were also improved (Table 6). Moisture values were found to be in the range from (3.26 to 8.50)%. The pH range for treated soils ranged from (3.72 to 9.12). According to pH values, it was found that the treated soil samples with biochar (rice husks) were acidic soils and with biochar (bamboo leaves) were acidic soil for S_c(I) and alkaline soils for S_c(II) and S_c(III). The major nutrients values of treated soil samples were higher than the contaminated soil samples (Table 6). Both biochars can remove the toxic metal (Pb and Cd) in all contaminated soil samples. The cadmium content in treated soil samples were decreased with time due to the absorption properties of biochar (rice husks and bamboo leaves). Lead contents in treated soil samples were found to be in the range of 83.040 ppm to 0,189 ppm within three months (Table 9).

Table 5 Moisture, pH, Organic Carbon and Humus Contents of the Treated Soil Samples (at Average Temperature 27°C)

Sample	Moisture (%)	pH	Organic Carbon (%)	Humus (%)	Time
S_c(I)+R	5.61	6.26	0.52	0.89	after one month
S_c(II)+R	5.74	5.40	1.44	2.48	
S_c(III)+R	5.86	3.72	1.69	2.92	
S_c(I)+B	3.38	8.54	0.56	0.96	
S_c(II)+B	4.55	6.11	1.42	2.45	
S_c(III)+B	3.45	4.47	1.81	3.13	
S_c(I)+R	5.12	6.39	0.60	1.03	after two months
S_c(II)+R	7.02	5.55	1.36	2.34	
S_c(III)+R	7.57	3.76	1.70	2.94	
S_c(I)+B	5.04	9.06	0.40	0.69	
S_c(II)+B	5.03	6.64	1.32	2.27	
S_c(III)+B	4.59	4.23	1.59	2.74	
S_c(I)+R	6.60	6.27	0.44	0.76	after three months
S_c(II)+R	5.04	5.34	1.32	2.29	
S_c(III)+R	8.50	3.81	1.71	2.96	
S_c(I)+B	3.91	9.12	0.40	0.69	
S_c(II)+B	3.27	6.45	1.32	2.29	
S_c(III)+B	5.73	3.99	1.37	2.35	

Removal of Heavy Metals by Using Biochar

In this study, lead contents in three soil samples were found to be the range of 4.920 ppm to 91.02 ppm (Table 8). Both biochars removed the toxic metals (Pb and Cd) in all contaminated soil samples. The cadmium content in treated soil samples were decreased with time due to the absorption properties of biochar (rice husks and bamboo leaves). Lead contents in treated soil samples were found to be reduced range from 83.040 ppm to 0.189 ppm within three month (Table 9).

Table 6 Available Nutrients (N , P and K) Contents of the Treated Soil Sample

Sample	Total Nitrogen (%)	Available (P) ppm	Available (K_2O) mg/100 g	Time
S_c(I)+R	0.13	8.21	18.43	after one month
S_c(II)+R	0.17	31.19	12.72	
S_c(III)+R	0.11	187.92	10.20	
S_c(I)+B	0.11	46.17	416.77	
S_c(II)+B	0.17	48.05	115.00	
S_c(III)+B	0.18	158.36	115.65	
S_c(I)+R	0.13	9.10	23.39	after two months
S_c(II)+R	0.15	37.00	17.43	
S_c(III)+R	0.13	239.81	12.33	
S_c(I)+B	0.11	56.41	424.23	
S_c(II)+B	0.17	30.28	193.46	
S_c(III)+B	0.17	234.34	226.40	
S_c(I)+R	0.09	9.99	23.77	after three months
S_c(II)+R	0.13	55.11	19.60	
S_c(III)+R	0.15	291.7	14.45	
S_c(I)+B	0.11	53.05	38.09	
S_c(II)+B	0.18	54.54	161.94	
S_c(III)+B	0.15	310.30	337.15	

Table 7 Exchangeable Cations (Calcium, Magnesium and Potassium) Contents in the Treated Soil Samples (at Average Temperature 27°C)

Sample	Exchangeable Cations (meq/100 g)			
	K^+	Ca^{2+}	Mg^{2+}	Time
S_c(I)+R	0.39	5.65	2.82	after one month
S_c(II)+R	0.27	4.95	0.71	
S_c(III)+R	0.22	3.54	1.12	
S_c(I)+B	8.88	6.21	0.69	
S_c(II)+B	2.45	6.29	0.70	
S_c(III)+B	2.46	3.45	0.68	
S_c(I)+R	0.50	6.32	non	after two months
S_c(II)+R	0.37	5.74	2.15	
S_c(III)+R	0.26	2.89	2.16	
S_c(I)+B	9.04	5.62	0.70	
S_c(II)+B	4.12	5.62	non	
S_c(III)+B	4.82	4.89	0.69	
S_c(I)+R	0.51	8.56	non	after three months
S_c(II)+R	0.42	3.51	non	
S_c(III)+R	0.30	2.24	2.31	
S_c(I)+B	0.81	7.63	0.72	
S_c(II)+B	3.45	6.20	non	
S_c(III)+B	7.18	6.33	0.72	

Table 8 Removal of Heavy Metals (Lead and Cadmium) from the Contaminated Soil Samples by Atomic Absorption Spectrophotometer

Toxic Element	Contaminated soil		
	S_c(I)	**S_c(II)**	**S_c(III)**
Cd (ppm)	0.287	0.276	0.447
Pb (ppm)	4.920	5.935	91.02

Table 9 Removal of Heavy Metals (Lead and Cadmium) from the Treated Soil Samples by Atomic Absorption Spectrophotometer

Element	Treated soil samples with biochar (rice husks and bamboo leaves) (**after one month**)					
	S_c(I)+B	S_c(I)+R	S_c(II)+B	S_c(II)+R	S_c(III)+B	S_c(III)+R
Cd(ppm)	0.227	0.231	0.234	0.219	0.289	0.257
Pb(ppm)	4.709	4.108	3.689	3.053	75.460	83.040
	(after two months)					
	S_c(I)+B	S_c(I)+R	S_c(II)+B	S_c(II)+R	S_c(III)+B	S_c(III)+R
Cd(ppm)	0.162	0.161	0.182	0.194	0.215	0.199
Pb(ppm)	2.761	2.582	1.588	0.896	46.342	43.023
	(after three months)					
	S_c(I)+B	S_c(I)+R	S_c(II)+B	S_c(II)+R	S_c(III)+B	S_c(III)+R
Cd(ppm)	0.108	0.123	0.104	0.067	0.014	0.068
Pb(ppm)	0.245	1.498	0.365	0.189	13.371	12.140

Both biochars can remove the toxic metals (Pb and Cd) in all contaminated soil samples. The cadmium content in treated soil samples were decreased with time due to the absorption properties of biochar (rice husks and bamboo leaves). Lead contents in treated soil samples were found to be the range from 83.040 ppm to 0.189 ppm within three month. Among the treated soil samples, the maximum value of lead content were 83.040 ppm with biochar (rice husks) and 75.460 ppm with biochar (bamboo leaves) and minimum value of lead content were 0.189ppm with biochar (rice husks) and 0.245ppm with biochar (bamboo leaves) respectively.

ED XRF Analysis of Relative Composition of Elements in the Contaminated Soil Samples

Relative compositions of elements were measured by ED XRF. The results are shown in Table 10.

The content of silicon in three contaminated soil samples were the highest relative to that of other elements and iron content is the second highest. The other elements such as Pb, K, Ca, Ti, Mn, Ba, Cu, Zn, V, Rb, W, Y, Sn and Se were also investigated. The contents of toxic metal: Pb were 0.155 %, 0.148 % and 7.230 % in the contaminated soil samples (I, II, III) respectively. Among the three contaminated soil samples, lead content of S_c(III) was the highest percent because the soil samples S_c(III) was collected from Battery service area.

Study on Soil Fertility by Using the Treated Soil Sample S_c(III) with Biochars

Removal of heavy metals and soil fertilization activities of biochars were also determined by growing plants (lettuce plants) within one month (Figure 4). It was found that the treated soil samples S_c(III)+B and S_c(III)+R were improved the soil fertility than the contaminated soils by studying the growth of lettuce plants within one month. Therefore, the soil fertility was improved when the soil samples were treated with biochar (rice husks and bamboo leaves) (Table 11).

Table 10 Relative Composition of Elements in the Treated Soil Samples Using Biochar

Sample	Elemental Composition (%) of Samples																		
	Si	Fe	K	Ti	W	Rb	Mn	Zr	Zn	Al	V	Y	Se	Au	Cu	Ca	Cr	Pb	Sn	
S_c(I)+R	40.504	41.07	12.747	2.802	0.485	0.500	0.752	0.334	0.175		0.104	0.082	-	0.186	-	-	-	-	0.256	
S_c(II)+R	55.230	31.46	9.049	2.717	-	0.140	0.348	0.407	0.132	0.174	-	0.055	0.194	0.092	-	-	-	-	-	one
S_c(III)+R	52.828	34.77	4.078	3.581	-	-	0.436	0.922	-	-	0.109	-	-	0.272	-	-	-	3.004	-	month
S_c(I)+B	47.003	32.33	14.841	-	1.085	0.624	0.672	0.255	0.107	0.160	0.104	0.071	0.086	0.104	-	-	-	-	-	
S_c(II)+B	49.437	35.97	9.440	2.650	-	0.067	0.387	0.383	0.128	0.182	0.099	0.048	-	-	-	-	-	-	1.030	
S_c(III)+B	54.072	31.90	5.015	3.818	-	-	0.340	0.578	0.120	-	-	-	-	-	-	-	-	3.091	-	
S_c(I)+R	34.729	45.14	12.606	3.074	0.660	1.150	0.918	0.602	0.242	0.229	0.154	0.123	-	0.101	0.260	-	-	-		
S_c(II)+R	53.043	33.50	9.312	2.595	-	0.148	0.376	0.534	0.114	0.217	0.111	0.051	-	-	-	-	-	-		two
S_c(III)+R	67.914	4.527	0.820	0.880	-	0.158	0.064	0.034	0.010	25.41	0.028	-	-	-	0.010	0.09	0.017	0.220		months
S_c(I)+B	34.402	39.30	17.076	3.022	1.771	1.350	0.962	0.488	0.164	0.162	0.140	0.112	0.049	0.019	-	-	-	-	0.061	
S_c(II)+B	52.646	32.05	10.774	2.761	-	0.152	0.387	0.527	0.109	0.324	-	0.069	-	0.194	-	-	-	-	-	
S_c(III)+B	65.197	4.739	1.459	0.935	-	0.239	0.069	0.054	0.010	27.05	0.027	-	-	-	0.010	0.065	0.011	0.239	-	
S_c(I)+R	34.729	45.14	3.736	3.074	0.660	1.150	0.918	0.602	0.242	0.229	0.154	0.123	-	0.101	0.260	-	-	-	-	
S_c(II)+R	53.043	33.50	2.801	2.595	-	0.148	0.376	0.534	0.114	0.217	0.111	0.051	-	-	-	-	-	-	-	three
S_c(III)+R	67.914	4.527	0.724	0.880	-	0.158	0.064	0.034	0.010	25.41	0.028	-	-	-	0.010	0.090	0.017	0.220	-	months
S_c(I)+B	34.402	39.30	4.554	3.022	1.771	1.350	0.962	0.488	0.164	0.162	0.140	0.112	0.049	0.019	-	-	-	-	-	
S_c(II)+B	52.646	32.05	2.467	2.761	-	0.152	0.387	0.527	0.109	0.324	-	0.069	-	0.194	-	-	-	-	-	
S_c(III)+B	65.197	4.739	1.320	0.935	-	0.239	0.069	0.054	0.010	27.05	0.027	-	-	-	0.010	0.065	0.011	0.239	0.061	

Table 11 Plant Height of Lettuce after Fertilizer Application Rate for Vegetables Production

No	Sample	Plant Height (cm) after Sowing			
		7 days	14 days	21days	28days
1	S(III)	1.1	1.9	3.5	5.6
2	S(III)+B	1.1	1.9	3.8	6.1
3	S(III) +R	1.1	1.9	3.9	6.7

Figure 4 The growth of lettuce plants within one month

Conclusion

In this research work, physicochemical properties of treated soil samples were determined by different time intervals. Generally, it was found that moisture, pH, organic carbon and humus were improved in treated soils and nutrients and exchangeable cations: K^+ and Ca^{2+} were also improved. The major nutrients values of treated soil samples were more than the contaminated soil samples. The minor nutrients such as exchangeable potassium and calcium, magnesium ions in treated soil samples were found to be in the range of 0.22 to 9.04 meq/100 g for K^+, 2.24 to 8.56 meq/100 g for Ca^{2+} and (0 to 2.82) meq/100 g for Mg^{2+}. Both biochars can remove the toxic metals (Pb and Cd) in all contaminated soil samples. The cadmium content in treated soil samples were decreased with time due to the absorption properties of biochar (rice husks and bamboo leaves). Lead contents in the treated soil samples were found to be decreased from 83.040 ppm to 0.189 ppm within three month. Among the treated soil samples, the maximum value of lead content was 83.040 ppm removed by biochar (rice husks) and 75.460 ppm with biochar (bamboo leaves) and minimum value of lead content were 0.189 ppm removed by biochar (rice husks) and 0.245 ppm with biochar (bamboo leaves) . In addition, it was the comparison of the soil fertility on the contaminated and treated soils were carried out within one month. It was found that the soil fertility in the treated soil samples: Sc (III) +B and Sc (III) +R were improved than the contaminated soil. Therefore, the soil fertility was improved when the soil sample was treated with biochar. It was concluded that treating the contaminated soil with biochar (rice husks and bamboo leaves) improved the soil fertility and using these biochars can remove the toxic metals in soils.

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PREPARATION OF PROTON EXCHANGE MEMBRANE (PSS) USED AS AN ELECTROLYTE IN FABRICATED DIRECT METHANOL FUEL CELL (DMFC)

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Abstract

Membrane is one of the key performances for Direct Methanol Fuel cell(DMFC). A new polymer electrolyte membrane based on polystyrene sulphonated has been developed. In this research, the polystyrene sulphonated membrane was a casted product that obtained by the sulphonation of polystyrene with concentrated sulphuric acid. The polystyrene sulphonated membranes have been characterized by properties of ion exchange capacity, and water uptake as a function of molar ratio of polystyrene to concentrated H_2SO_4, FTIR, SEM and EDXRF. The S content of the prepared membrane was determined using titration method. Fourier Transform Infrared (FTIR) was performed to verify the sulphonation reaction on the polystyrene polymer. Morphology of the prepared membranes were characterized by Scanning Electron Microscopy (SEM). The chemical constituents of polystyrene sulphonated membranes were detected by using Energy Dispersive X-ray Fluorescence (EDXRF). At the optimal temperature of 40 ± 2˚C for the time of 2 h, the optimal composition ratio of polystyrene to concentrated H_2SO_4 (w/v) was found to achieve as 1:2. The polystyrene sulphonated membrane has an ion exchange capacity of 0.79 meq/g and S content of 2.53 %. Therefore, the prepared polystyrene sulphonated membranes were suitable for the low temperature fuel cell, especially Direct Methanol Fuel Cell (DMFC) due to the range of ion exchange capacity 0.45-0.79 meq/g.

Keywords: Direct methanol fuel cell, polystyrene sulphonated membrane, ionhange capacity, water uptake

Introduction

Polystyrene Sulphonated Membrane

A variety of membrane materials have been used for fuel cell applications. Nafion (Du Pont), a perfluorosulphonic acid polymer, is the most widely used proton conductor in polymer electrolyte membrane fuel cells (PEMFCs). The membranes are made by copolymerization of tetrafluoroethylene and a substituted perfluorinated alkene (Motokawa, 2005). The stability and toughness of this type of membrane have led to its being the most commonly used in fuel cell as the electrolyte. However, the synthesis of the substituted monomer requires several steps and the ultimate cost of membrane fabrication is high (Larmine, 2002). This perfluorinated ionomer material is confronted with some challenges for PEMFC and DMFC applications. Therefore, many researchers are concerned to develop solid polymer electrolyte with low methanol permeation. Initially, polystyrene sulphonic acid and sulphonated-phenol formaldehyde membranes were used (Halacy, 1966). Membranes for the DMFC have been prepared from the film casting of solutions of homogeneously polymerized sulphonated polystyrene. The thin thermoplastic film, forming from polystyrene, may be used of several types, both as regard their chemical structures and other physical properties (Hogarth, 1996). The plastic film should be capable of being cast into a thin homogeneous film from an organic solvent casting solution. This plastic film is chemically stable, resistant to acids and alkalis and

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water insoluble (Geiger, 2002). The aim of this research work is to prepare the cheap proton exchange membrane to be used as an electrolyte instead of Nafion (Du Pont) in fabricated direct methanol fuel cell. To get polystyrene sulphonated membrane, sulphonation process are carried out by using the commercial polystyrene beads and 98 % concentrated sulphuric acid. In polystyrene sulphonated membrane, sulphonic acid ($-SO_3H$) are present as cation exchange radicals. These $-SO_3H$ radicals attached to the membrane matrix, with sufficient water being held in the membrane matrix by Vander Waals force so that the H^+ ion is extremely mobile in the membrane. Without external supply of water, these membranes will tend to dehydrate and shrink during operation (Carretta *et al*., 2000).

Materials and Methods

Materials

The main source of material for the preparation of the proton exchange membrane is polystyrene (commercial) that was purchased from Academy Chemical Group, 28th street, Pabedan Township, Yangon, Myanmar. The chemicals used in the experimental work were from the British Drug House Chemical Co.Ltd., Kanto Chemical Co.Ltd., Sigma Aldrich Co.Ltd., Walco Chemical Co.Ltd., All chemicals used were of analytical reagent grade. In all investigations, the recommended standard methods and techniques involving both conventional and modern methods were provided.

Preparation of Polystyrene Sulphonated Membrane

Five grams of polystyrene beads were weighed and put into a glass bottle. Then, 100 mL of solvent toluene was poured and the bottle was well stoppered and allowed to stand for overnight. The clear polystyrene solution was obtained and which is used for sulphonation process. The sulphonation process was carried out with different amount of concentrated sulphuric acid (5 mL,10 mL,15 mL, 20 mL), various sulphonation time (1h, 2h, 3h, 4h, 5h) and various sulphonation temperature (40 ± 2, 50 ± 2, 60 ± 2, 70 ± 2, 80 ± 2) $^{\circ}$C. The temperature within the reaction bottle was checked by immersing a thermometer. During sulphonation, stirring was continuously carried out by means of magnetic bar and the sulphonating agent was added drop by drop. The obtained brown color polymer solution was then cast into deionized water at room temperature. The resulted films were polystyrene sulphonated (PSS) membranes and named according to the added amount of concentrated sulphuric acid such as PSS5, PSS10, PSS15 and PSS20, respectively. The prepared membranes were used as electrolyte in fabricated direct methanol fuel cell.

C**haracterization of the Prepared PSS Membranes**

The prepared PSS membranes were characterized by modern techniques such as EDXRF, FT IR, and SEM. Moreover, the properties such as tensile strength, thickness, solubility, and the other parameters of the prepared PSS membranes were carried out by means of materials testing and titration methods.

Results and Discussion

Optimal Conditions of Sulphonation Process

In this investigation, the polystyrene used was pure polystyrene and was dissolved in toluene. Table 1 shows the weight of polystyrene in toluene with respect to the volume of concentrated sulphuric acid used for sulphonation. Based on sulphonation time of 2 h (Williams,1966), sulphonation process was done. Qualitatively polystyrene sulphonated membranes were with respect to 1:1, 2:1, 3:1, 4:1. Concentrated sulphuric acid volume to polystyrene weight shows the nature of the membrane sheets that were casted in deionized water. On the basis of the texture and nature of the casted membrane, it was found that 2:1 (v/w) indicates a very smooth uniform in texture and nature compared to those in all other membranes. Higher ratios of 3:1 and 4:1 showed very brittle and easily torn up type of membrane on this aspect.

Table 1 indicates those membranes possessing satisfactory results of sulphonation. The membrane designated as PSS10 that is membrane casted by using the ratio of 2:1(v/w) (concentrated sulphuric acid : polystyrene) in the sulphonation process, probably indicates the most soluble membrane to be used as an electrolyte. However, it is very possible that lower sulphonation temperature may produce more suitable types of membrane.

On this aspect, Table 2 and Figure 2 show the change in IEC (meq/g) as well as the S content in percentage achieved with respect to variation in temperature from 40 to 80℃. Sulphonation was done based on selected polystyrene mixture 2:1 (v/w). It can be seen from Figure 2 that the optimal sulphonation temperature was about 40℃. At this optimal temperature the IEC value as well as the S content of the prepared PSS membranes indicate the highest value compared to the values of the other prepared PSS membranes which were prepared at higher temperature.

Table 3 and Figure 3 show the various sulphonation times used in the sulphonation of PSS10 at the sulphonation temperature 40 ±2 ℃. It can be seen from Table 3 and Figure 3. The optimal sulphonation time was 2 h. The IEC and S content in percentage were the highest value corresponding to the polystyrene sulphonated membrane prepared at 40 ±2℃.

It can be inferred that the operational optimal conditions of the casted PSS membranes, the weight ratio of sulfuric acid to polystyrene should be 2:1 (v/w), temperature should be 40 ±2℃ and the time of sulphonation should be 2 h.The membrane obtained at these optimal conditions showed the highest IEC value of 0.79 meq/g with a S content (%) of 2.53. Regarding PSS10 membrane, it was also found to possess smooth surface texture and the more uniform cavitated pores. The prepared PSS10 membrane is shown in Figure1.

Figure 1 The prepared proton exchange membrane

Table 1 Sulphonation of Polystyrene with Different Amounts of Concentrated Sulphuric Acid

Membranes	Vol. of con. H_2SO_4(mL)	Sulphur content (%)	Remark
PSS 5	5	1.60	Satisfactory
PSS 10	10	1.66	very uniform smooth
PSS 15	15	1.32	torn-up
PSS 20	20	1.54	very brittle

Note: Amount of polystyrene = 5g, Sulphonation time = 2 h, Sulphonation temp. = 80 ± 2 °C

Table 2 Sulphonation of Polystyrene at Various Sulphonation Temperatures

Membranes	Sulphonation temp. (°C)	IEC (meq/g)	S-content (%)
PSS10	40±2	0.79	2.53
PSS10	50±2	0.70	2.24
PSS10	60±2	0.67	2.14
PSS10	70±2	0.71	2.27
PSS10	80±2	0.52	1.66

Note: Sulphonation time = 2 h

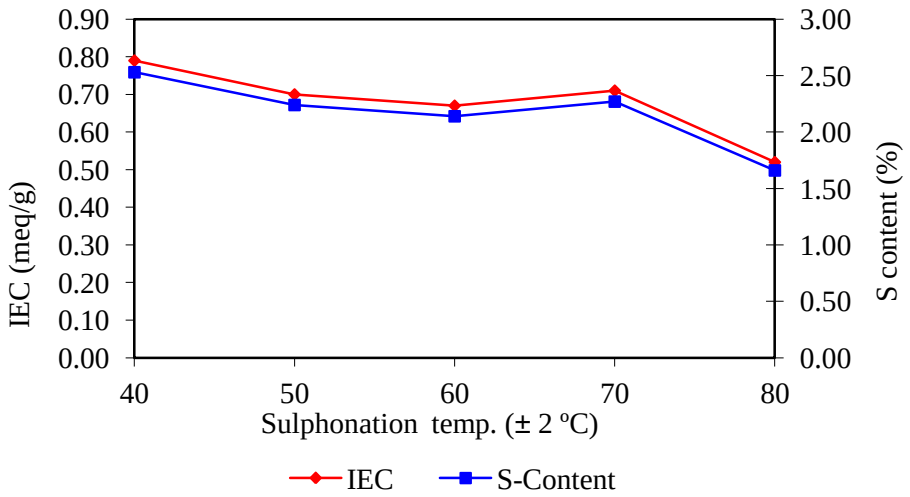


Figure 2 Variation of IEC and sulphur content as a function of sulphonation temperature

Table 3 Sulphonation of Polystyrene at Various Sulphonation Times

Membranes	Sulphonation Time(h)	IEC (meq/g)	S-content (%)
PSS10	1	0.45	1.44
PSS10	2	0.79	2.53
PSS10	3	0.78	2.49
PSS10	4	0.73	2.34
PSS10	5	o.74	2.37

Note: Sulphonation temp. = 40 ± 2°C

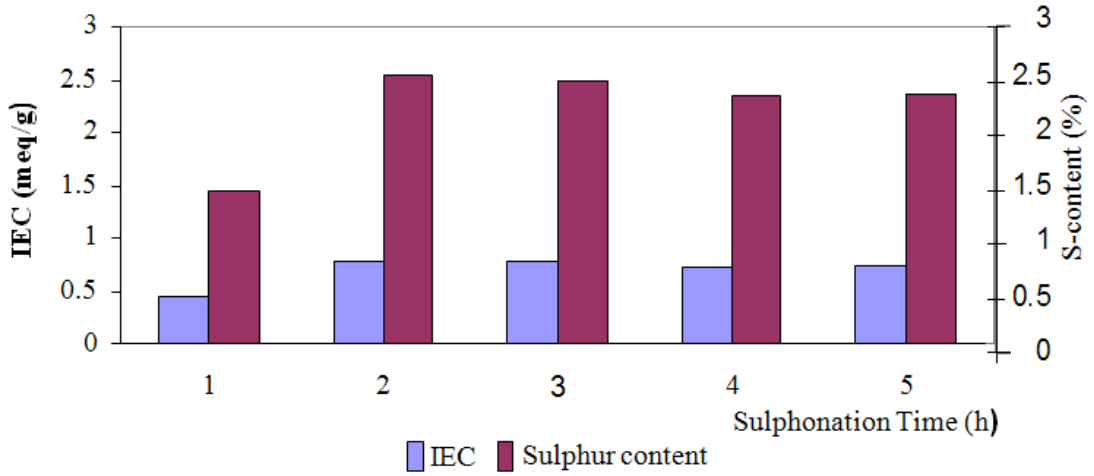


Figure 3 Variation of S content and IEC with various sulphonation time

Identification of Polystyrene Sulphonated Membranes (PSS10)

FT IR analysis of PSS10 membrane

Figure 4 shows the FT IR spectrum of PSS10 membrane. The corresponding FT IR spectrum assignment is presented in Table 4. The FT IR spectrum of PSS10 membrane observes the C-H stretching of CH_3, CH_2 and CH at 302 5, 2923, 2851 cm^{-1}. The band at 1068-1028 cm^{-1} indicates the S=O stretching of sulphonic acid group and the band at 756-699 cm^{-1} shows the C-S stretching. Therefore, the FT IR spectrum of polystyrene sulphonated membrane indicates the presence of sulphonic acid group.

Table 4 FT IR Spectral Assignment for PSS10 Membrane

Observed Frequency (cm^{-1})	Frequency Range (cm^{-1})	Assignment	Group and Class
3442	3400-3200	O-H stretch	$-SO_3H$ group
3025	3100-3000	aromatic C-H stretching vibration	
2923	3000-2800	Aliphatic C-H stretching vibration	$-CH_2-$ group
2851	2870-2840	CH_2 sym stretch	CH_2- group
1600-1492	1600-1450	Aromatic C=C stretching vibration	C=C group
1451	1470-1431	C-H deformation vibration	$-CH_3-$
1181-1155	1190-1120	SO_2 sym stretching vibration	$-SO_2$ group
1068-1028	1060-1020	S=O stretch	S=O group
756 - 699	720 - 660	C-H bending monosubstituted benzene	C-S group

(Silverstein, 1991)

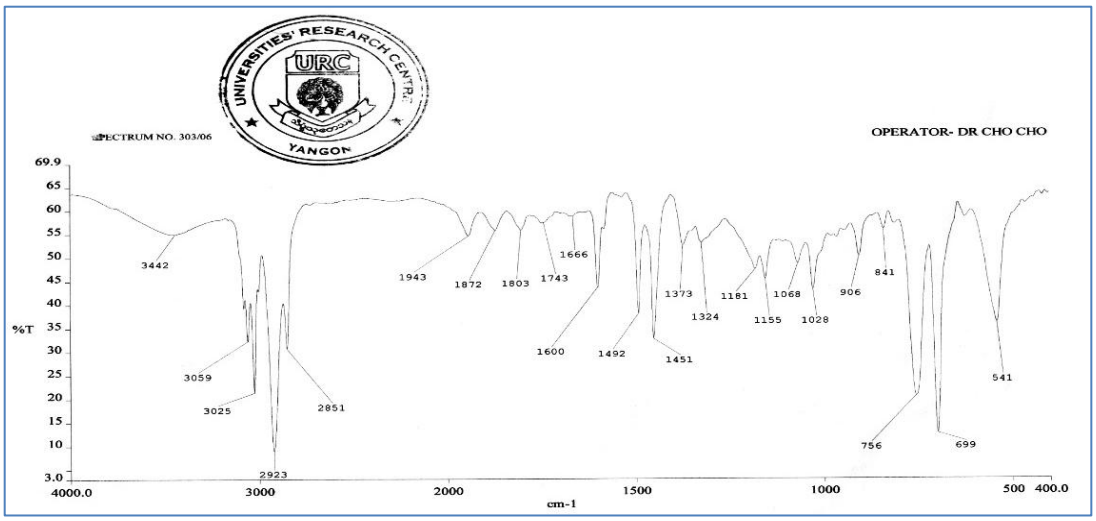


Figure 4 FT IR spectrum of PSS10 membrane

EDXRF analysis of PSS10 membrane

Figure 5 shows the EDXRF spectrum of PSS membrane. According to EDXRF spectrum, PSS membrane contains sulphur which was the main component, iron and copper were the trace constituents. The content of sulphur is relevant to any influencing factors on fuel cell performance.

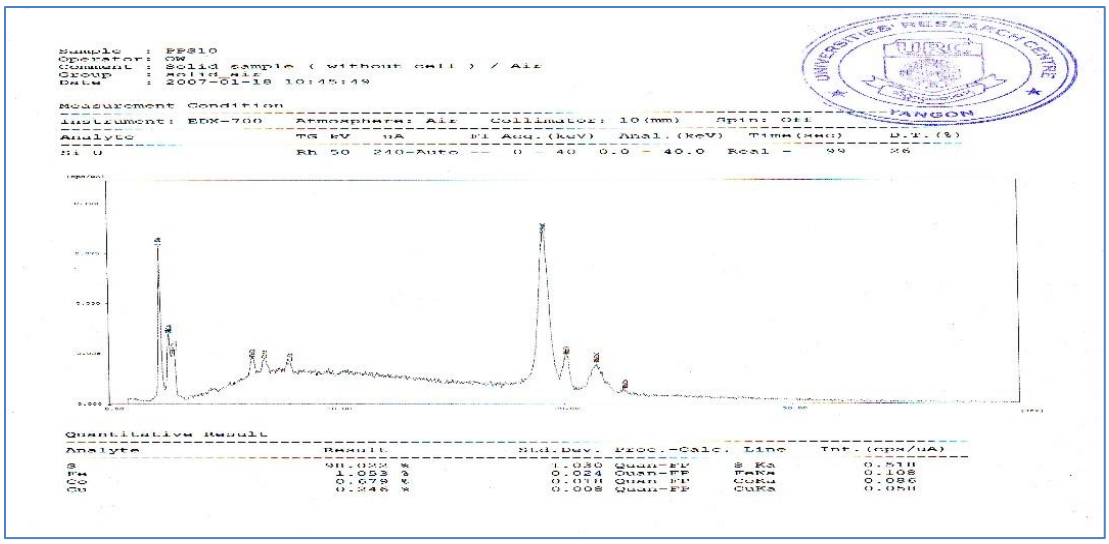


Figure 5 EDXRF spectrum of PSS10 membrane

Scanning electron microscopy of the prepared PSS membranes

SEM micrographs Figures 6(b), (c), and (d) show the surface morphology of PSS membranes and Figure 6(a) is the SEM micrograph of PS membrane which is not sulphonated. From the SEM results, it is clear that the surface texture of PSS membranes were different with different amount of concentrated sulphuric acid. Among them, PSS10 membrane shows uniform pores size. Surface morphology of these membranes were also relevant to the sulphonation time. According to SEM micrographs Figures 7(d), and (e) are typical, they are brittle and their pores size are not uniformed. In addition, the surface texture of Figure 7(b) is characterized by the presence of imperfections, probably due to the presence of aggregates of sulphonic groups at the

film surface. Hence, in accordance to the spectra and resulting data of FT IR, EDXRF and the results presented by SEM,can be inferred that sulphonation of polystyrene was successfully achieved.

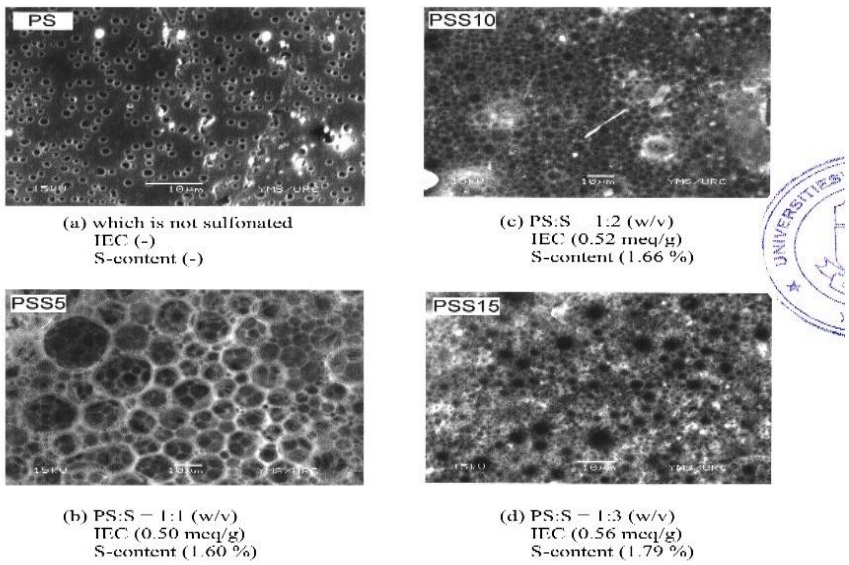


Figure 6 SEM micrographs of PSS membranes with various amounts of con.H_2SO_4

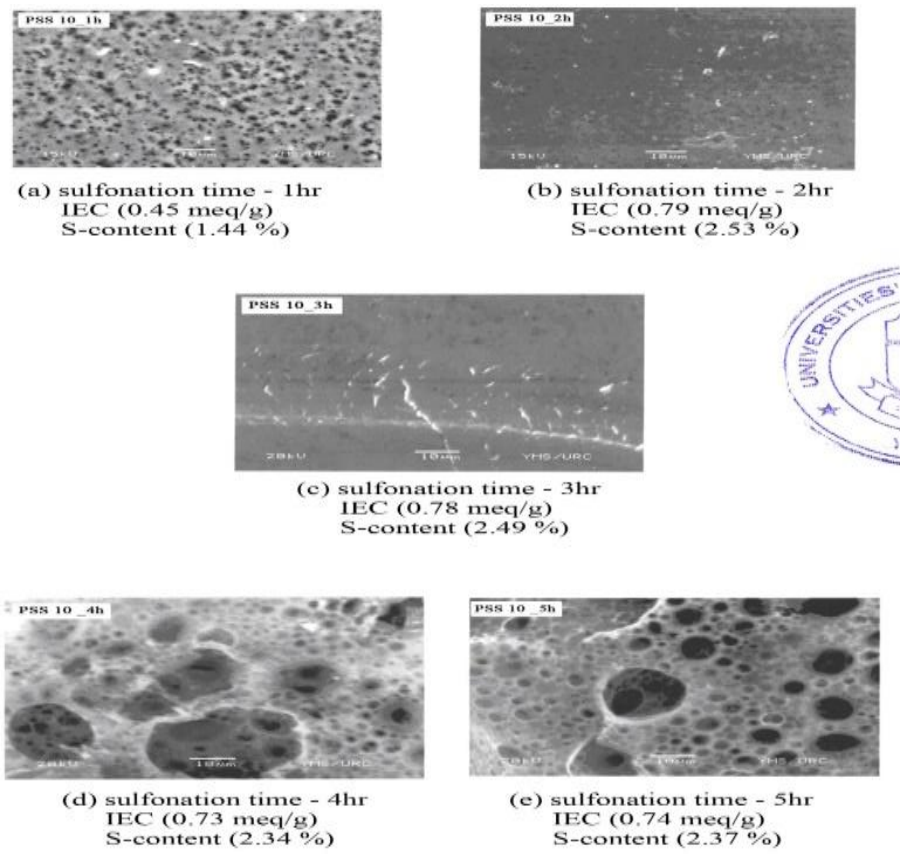


Figure 7 SEM micrographs of PSS10 membrane with various sulphonation times

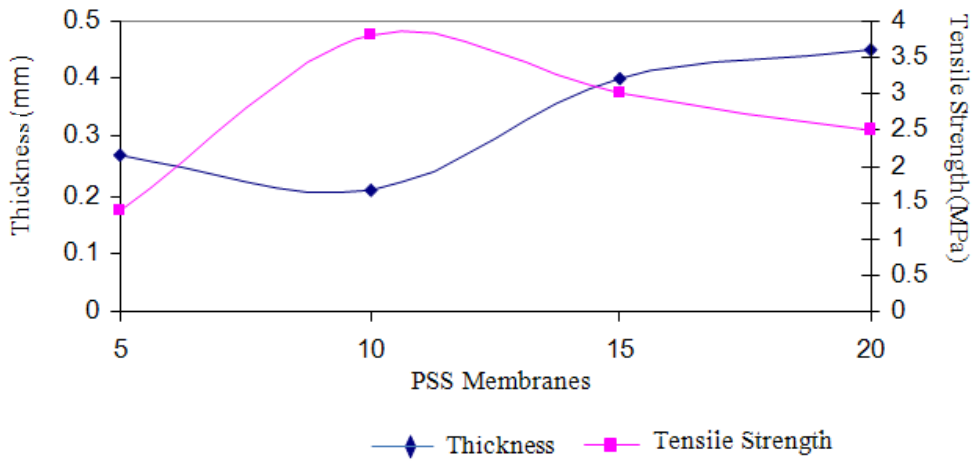
Evaluation of Physicochemical and Physicomechanical Properties of the Prepared Membranes

Tables 5, 6, 7 describe the data obtained from the determination of ion exchange capacity of PSS membranes. From the experimental point of view, amount of sulphonating agent, sulphonation temperature and duration of sulphonation are the most important factors, which play the role of preparation of ion exchange membranes. It has been found that the PSS membrane which has been prepared by sulphonation of polystyrene with increasing amounts of concentrated sulphuric acid. At the high amount of sulphuric acid, water uptake decreases with increasing IEC value. The samples also have become more fragile.These results are shown in Table 5 and Figures 8 and 9. From the results, PSS20 showed a high IEC value (0.59 meq/g), but its water uptake had decreased (i.e. 50.26%).The sample has become more brittle,so tensile strength was lowered (2.5 MPa).The IEC value of PSS10 (0.52 meq/g) was much lower than that of PSS20, but it has a high water uptake property.

Table 5 Physical Properties of PSS Membranes with Different Amounts of Con.H_2SO_4

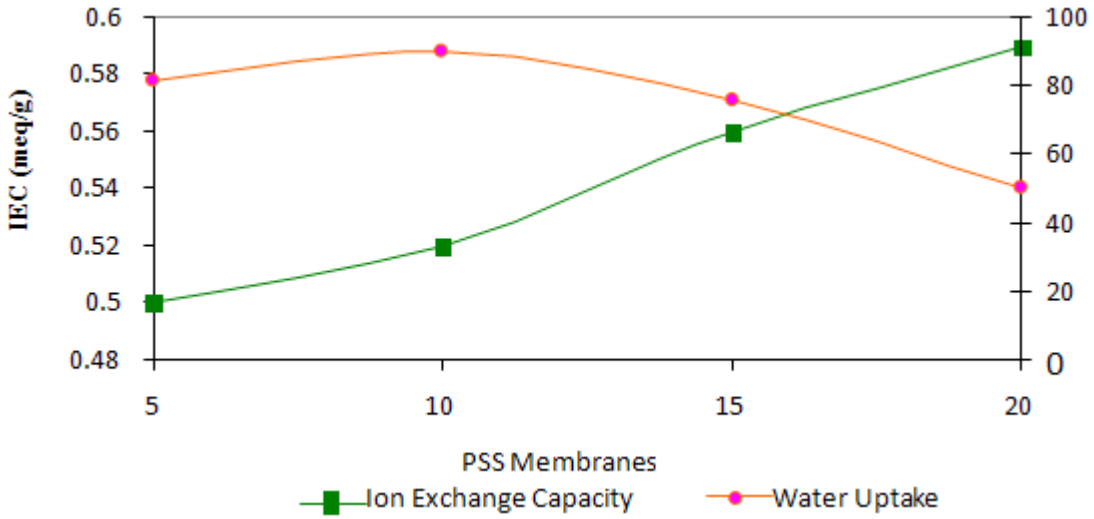
Type	Thickness (mm)	Tensile Strength (MPa)	Ion Exchange Capacity (meq/g)	Water Uptake Wt (%)
PSS 5	0.27	1.40	0.50	81.22
PSS 10	0.21	3.80	0.52	90.32
PSS 15	0.40	3.00	0.56	75.68
PSS 20	0.45	2.50	0.59	50.26

Note: Sulphonation temperature was controlled at (80±2)ºC for 2 h.



5 = PSS5 membrane prepared with 5 % conc: H_2SO_4
10 = PSS10 membrane prepared with 10 % conc: H_2SO_4
15 = PSS15 membrane prepared with 15 % conc: H_2SO_4
20 = PSS20 membrane prepared with 20 % conc: H_2SO_4

Figure 8 Thickness and tensile strength vs. prepared membranes



5 = PSS5 membrane prepared with 5 % conc: H_2SO_4
10 = PSS10 membrane prepared with 10 % conc: H_2SO_4
15 = PSS15 membrane prepared with 15 % conc: H_2SO_4
20 = PSS20 membrane prepared with 20 % conc: H_2SO_4

Figure 9 IEC and water uptake vs. prepared membranes

The variation of ion exchange capacity as a function of sulphonation temperature under controlled temperatures; (40±2) ˚C, (50±2) ˚C, (60±2) ˚C, (70±2) ˚C, (80±2) ˚C are presented in Table 6 and Figures 10 and 11. It can be seen that the ion exchange capacity of polystyrene sulphonated membrane at low temperatures showed maximum IEC values and then decreases with increasing sulphonation temperature.

Table 6 Physical Properties of PSS10 Membranes with Various Sulfonation Temperature

Membranes	Sulfonation Temp. (˚C)	Thickness (mm)	Tensile Strength (MPa)	IEC (meq/g)	Water Uptake (Wt.%)
PSS10	40 ± 2	0.25	3.5	0.79	95.50
PSS10	50 ± 2	0.23	3.3	0.70	92.44
PSS10	60 ± 2	0.28	3.5	0.67	98.44
PSS10	70 ± 2	0.26	3.9	0.71	96.50
PSS10	80 ± 2	0.21	3.8	0.52	90.32

Note: Sulfonation time was controlled at 2 h.

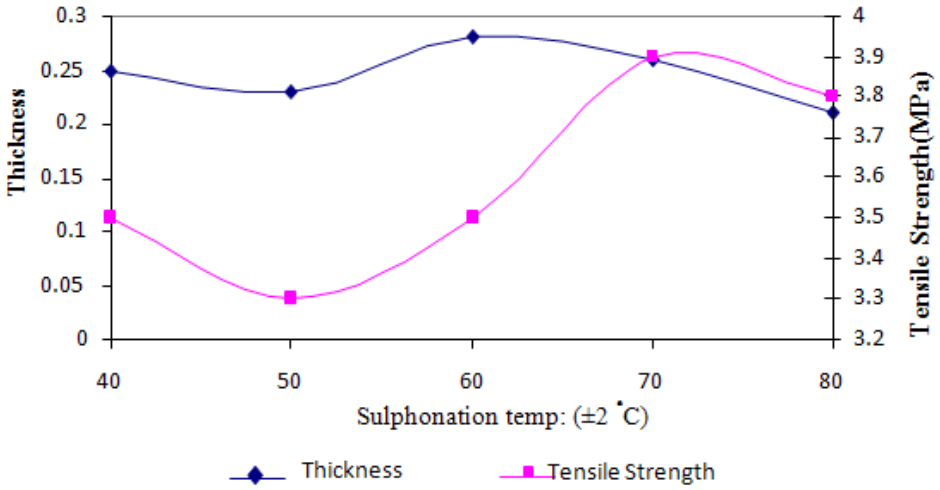


Figure 10 Thickness and tensile strength vs. sulphonation temperature

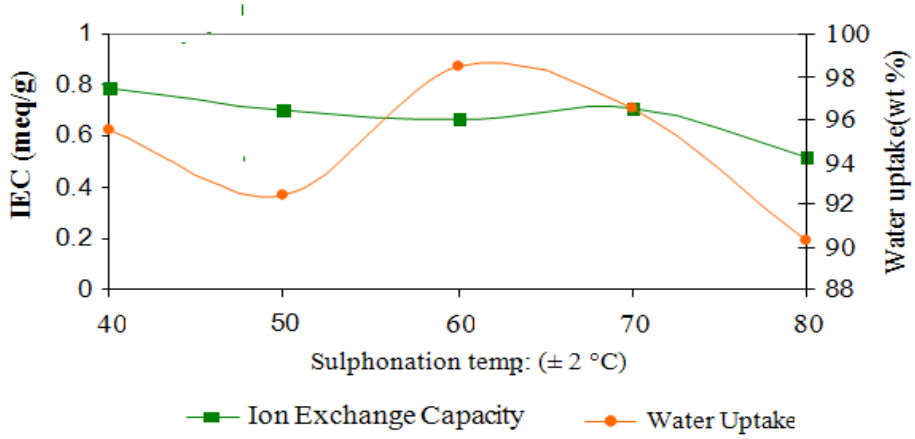


Figure 11 IEC and water uptake vs. sulphonation temperature

In sulphonation of polystyrene, sulphonation temperature that close to the room temperature are suitable, thus it is preferably kept under 50 ˚C or less. IEC value depends on sulphonation time. These results are shown in Table 7 and Figures 12 and 13. From the results, IEC values of PSS10 membranes except PSS10 (1 h) membrane, were slightly different but water uptake were different and lesser because of surface texture was more fragile.

Table 7 Physical Properties of PSS10 Membranes with Various Sulfonation Time

Membranes	Sulphonation Tememperature (h)	Thickness (mm)	Tensile Strength (MPa)	Ion Exchange Capacity (meq/g)	Water Uptake (Wt. %)
PSS10	1	0.24	3.3	0.45	90.55
PSS10	2	0.25	3.5	0.79	95.50
PSS10	3	0.45	3.5	0.78	85.60
PSS10	4	0.45	3.0	0.73	66.67
PSS10	5	0.40	3.2	0.74	65.63

Note: Sulphonation temperature was controlled at (40±2) °C

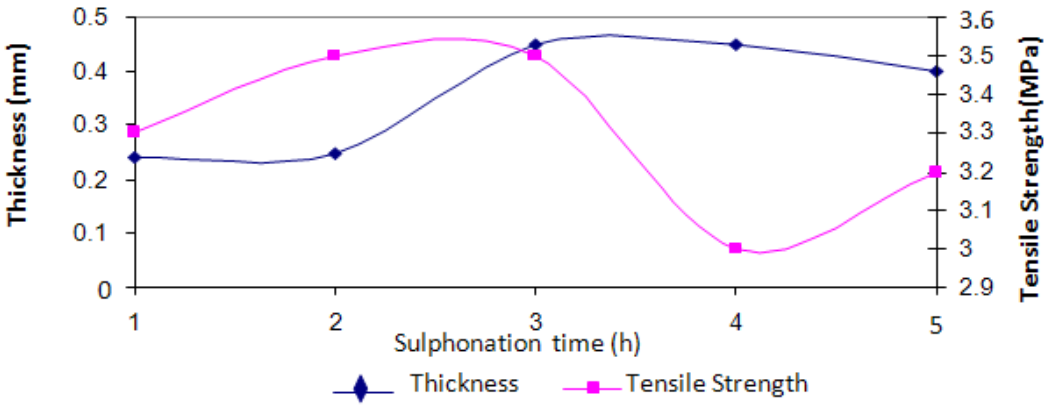


Figure 12 Thickness and tensile strength vs. sulphonation time

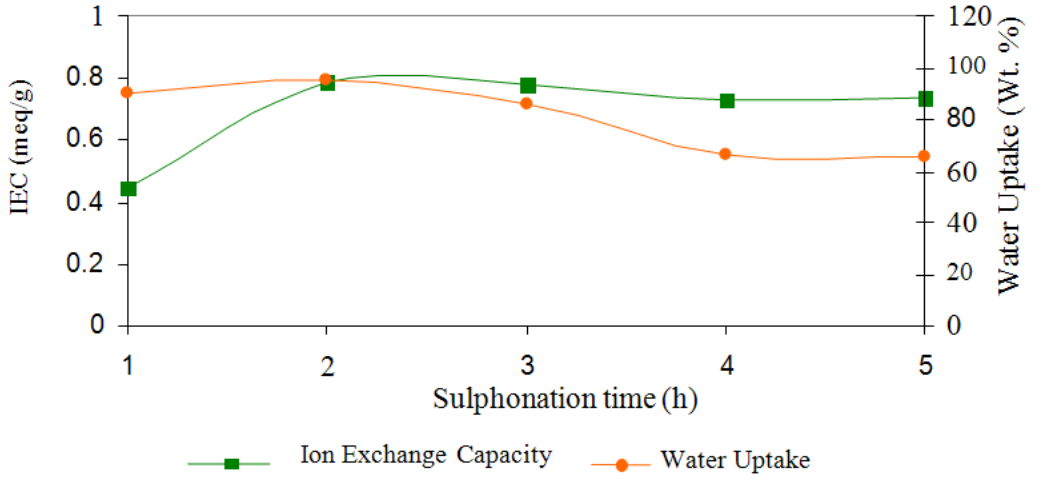


Figure 13 IEC and water uptake vs. sulphonation time

The solubility characteristics of prepared PSS membranes are shown in Table 8. From the experimental point of view, PSS membrane is highly hydrophobic and insoluble in water and in most organic solvents such as ethanol, methanol, petether. They are soluble in butanone, toluene and slightly soluble in acetone. Moreover, these membranes are not soluble in sulfuric acid, hydrochloric acid, nitric acid, potassium hydroxide and sodium hydroxide at room temperature. According to the results, The optimum conditions for preparing sulphonated polystyrene membrane (PSS10) were found to be ; 5 g of polystyrene, 10 mL of 98 % concentrated sulphuric acid,100 mL of toluene as solvent, sulphonation time of 2 h, and sulphonaton temperature of 40 ± 2 ˚C. The photograph of the stack of unit cells in series are shown in Figure 14. The stack of unit cell was able to produce only about 2.6 V. To produce stabilized output voltage.The assembly needs to be modified by using bipolar plates.

Solubility of the Prepared PSS10 Membrane

Table 8 Solubility of the Prepared PSS10Membrane

Solvents	Prepared PSS10 Membranes
Water	-
Sulphuric acid (1M)	-
Hydrochloric acid (1M)	-
Nitric acid (1M)	-
Potassium hydroxide (1M)	-
Sodium hydroxide (1M)	-
Methanol	-
Ethanol	-
Petether	-
Acetone	±
Butatone	+
Toluene	+

Note : + Soluble, - Insoluble, ± Slightly soluble

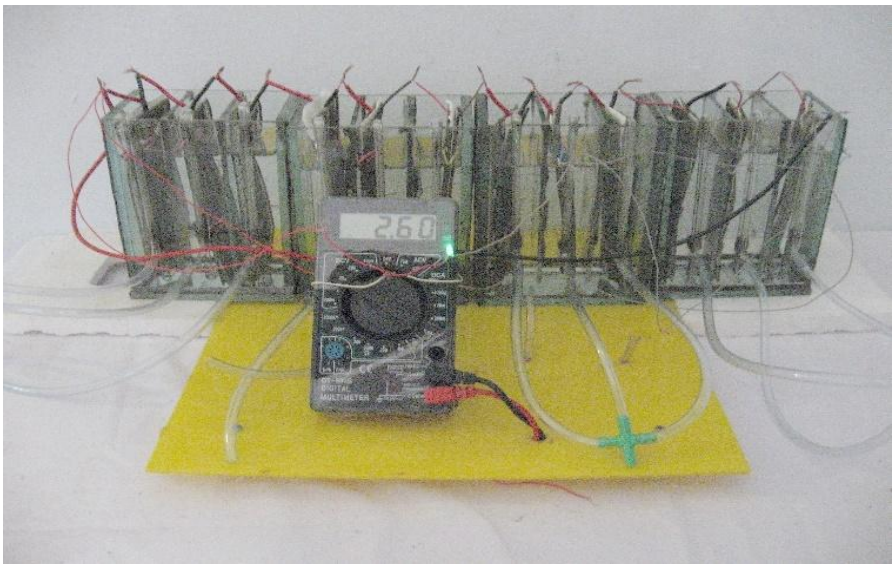


Figure 14 Photograph of constructed DMFC in series

Conclusion

PSS membranes were characterized in terms of its quality, intrinsic properties such as ion exchange capacity (IEC), water uptake and tensile strength. All these parameters can be adjusted to the PSS membrane suitable for its end uses. The determination of IEC is one of the most important characteristic and most widely used in preparation of ion exchange membrane. Several methods are available for the determination of IEC of ion exchange membrane. From the experimental point of view, amount of sulphonating agent, sulphonation temperature and duration of sulphonation are the most important factors, which play the role of preparation of ion exchange membranes.This investigation has shown that it is feasible to prepare sulphonated polystyrene by using 98 % concentrated sulphuric acid as a sulphonating agent. The optimum

conditions for preparing sulphonated polystyrene membrane (PSS10) were found to be ; 5 g of polystyrene, 10 mL of 98 % concentrated sulphuric acid, 100 mL of toluene as solvent, sulphonation time of 2 h and sulphonation temperature of 40 ± 2℃.

The desired membranes were prepared by casting in deionized water at ambient temperature and were immersed in 1M H_2SO_4 for about 24 h so that the conductivity of prepared membranes can be promoted. The presence of $-SO_3H$ groups were able to achieve by cation exchange capacity (titration method). IEC and water uptake of prepared PSS membranes were calculated by using the formula. The maximum cation exchange capacity of PSS10 membrane was 0.79 meq/g. Using the sulphonation process a polymer that can perform an ion exchange capacity of from 0.50 to 4.10 (From Literature) can be obtained. From the results, it clearly showed that all the PSS membranes have ion exchange capacity ranging from 0.45-0.79 meq/g. Therefore, the prepared polystyrene sulphonated membranes were suitable for the low temperature fuel cell, especially direct methanol fuel cell.

Acknowledgements

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STUDY ON THE PHYSICOCHEMICAL PROPERTIES OF NATURAL SOIL FROM THE SELECTED AREAS OF LOIKAW TOWNSHIP, KAYAH STATE

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Abstract

This study focuses on physicochemical properties of soils from the selected areas of Loikaw Township, Kayah State where many cereal fields have and the farmers always cultivate the subsidiary crops. In this work, soil samples were collected from the surrounding areas of Lin Phone Lay Village, Moh Pyar Village and Law Pi Ta Village in Loikaw Township, Kayah State at the depth of 0-6 inches in a zigzag manner. Physical parameters like moisture content, water holding capacity, pH, electrical conductivity, texture, organic carbon and humus and chemical parameters such as available P_2O_5, available K_2O, total nitrogen, exchangeable Ca^{2+}, Mg^{2+}, K^+, Na^+, H^+ and Al^{3+} were determined according to standard procedures. From this study, it revealed that the Moh Pyar soil sample was moderately acidic nature, Lin Phone Lay soil sample was alkaline nature and Law Pi Ta soil sample was neutral. The soil texture from Moh Pyar and Law Pi Ta soil samples were found to be sandy clay loam and Lin Phone Lay soil sample was silt loam. The contents of electrical conductivity, organic carbon and humus were found to be low level in Moh Pyar and Law Pi Ta soil samples. Among these three samples, the high content of macronutrients like available phosphorus (97.53) and available potassium (803 ppm) were found in Lin Phone Lay soil sample which were found to have sufficient value for agricultural use. The relative abundances of elements of selected soil samples were aslo analyzed by energy dispersive X-ray fluorescence (EDXRF) technique. The results obtained from this analysis revealed that seventeen elements in Moh Pyar soil, sixteen elements in Lin Phone Lay soil and Law Pi Ta soil were contained in these soil samples. The results from this research work indicate the types of soil as well as the nutritional values of selected soil samples needed for crop production.

Keywords: Soil samples, soil types, physical parameters, chemical parameters, agricultural use

Introduction

The word soil represents one of the most active and complex natural systems on the earth's surface. It is essential for the existence of many forms of life and provides medium for plant's growth and also supplies the organisms with most of their nutritional requirements (Gaur, 1997). The physicochemical properties of soil play a big role in the plant's ability to extract water and nutrients. High quality soils not only produce better food and fiber, but also help to establish natural ecosystems and enhance air and water quality (Griffith, 2010). The physical properties of the soil depend upon the amount, shape, structure, size, pore spaces, organic matter and mineral composition of soil. The chemical properties of the soil are the interactions of various chemical constituents among soil particles and the soil solution. These physicochemical properties are soil texture, bulk density, soil structure, soil colour, pH, electrical conductivity, cation exchange capacity, organic carbon, organic matter and soil nutrients. All soils have different properties and working with them requires understanding of these properties. The knowledge of the physicochemical properties of soil helps in managing resources while working with a particular soil (Brady, 2002). They need to be studied for agricultural purpose, to increase

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the productivity and to improve the workability of the soil mass. The study of up-to-date status of soil properties is a very important tool to enhance production on a sustainable basis. Therefore, the study was aimed to find out the difference in health of the soil under different location systems.

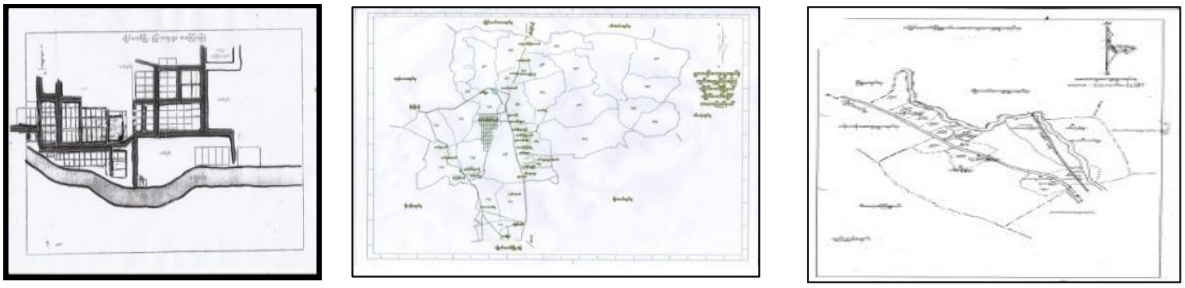
Materials and Methods

This part deals with all experimental procedures. The chemicals used were from the chemical suppliers; 'British Drug House Chemicals Ltd., Poole, England' and 'Kanto Chemical Co., Ltd., Tokyo, Japan', unless otherwise stated.

Various conventional and modern techniques and instruments were used throughout the experimental procedures. All analytical works were according to recommended standard texts (Vogel, 1968). All experimental data were computed on the statistical basis. The apparatus consists of both conventional labware and glassware and modern equipment.

Sample Collection and Handling

Soil samples were collected from the surrounding areas of agriculture farm near Moh Pyar Village, Lin Phone Lay Village and Law Pi Ta Village in Loikaw Township, Kayah State in December, 2018. All soil samples were taken from the depth of 0-6 inches of the surface in a zigzag manner by using GPS (Global Positioning System), mixed thoroughly to homogenize and dried in the shade before sieving. Afterwards, gravel, roots, etc, were discarded. Then, the collected soil samples were passed through the mesh size (2 mm) and stored in polyethene bags and then clearly labeled. The sampling sites are presented in Figure 1.



(i) (ii) (iii)

Figure 1 Sampling sites(i) Moh Pyar Village (ii)Lin Phone Lay Village(iii) Law Pi Ta Village

Determination of Physicochemical Properties

The texture of the soil sample was determined by pipette method (Vogel, 1968). Soil pH was measured in water (1:1.25 w/v) using pH meter (Nathan, 1998). Moisture was determined by using oven-dried method. Organic carbon content in the sample was determined by Walkey - Black method (Nathan and Beegle, 1998). Total nitrogen was determined by the Micro-Kjeldhal digestion - distillation method, available phosphorus was determined by using UV- visible spectrophotometry and available K_2O was determined by flame photometer. The exchangeable cations were extracted with 1M ammonium acetate solution. The extract was then analyzed for calcium (Ca) and magnesium (Mg) by EDTA titration method (Gupta, 2000), and for potassium K and sodium Na, by flame photometer. Total aluminum was determined by Sokoiov's Method (Bower and Fireman,1952).

Results and Discussion

This study focuses on soil properties from the selected areas of the Moh Pyar Village, Lin Phone Lay Village and Law Pi Ta Village in Loikaw Township, Kayah State. In this work, the surface horizon of soil samples were collected from cultivated areas in Loikaw Township, Kayah State in December, 2018. The samples were collected during this period because it was the time when the field was being prepared to cultivate the subsidiary crop after the harvest of corn. It was the time when the nutrients in the soil can be stable.

Physicochemical Properties of Soil Samples

Texture of the selected soil samples

Texture indicates the relative contents of particles of various sizes, such as sand, slit and clay in the soil. Soil texture is an important soil characteristic that influences storm water infiltration rates. In this research, the soil types of selected soil samples are shown in Table 1. According to data, the soil texture for Moh Pyar soil sample was sandy clay loam which contains (41.76 % sand, 24.00 % silt and 34.24 % clay), Lin Phone Lay soil sample was slit loam (13.00 % sand, 65.60 % silt and 20.30 % clay) and Law Pi Ta soil sample was sandy clay loam (69.76 % sand, 10.00 % silt and 20.24 % clay). Generally, the best agricultural soils contained 10-20 % clay. Clay particles tend to retain or fix nutrient in soil.

Table 1 Physicochemical Properties of the Selected Soil Samples

Parameter	Results of different soil samples		
	Moh Pyar	**Lin Phone Lay**	**Law Pi Ta**
Moisture (%)	3.24	1.46	1.12
pH	5.61	8.43	6.94
Electrical conductivity ($mScm^{-1}$)	0.09	0.23	0.05
Texture	sandy clay loam	slit loam	sandy clay loam
Sand (%)	41.76	13.00	69.76
Silt (%)	24.00	65.60	10.00
Clay (%)	34.24	20.30	20.24
Water holding capacity (%)	52.00	58.00	50.00
Organic Carbon (%)	1.54	2.39	1.37
Humus (%)	2.65	4.13	2.35

The effect of pH and electrical conductivity on soil

The soil pH is a measure of the acidity or alkalinity in soil. Soil pH is considered variable in soil as it controls many chemical processes that take place. It specifically affects availability by controlling the chemical forms of the nutrient. The optimum pH range for most soil falls between 5.5 and 7.0, however many plants have adapted to thrive at pH value outside. Soils with high acidity tend to have toxic amounts of aluminium and manganese. Plants need calcium with moderate alkalinity, but most minerals are more soluble in acid soils. Soil organisms are hindered by high acidity, and most agricultural crops do best with mineral soils of pH 6.5 and organic soils of pH 5.5 (Hazelton, 2007).

The pH value of the selected soil samples from Moh Pyar Village, Lin Phone Lay Village and Law Pi Ta Village in Kayah State are shown in Table 1. The data indicated that the soil pH

range from 5.6 to 8.4. Soil sample from Moh Pyar Village was moderately acid nature (pH 5.61), Lin Phone Lay Village was alkaline nature (pH 8.43) and Law Pi Ta Village was neutral (pH 6.94). From this result, it was seen that soil sample from Lin Phone Lay Village had higher pH as compared to the soil samples from Moh Pyar Village and Law Pi Ta Village. Soil electrical conductivity measures the dissolved material in an aqueous solution which relates to the ability of the material to conduct electric current through it. The values of electrical conductivity of selected soil samples were observed to be in the range from 0.05 mS cm^{-1} to 0.23 mS cm^{-1} which was not very high. Hence, suitable fertilizers should be added to the soils for ensuring the maximum crop production.

The moisture contents, organic carbon and humus of the selected soil samples

Humus is the dark organic matter that forms in the soil when plant and animal matter decay which contains many useful nutrients for healthy soil, nitrogen being the most important of all. Humus can participate in aggregate stability and nutrient and water holding capacity. The contents of moisture, organic carbon and humus of the selected soil samples are shown in Table 1. In this research, the moisture content of the soil samples from the Moh Pyar Village, Lin Phone Lay Village and Law Pi Ta Village were found to be 3.24 %, 1.46 % and 1.12 %. The values of organic carbon and humus were 1.54 % and 2.65 % in Moh Pyar Village, 2.39 % and 4.13 % in Lin Phone Lay Village, 1.37 % and 2.35 % in Law Pi Ta Village. From this result, it was found that soil sample from Lin Phone Lay Village has high fertility due to the highest percentage of humus and organic carbon. This implies that the higher the organic carbon content of a soil, the higher the nitrogen content of the soil, and the higher fertile the soil will be. The contents of humus and organic carbon of the soil samples from Moh Pyar Village and Law Pi Ta Village were found to be low which was suggested that the soils can be classified as low level organic soil (3 to 19 % humus) (Magdoff, 2000).

Distribution of cation exchange capacity and water holding capacity in the selected soil samples

The cation exchange capacity is the number of exchangeable cations per dry weight that soil is capable of holding. It is very important soil property influencing soil structure, stability, nutrient availability, soil pH and soil's reaction toward fertilizers. The exchangeable cations of the collected soil samples are summarized in Table 2 and Figure 2. Result of the study indicated that the soil samples were contained Ca^{2+} (13.10 meq/100 g), Mg^{2+} (1.38 meq/100 g), Na^{+} (0.50 meq/100 g), K^{+} (0.19 meq/100 g), H^{+} (0.05 meq/100 g) and Al^{3+} (0.08 meq/100 g) in Moh Pyar Village, Ca^{2+} (79.11 meq/100 g), Mg^{2+} (4.06 meq/100 g), Na^{+} (0.42 meq/100 g) and K^{+} (1.71 meq/100 g) in Lin Phone Lay Village and Ca^{2+} (6.01 meq/100 g), Mg^{2+} (1.50 meq/100 g), Na^{+} (0.66 meq/100 g) and K^{+} (1.12 meq/100 g) in Law Pi Ta Village. The result reveals that high cation exchange capacity (85.3 meq/100 g soil) from Lin Phone Lay soil sample indicated that the soil contained more organic matter and also had high water holding capacity. The water holding capacity increases with increasing level of organic carbon and with increasing percentage of silt and clay particles in the soil because silt and clay particles have much higher surface area to hold greater quantity of water. The greater water holding capacity was recorded in Lin Phone Lay soil sample (58.00 %). Cations Al^{3+} and H^{+} were detected in soil sample from Moh Pyar Village. It indicated that the problem associated with acidic soil (pH 5.6) due to aluminium toxicity (Hazelton, 2007).

Table 2 Exchangeable Cation Contents in the Selected Soil Samples

Cation	Contents (meq/100 g)		
	Moh Pyar	Lin Phone Lay	Law Pi Ta
Ca^{2+}	13.10	79.11	6.01
Mg^{2+}	1.38	4.06	1.50
K^{+}	0.19	1.71	1.12
Na^{+}	0.50	0.42	0.66
H^{+}	0.05	Not detected	Not detected
Al^{3+}	0.08	Not detected	Not detected

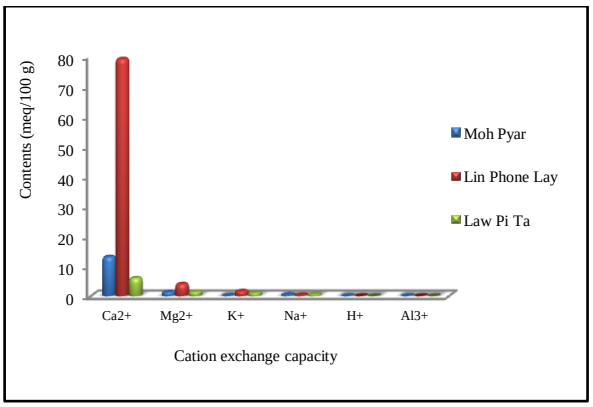


Figure 2 Histogram of exchangeable cations contents in the selected soil samples

Macronutrients (N, P, K) contents of the selected soil samples

Total nitrogen (N), available phosphorus (P_2O_5) and available potassium (K_2O) are referred to as the primary macronutrients of soil and most important to provide the vitality and performance of plant grown. Therefore, it is a vital work to analyze these elements not only in qualitative but also in quantitative. Macronutrients contents in soil samples are shown in Table 3. Total nitrogen (N) contents of the selected soil samples were found to be 0.18 % in Moh Pyar Village, 0.23 % in Lin Phone Lay Village and 0.14 % in Law Pi Ta Village. The contents of available phosphorus (P_2O_5) and available potassium (K_2O) in Moh Pyar Village, Lin Phone Lay Village and Law Pi Ta Village were (1.25 ppm and 869 ppm), (97.53 ppm and 803 ppm) and (1.69 ppm and 527 ppm), respectively. From these results, the soil samples contained a good amount of potassium contents. Significant positive correlation has been observed between available potassium and clay contents which might be due to the presence of most of mica in the finer fraction. Soil that has adequate potassium allows plant roots to proliferate, increasing the plants' ability to grow rapidly.

Table 3 Contents of Macronutrients in the Selected Soil Samples

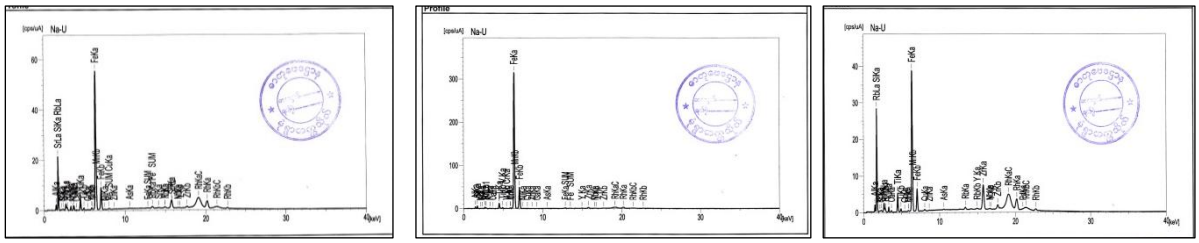
Parameter	Results		
	Moh Pyar	Lin Phone Lay	Law Pi Ta
Total Nitrogen (%)	0.18	0.23	0.14
Available Phosphorus (ppm)	1.25	97.53	1.69
Available Potassium (ppm)	869	803	527

Relative abundance of some elements in the selected soil samples

The relative abundance of some elements in the selected soil samples were analyzed by EDXRF technique. The resultant compositions of the elements in the selected soil samples are presented in Table 4 and Figure 3. According to EDXRF data, the selected soil samples contained the high amounts of silicon 72.346 % (Moh Pyar soil), 47.962 % (Lin Phone Lay soil) and 79.054 % (Law Pi Ta soil). It was followed by Al, Fe, Ti, S, K, Ca, Mn, Zr, Pd, Cr, Y, Zn, Rb, Ni, Sr, Nb, Ga and Cu.

Table 4 The Relative Abundance of Elements in the Selected Soil Samples by EDXRF Technique

Elements	Relative Abundance of Elements in soil samples (%)		
	Moh Pyar	**Lin Phone Lay**	**Law Pi Ta**
Si	72.346	47.962	79.054
Al	21.390	28.007	16.745
Fe	3.093	20.090	1.823
Ti	0.949	2.112	0.873
S	0.810	0.836	0.636
K	0.726	0.359	0.562
Ca	0.539	0.201	0.148
Mn	0.051	0.116	0.014
Zr	0.051	0.070	0.088
Cr	0.018	0.046	0.011
Y	0.006	0.012	0.003
Zn	0.006	0.013	0.004
Rb	0.006	-	0.007
Ni	0.005	0.024	-
Sr	0.004	-	-
Nb	0.002	0.006	0.002
Cu	0.002	0.028	0.011
Pd	-	-	0.020
Ga	-	0.010	-



(i) (ii) (iii)

Figure 3 EDXRF spectra for the selected soil samples
(i) Moh Pyar (ii) Lin Phone Lay (iii) Law Pi Ta

Conclusion

The study of physicochemical parameters is important to agricultural chemists for plant growth and soil management. From the present work, on the study of physicochemical properties of soil from the selected areas of Loikaw Township in Kayah State, the following inferences could be deduced. The results of pH value showed that the soil sample from Moh Pyar Village was moderately acidic (5.61), Lin Phone Lay Village was alkaline nature (8.43) and Law Pi Ta Village was neutral (6.94). Soil types were found to be Moh Pyar and Law Pi Ta soil samples were sandy clay loam and Lin Phone Lay soil sample was slit loam. According to EDXRF data,

high amounts of silicon were present in the selected soil samples. This study indicates that the physicochemical properties of the soil such as electrical conductivity, humus, organic carbon, cation exchange capacity, available nitrogen and available phosphorus are negatively affected in Moh Pyar and Law Pi Ta soil systems as compared to the Lin Phone Lay soil due to frequent tillage practices. The high quantities of water holding capacity, available nitrogen, available phosphorus and available potassium in the Lin Phone Lay soil indicate that this soil ecosystem has considerable impact on soil nutrient build up and accumulation by reducing the loss through soil erosion and leaching due to dense canopy. Results show that the selected soil samples have medium as well as high mineral contents. This information will help the villagers who cultivated in this area to solve the problem related to soil nutrients which amount of fertilizer to be added to increase the yield of crops.

Acknowledgements

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SCREENING OF SOME BIOACTIVITIES AND ISOLATION OF MANGOSTIN FROM *GARCINIA MANGOSTANA* L. (MANGOSTEEN) PERICARP

Mie Mie Aye[1], Wyine Myat Noe Oo[2]

Abstract

The aim of present study is to screen some bioactivities and to isolate mangostin from the pericarp of *Garcinia mangostana* L.(Mangosteen). Firstly, the phytochemical constituents were investigated by the reported chemical methods. The qualitative elemental analysis was done by EDXRF technique. The extractable matter contents were determined by extracting sample with polar solvents such as ethanol and water. In the study of the antioxidant activity, ethanol and watery extracts were screened by DPPH radical scavenging assay. The ethanol extract (IC_{50} = 8.15 µg/mL) was found to be more potent than watery extract (IC_{50} = 12.3 µg/mL) in antioxidant activity. In addition, the antitumor activity of ethanol and watery extracts was investigated by Potato Crown Gall (PCG) test and both extracts exhibited antitumor activity with the doses of 0.15, 0.1 and 0.05 g/disc. Furthermore, the mangostin (0.09 %, 180-181°C) was isolated from the ethanol extract by column chromatographic separation technique. The isolated compound was identified by spectroscopic techniques compared with authentic mangostin.

Keywords: *Garcinia mangostana* L., antioxidant activity, antitumor activity, mangostin

Introduction

Nowadays, the government has encouraged in traditional medicine products with the genuine quality, safety and efficacy. In Myanmar, many indigenous medicinal plants have been used in traditional medicine. Among them *Garcinia mangostana* L. (mangosteen) is widely used for medicinal purposes. Mangosteen is a tropical fruit originated from South East region. It is dark purple to red purple fruit. The edible fruit aril is white, soft and juicy with a sweet, slightly acid taste and a pleasant aroma. It is also known as " Queen of fruits " (Martin, 1980). It has been established that mangosteen contains a variety of active ingredients, including xanthones, phenolic acids, polysaccharides and pigments. Xanthones are the main active substances in mangosteen, where in α–mangostin is one of the most important natural xanthone derivatives (Ibrahim *et al*., 2016). Scientists who studied the properties of xanthones in mangosteen found that they exhibited potent free radical scavenging activity. Xanthones absorb free radicals and stop cellular damage that leads to many types of diseases. Several studies have shown that obtained xanthones from mangosteen have remarkable bioactivities such as antioxidant, antitumor, anti-inflammatory, antiallergy, antibacterial, antifungal and antiviral activities (Suksamrarn *et al*., 2006). The presence of these bioactivities in mangosteen makes it very potent immune system booster that promotes good health and wellness. Recently, products such as mangosteen juices or dietary supplements have begun to be wide spread around the world. In Myanmar, mangosteen is widely cultivated in Mon State and Tanin-thayi Region. The photograph of fruits *Garcinia mangostana* L. (mangosteen) is described in Figure 1. The research has focused on screening of some bioactivities and isolation of mangostin from *Garcinia mangostana* L. (mangosteen) pericarp.

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Botanical Aspects of *Garcinia mangostana* L. (mangosteen)

Genus	:	*Garcinia*
Species	:	*mangostana*
Family	:	Clusiaceae
Botanical name	:	*Garcinia mangostana* L.
English name	:	Mangosteen
Myanmar name	:	Mang khut thee
Part used	:	pericarp

Figure 1 Photograph of fruits of *Garcinia mangostana* L. (Mangosteen)

Materials and Methods

In this research work, *Garcinia mangostana* L. (Mangosteen) fruit was collected from Mawlamying Township, Mon State, during the period of September to November, 2017. After collection, the sample was identified at Botany Department, Taungoo University. The pericarp of the fruit was separated from edible part and dried at room temperature. The dried sample was ground into powder form and stored in airtight container to prevent moisture changes and other contaminations. The reagents used in this research were analar grade chloroform, methanol, ethanol, 2,2-diphenyl-1-picryl hydrazyl, DPPH (BDH), ascorbic acid (BDH), dimethyl sulfoxide, DMSO (BDH), sodium hypochlorite, iodine and agar powder. The instruments used were UV - visible Spectrophotometer (Shimadzu UV-240) , Shimadzu FT IR- 8400 Spectrophotometer and EDXRF (Shimadzu EDX-7000 spectrometer).

Phytochemical Investigation of Pericarp of *Garcinia mangostana* L.

In order to find out the types of phytochemical constituents present in pericarp of *G.mangostana*, phytochemical investigation was carried out by chemical methods (Harborne, 1984; Robinson, 1983; Vogel, 1966).

Qualitative Elemental Analysis of Pericarp of *Garcinia mangostana* L.

The elemental contents in pericarp of *G.mangostana* were determined by EDXRF spectrometer. For this measurement, pellet of the sample (2.5 cm diameter) was first made. X-ray fluorescence spectrometer (Shimadzu EDX-7000) can analyze the element from Na to U under vacuum condition. It uses x-ray to excite an unknown sample. The sample was placed in the chamber and pumped up to vacuum. The vacuum pressure was about 38 Pa and the detector temperature is about 170 ºC. Therefore, liquid nitrogen needs to be added at the time of analysis. The measurement condition of x-ray spectrometer was used Rh target. The sample was run for a counting time of about 100 s and the spectrum obtained was stored and analyzed in PC based multi-channel analyzer using EDX-7000 software.

Determination of Extractable Matter Contents of Pericarp of *Garcinia mangostana* L.

Dried powdered sample (100 g) was percolated with ethanol (400 mL) for three days and then filtered. The same procedure was repeated three times. The combined filtrate was

concentrated under vacuum rotatory evaporator. The dried filtrate was transferred to a weighed porcelain basin and evaporated to dryness on water-bath to obtain ethanol extract.

For water soluble extractable matter, dried powdered sample (100 g) was boiled with distilled water (300 mL) for 2 h. The extract was then filtered and transferred to a weighed porcelain basin and evaporated to dryness on water-bath. The dried filtrate was placed in oven, maintained till constant weight, at 100 °C. The two extracts were stored in a desiccator containing dry silica gel prior using in each experiment.

Investigation of Bioactivities of Pericarp of *Garcinia mangostana* L.

Investigation of antioxidant activity by DPPH assay

The antioxidant activity of ethanol and watery extracts of pericarp of *G. mangostana* was determined by radical scavenging (DPPH) assay. The control solution was prepared by mixing 1.5 mL of 0.002 % DPPH solution and 1.5 mL of ethanol. The sample solution was also prepared by mixing thoroughly 1.5 mL of 0.002 % DPPH solution and 1.5 mL of test sample solution. The solutions were allowed to stand at room temperature and shaken on shaker for 30 min. The absorbance was measured at 517 nm using UV-7504 spectrophotometer. Absorbance measurements were done in triplicate for each sample and the mean values so obtained were used to calculate % radical scavenging activity (% RSA) by the following equation:

$$\% \text{ RSA} = [\{(A_{DPPH} - A_{Sample}) - A_{Blank}\} / A_{DPPH}] \times 100$$

A_{DPPH} = the absorbance of the control solution

A_{Sample} = the absorbance of the sample solution

A_{Blank} = the absorbance of the blank solution

Then, IC_{50} value was calculated by linear regressive excel program and the mean % RSA and standard deviation was also calculated by excel program.

Investigation of antitumor activity by Potato Crown Gall test

Tumor producing bacteria, *Agrobacterium tumefaciens* (Smith and Townsend) Conn. was obtained from the extraction of the leaf of *Sandoricum koetjape* Merr. (Thitto), family Meliaceae. All of these strains have been maintained as solid slants under refrigeration. For inoculation into the potato discs, 48 h broth culture containing 9×10^9 cell/mL was used by the method of Galsky *et al*., 1980. Tubers of moderated size obtained from fresh potato were surface sterilized by immersion in 50 % sodium hypochlorite (clorox) for 20 min. The ends were removed and soaked for 10 min more in clorox. A core of the tissue was extracted from each tuber by using surface sterilized (ethanol and flame) 1.0 cm wide cork borer. Pieces of 2 cm were removed from each end and discarded. The remainder of the cylinder is cut into 0.5 cm thick discs with a surface sterilized cutter. The discs were then transferred to agar plants (1.5 g of agar was dissolved in 100 mL distilled water, autoclaved for 20 min at 121°C, 20 mL was poured into each petri dish). Each plate contained three potato discs and three plates were used for each sample dilution.

Samples (0.15 g, 0.1 g, 0.05 g) of ethanol and watery extracts were respectively dissolved in dimethyl sulphoxide (DMSO) (2 mL) and filtered through Millipore filter (0.22 μm) into sterile tube. Each solution (0.5 mL) was added to sterile distilled water (1.5 mL) and broth culture (2 mL) of *A.tumefaciens* strains. Controls were made in this way, DMSO (0.5 mL) and

sterile distilled water (1.5 mL) were added to the tube containing broth culture (2 mL) of *A.tumefaciens* strains. By using a sterile disposable pipette, one drop (0.5 mL) from these tubes was used to inoculate each potato disc spreading it over the discs surface. After inoculation, petri dishes were sealed by paraffin and incubated at 27-30 °C for 3 days. Tumors were observed on potato discs after 3 days under stereomicroscope followed by staining with Lugol's solution (5 % I_2 and 10 % KI) after 30 min and compared with control. The antitumor activity was examined by observation of crown gall produced or not.

Isolation and Identification of Phytoconstituent from the Ethanol Crude Extract

The ethanol crude extract was subjected to chromatography over a silica gel column. The column was initially eluted with $CHCl_3$:CH_3OH (19:1 v/v) solvent system and the fractions were collected at the rate of one drop per second. Gradient elution was performed successively with $CHCl_3$:CH_3OH (19:1, 9:1, 4:1 and 1:1). A quantity of 10 mL was collected for each fraction and chromatographic separation was monitored by TLC. Spots on TLC were examined under UV lamp (254 and 365 nm). Fractions that showed similar TLC pattern were combined to provide four main fractions. Fraction F_2 was crystallized in benzene to provide yellow crystalline solid compound.

The isolated compound was characterized by melting point determination, TLC examination and spectroscopic techniques such as Shimadzu UV-240 UV–visible Spectrophotometer and Shimadzu FT IR-8400 Fourier Transform Infrared Spectrophotometer.

Results and Discussion

Phytochemicals Present in Pericarp *Garcinia mangostana* L.

Phytochemicals or phytoconstituents are non-nutrient plant chemical compounds and are responsible for protecting the plant against microbial infections (Doughari *et al*., 2009). The phytochemical investigation of pericarp of *G.mangostana* showed that α-amino acids, carbohydrates, flavonoids, glycosides, organic acids, phenolic compounds, saponins, tannins, steroids and terpenoids were present. However, alkaloids, cyanogenic glycosides, reducing sugars and starch were not detected.

Some Elements Present in Pericarp of *Garcinia mangostana* L.

The relative abundance of elements in dried powder sample was determined by using EDXRF spectrometer and the EDXRF spectrum is described in Figure 2. It can be found that the sample contains K (0.354 %), S (0.074 %), Ca (0.046 %), Mn (0.003 %), Fe (0.003 %), Cu (0.001 %) and Zn (0.001 %) respectively. According to the results, potassium was found to be the principal element. Potassium is a vasodilator, meaning that it relaxes blood vessels and by rigidity, it increases blood flow and reduces the strain on the cardiovascular system. It is an essential component of neural activity and the passage of fluid and blood in the brain.

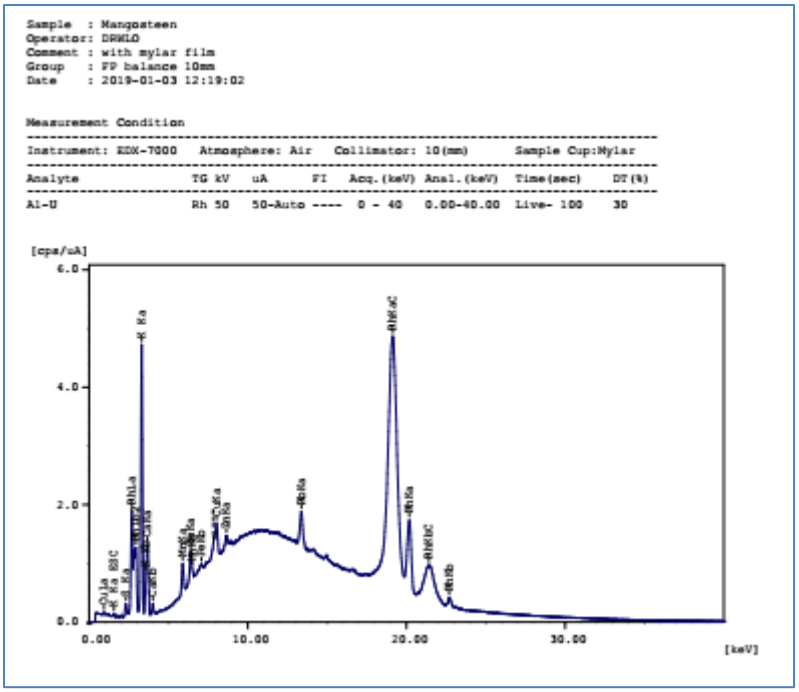


Figure 2 EDXRF spectrum of pericarp of *Garcinia mangostana* L.

Table 1 Some Elements Present in Pericarp of *Garcinia mangostana* L.

No.	Elements	Relative Abundance (%)
1	K	0.354
2	S	0.074
3	Ca	0.046
4	Mn	0.003
5	Fe	0.003
6	Cu	0.001
7	Zn	0.001

Extractable Matter Contents of Pericarp of *Garcinia mangostana* L.

After performing the preliminary phytochemical tests, it is required to investigate some organic constituents present in pericarp of *G.mangostana*. Ethanol and watery crude extracts were prepared and the percentage of crude extract yield was derived from the weight of dried and ground plant material. According to the results, it was observed that the yield of ethanol extract (15.2 %) was higher than that of watery extract (12.3 %). It can be concluded that the amount of active constituents contained in ethanol extract was higher than watery extract.

Antioxidant Activity of Pericarp of *Garcinia mangostana* L.

Antioxidant activity screening of ethanol and watery extracts of pericarp of *G. mangostana* was carried out by determining the DPPH free radical scavenging property using the UV spectroscopic method. DPPH free radical scavenging assay has been widely used especially evaluation of antioxidant potential in food system. DPPH radical is reduced to the corresponding to hydrazine when it reacts with hydrogen donors that can be phenolic compounds. Plant phenolic contributes one of the major groups of antioxidants acting as free

radical terminators. In this study, six different concentrations (50, 25, 12.5, 6.25, 3.125 and 1.5625 µg/mL) of each crude extract were prepared by serial dilution method. Ascorbic acid was used as standard to be compared with the samples and ethanol without sample was employed as control. After mixing with the DPPH solution, the absorbance of each solution was measured at 517 nm using UV-visible spectrophotometer.

For both extracts, the percent inhibition of DPPH radical was plotted as a function of concentration in order to determine the IC_{50} value, which is defined as the necessary sample concentration to reduce 50. The results of % RSA and IC_{50} were shown in Figure 3 and Table 2. According to the results, it was observed that ethanol extract (IC_{50} = 8.15 µg/mL) of pericarp of mangosteen contained higher scavenging activity of DPPH radical in comparison with the watery extract (IC_{50} = 12.3 µg/mL), due to the lower value of IC_{50}. The ethanol extract contains a high quantity of bioactive compounds able to capture free radicals like DPPH. However, it was observed that ethanol extract has the lower radical scavenging activity than standard ascorbic acid (IC_{50} = 4.52 g/mL).

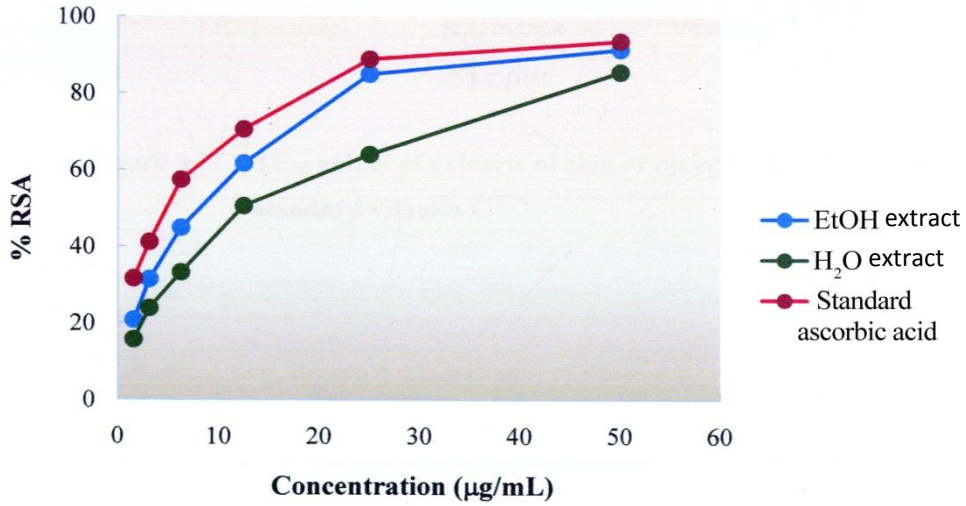


Figure 3 Percent radical scavenging activity vs concentration of crude extracts of *Garcinia mangostana* L. pericarp and standard ascorbic acid

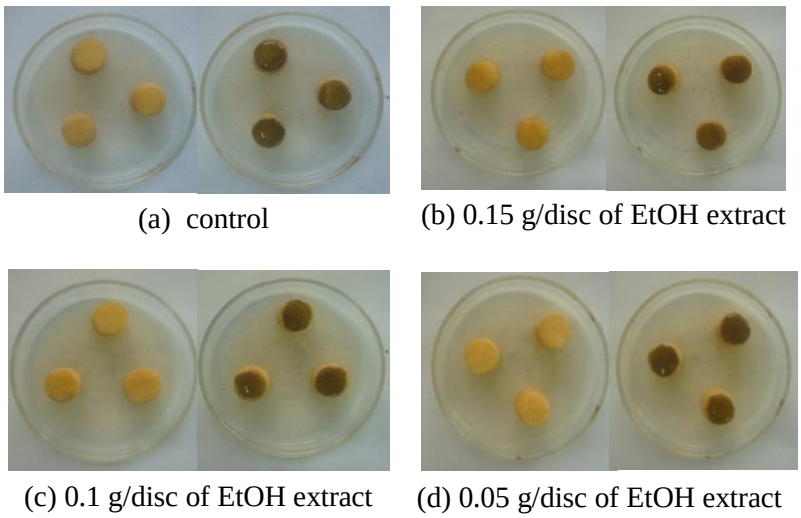
Table 2 Percent Radical Scavenging Activity and IC_{50} Values of Crude Extracts of *Garcinia mangostana* L. Pericarp and Standard Ascorbic acid

Sample	% RSA ± SD at different concentrations (µg/mL)						IC_{50} (µg/mL)
	50	25	12.5	6.25	3.125	1.5625	
EtOH extract	91.46 ± 0.39	84.99 ± 0.97	61.71 ± 0.78	44.90 ± 1.56	31.40 ± 3.51	20.80 ± 2.92	8.15
H_2O extract	85.54 ± 0.58	64.05 ± 2.14	50.55 ± 1.75	33.20 ± 2.92	23.83 ± 3.31	15.70 ± 3.51	12.3
Ascorbic acid	93.66 ± 0.78	88.98 ± 0.39	70.66 ± 0.97	57.44 ± 2.53	41.18 ± 3.31	31.68 ± 2.34	4.52

Antitumor Activity of Pericarp of *Garcinia mangostana* L.

The antitumor activity of ethanol and watery extracts of mangosteen pericarp was investigated by Potato Crown Gall test with *Agrobacterium tumefaciens* (Smith and Townsend) Conn. isolated from *Sandoricum koetjape* Merr. (Thitto) leaf. For inoculation of the potato discs, 48 h broth cultures containing $5x10^9$ cells/ mL were used. The tested samples were dissolved in DMSO and the diluted samples were mixed with the bacterial culture for inoculation. After preparing the inoculations, the bacterial suspensions was inoculated on the cleaned and sterilized potato discs, and incubated for 3 days at room temperature. After that, the tumor on potato discs were checked by staining the Knob with Lugol's solution (5 % I_2 and 10 % KI). In control disc, formation of white Knob on the blue background indicated the presence of tumor cells because there is no protein in tumor cells. The tested samples have no tumor formation on the potato discs and their surfaces remained blue as shown in Figures 4 and 5. In this study, it was found that ethanol and watery extracts of mangosteen pericarp were effective for preventing the tumor formation with the doses of 0.15, 0.1 and 0.05 g/disc and the results are shown in Tables 3 and 4. This experiment revealed that ethanol and watery extracts of mangosteen pericarp can inhibit the tumor growth.

Antitumor potato disc assay is valuable tool that indicates antitumor activity of test by their inhibition of formation of characteristic crown galls induced in wounded potato tissues by *A.tumefaciens* (Smith and Townsend) Conn. Tumor formations were inhibited by the plant extracts only for the presence of bioactive compounds (Inayatullah *et al*., 2007). Potato disc bioassay was based on *A.tumefaciens* (Smith and Townsend) Conn. infection on potato disc; it becomes useful for checking antitumor properties of biological and synthetic bioactive compounds. This assay has advantages of being short period, inexpensive, simple and reliable pre-screen for antitumor activity.



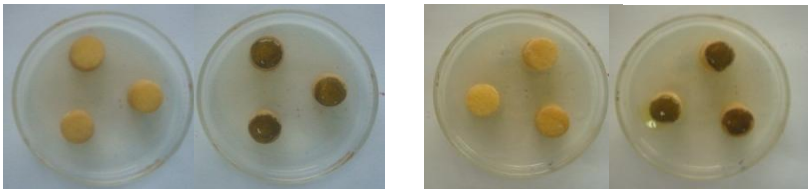
(a) control (b) 0.15 g/disc of EtOH extract

(c) 0.1 g/disc of EtOH extract (d) 0.05 g/disc of EtOH extract

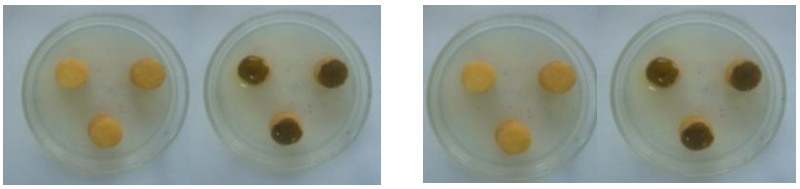
Figure 4 Tumor inhibition by EtOH extract of pericarp of *Garcinia mangostana* L.

Table 3 Antitumor Activity of EtOH Extract of *Garcinia mangostana* L. Pericarp

No.	Tested sample	Concentration of sample (g/disc)	Tumor	Remarks
1.	Control	0	++	Tumor occur significantly
2.	EtOH	0.15	-	No tumor occur
3.	EtOH	0.1	-	No tumor occur
4.	EtOH	0.05	-	No tumor occur



(a) control (b) 0.15 g/disc of H_2O extract



(c) 0.1 g/disc of H_2O extract (d) 0.05 g/disc of H_2O extract

Figure 5 Tumor inhibition by H_2O extract of pericarp of *Garcinia mangostana* L.

Table 4 Antitumor Activity of H_2O Extract of *Garcinia mangostana* L. Pericarp

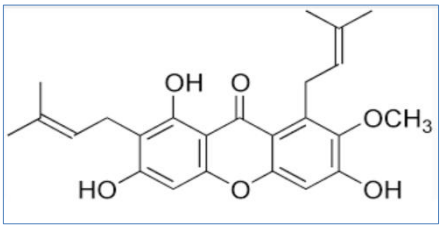
No.	Tested sample	Concentration of sample (g/disc)	Tumor	Remarks
1.	Control	0	++	Tumor occur significantly
2.	H_2O	0.15	-	No tumor occur
3.	H_2O	0.1	-	No tumor occur
4.	H_2O	0.05	-	No tumor occur

Identification of the Isolated Compound

The yellow crystalline solid compound (0.09 % yield) was isolated from fraction F_2 of the ethanol extract on silica gel by column chromatography using $CHCl_3$:MeOH (9:1) solvent system. The melting point of isolated compound was found to be 180-181 °C which consistent with reported value of mangostin (Windholz, 1983). Isolated compound provided deep blue colour with 5 % ferric chloride solution and its R_f value was 0.44 in $CHCl_3$:CH_3OH (9:1) solvent system. The ultraviolet spectrum of isolated compound in methanol solvent is shown in Figure 6. The wavelengths of maximum absorption were found to be 241, 257, 319 and 353 nm which agreed with reported value of mangostin (Windholz, 1983).

The FT IR spectrum of isolated compound is shown in Figure 7. Absorption bands appeared at 3419 cm^{-1} and 3255 cm^{-1} were attributed to O-H stretching vibration of phenolic OH

group. Aliphatic C-H stretching vibrations of CH_3 and CH_2 groups were observed at 2962 cm^{-1}, 2918 cm^{-1} and 2852 cm^{-1}, respectively. The C=O stretching vibration of xanthone ring observed at 1643 cm^{-1}. Absorption bands at 1610 cm^{-1} and 1460 cm^{-1} were assigned to the aromatic ring stretching vibration. The O-H bending vibration was observed at 1377 cm^{-1} and stretching vibration of C-O group was observed at 1284 cm^{-1}. The absorption band at 852 cm^{-1} was assigned as aromatic C-H out of plane bending (Silverstein and Webster, 1998). According to the melting point determination, UV and FT IR spectral data, isolated compound may be identified as mangostin with the following molecular structure. It was further confirmed by Co TLC with authentic mangostin.



(Mangostin)

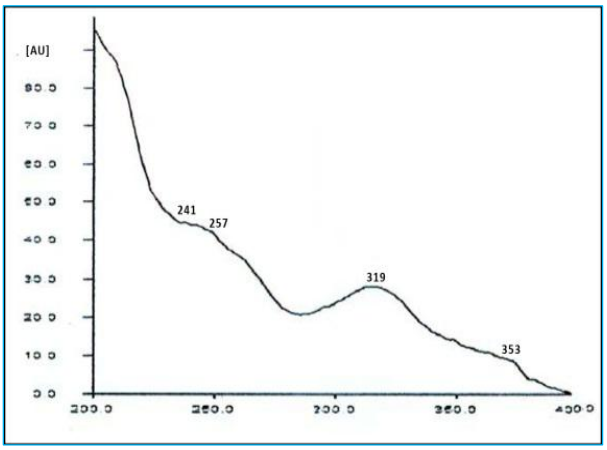


Figure 6 UV spectrum of the isolated compound (MeOH)

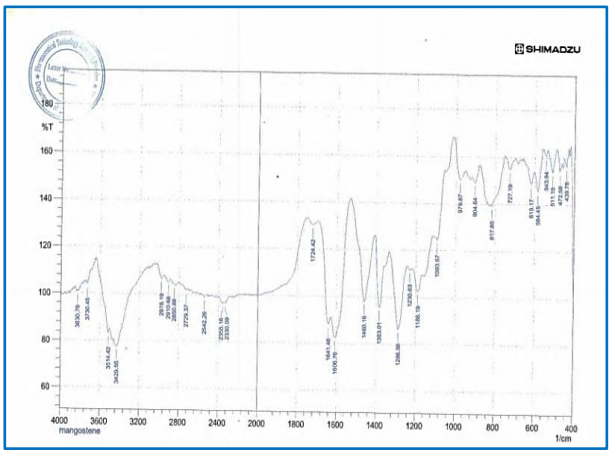


Figure 7 FT IR spectrum of the isolated compound (KBr)

Conclusion

From the preliminary phytochemical investigation, it was found that α-amino acids, carbohydrates, flavonoids, glycosides, organic acids, phenolic compounds, saponins, tannis, steroids and terpenoids were present in pericarp of mangosteen. However alkaloids, cyanogenic glycosides, reducing sugars and starch were not detected. In the qualitative elemental analysis, content of K is more significant than other elements such as S, Ca, Mn, Fe, Cu and Zn. In extractable matter contents, the percentage of ethanol extract (9.34 %) was found to be higher than watery extract (5.98 %). In screening of antioxidant activity using DPPH assay, it was observed that free radical scavenging efficacy of ethanol extract (IC_{50} = 8.15 µg/mL) was better than watery extract (IC_{50} = 12.3 µg/mL). The high antioxidant activity of the ethanol extract at low concentration makes the pericarp of mangosteen as a potential source of antioxidant. In addition, the antitumor activity of ethanol and watery extracts was also screened by Potato Crown Gall test. In this study, both extracts were effective for preventing the tumor growth with

the doses of 0.15 , 0.1 and 0.05 g/disc. On silica gel column chromatographic separation, mangostin (0.09 %, 180-181 °C) was isolated from ethanol extract and then identified by UV and FT IR spectroscopic methods compared with authentic mangostin. According to the results obtained from this research, it can be hoped that the waste product of mangosteen pericarp is beneficial to use for the people. In Myanmar, mangosteen is locally abundant and seasonally available in markets. So, mangosteen should be consumed for health.

Acknowledgements

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BIOSYNTHESIS OF THE SILVER NANOPARTICLES USING TWO LEAVES EXTRACTS FROM *E.ODORATUM* (TBAGNPS) AND *C. CITRATUS* (LGAGNPS) AND ITS BIOLOGICAL ACTIVITIES

Nwe Thin Ni[1], Aung Kyaw Moe [2]

Abstract

The fabrication of silver nanoparticles by using watery extracts of *Eupatorium odoratum* L. (Taw-Bizat) and *Cymbopogon citratus* Stapf (Lemon Grass) leaves was mainly conducted. Silver nitrate was used as a metal precursor and two selected leaves extracts were applied as biomimetic routes of reducing agent for synthesis of silver nanoparticles (AgNPs). The surface morphology of fabricated silver nanoparticles (AgNPs) were performed with FESM (Field Emission Scanning Electron Microscopy). From the result of XRD (X-ray diffraction), the average particle size of TBAgNPs (32 nm) from *E. odoratum* and LGAgNPs (38 nm) from *C. citratus* watery extracts were observed. The size distribution of each prepared (AgNPs) was analysed by using advanced techniques Zeta potential-DLS (Dynamic Light Scattering). The localized surface plasmon resonance bands for formation of TBAgNPs (440 nm) and LGAgNPs (435 nm) in the ratio of 1:3 and 1:5 were exhibited in UV-visible spectra. The role of selected stabilizing agent of *E. odoratum* and *C. citratus* leaves extracts and the effect of stirring time on synthesis of AgNPs was reported. Furthermore, the laser beam of reflected rays as observed in the fabrication of AgNPs by the Tyndall effect. In addition, the antioxidant activities of TBAgNPs and LGAgNPs (49.27 μg/mL, 94.32 μg/mL) were determined by DPPH radical scavenging assay method. The antimicrobial activities of two kinds of leaves extracts and the prepared TBAgNPs and LGAgNPS were discussed against different strains of microorganisms.

Keywords: fabrication, biosynthesis, morphology, average particle size, antioxidant, antimicrobial activity

Introduction

Nano-chemistry deals with synthesis of nanoscale building blocks with controlled size, shape, structure and their composition and their organization into functional architecture using self-assembly templating and lithographic techniques. A length scale is used by a nanometer, nanoscience and nanotechnology have been around for several decades, particularly in research, development and manufacturing in information technology, where film layers and lithographically defined features in the nanometer range are needed (Guzman, 2008). Nowadays, the nanomaterials become critically important because of their unique properties such as physical, magnetic, structural, thermal, mechanical, chemical and electronic properties. Nanoparticles can be divided into two groups: (i) organic nanoparticles and (ii) inorganic nanoparticles (Kumar and Yadav, 2009). Organic nanoparticles contain carbon nanoparticles. Inorganic nanoparticles involve magnetic nanoparticles, noble nanoparticles (like gold and silver), semiconductor nanoparticles (like titanium dioxide) possess optical properties (Albrecht, 2006). The synthesis of NPs are broadly divided into two main classes: (1) bottom-up approach and (2) top-down approach. The breakdown (top-down) method by which an external force is applied to a solid that leads to its break-up into smaller particles. The build-up (bottom-up) method that produces nanoparticles starting from atoms or gas or liquid based on atomic transformation or molecular condensation (Guzman , 2008; Veerasamy *et al.*, 2011). Silver

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nanoparticles are used in various fields, especially in biomedical industry or diagnosis, drug delivery, cell imaging, and implantation. Biosynthesis of silver nanoparticles could be advantages than photochemical reduction and chemical reduction methods (Saxena *et al.*, 2012). In biosynthesis including plant, bacteria, fungi and yeast have been used to prepare nanoparticles (Fatma and Nivien, 2015; Kumar *et al.,* 2014). Therefore, in this research proposal, biosynthesis of obtained silver nanoparticles (TBAgNPs, LGAgNPs) in the environmental friendly were conducted by using the two selected plants namely: *Eupatorium odoratum* Linn. and, *Cymbopogon citratus* Stapf. These two plants were widely distributed in the tropical and subtropical regions of Africa, Asia and America. In Myanmar, they are widely distributed throughout the country (Emmanuel and Anthony, 2017). Then, characterization of fabricated silver nanoparticles was studied by applying advanced modern techniques (Masurkar *et al.*, 2011). The antioxidant activity of fabricated AgNPs was also studied by using DPPH assay method. After that, the determination of inhibitory effect of reducing agent and prepared silver nanoparticles were studied against six kinds of microorganisms.

Materials and Methods

Sample Collection

The leaves of *E. odoratum* and *C. citratus* were collected from Hmone Pya village, Daw Oo Khu Quarter, Loikaw Township, Kayah State, Myanmar in the middle of November, 2017 [Figures 1 (a), (b)]. After cleaning, the leaves were air-dried at room temperature for three weeks and the dry sample were ground into powder and stored separately in air-tight containers to prevent moisture changes and other contamination. These selected plants were identified at the Department of Botany, University of Yangon.

(a) (b)

Figure 1 Plant of (a) *E. odoratum* L. (b) *C. citratus* Stapf

Preparation of Silver Nanoparticles (TBAgNPs, LGAgNPs)

Each of dried powdered sample of *E.odoratum* and *C. citratus* (30 g) was boiled in 150 mL of distilled water for 48 h to obtain the respective extract. This obtained extract was filtered through Whatman filter paper No.1. These filtrates were cooled down at 4 °C. Then, 0.017 g of $AgNO_3$ was dissolved in a volumetric flask with 10 mL of distilled water and made up to 100 mL of the solution to give 0.001 M of $AgNO_3$ solution. It was used in this biosynthesis of silver nanoparticles. 20 mL each of the prepared extract was added to the 0.001 M of $AgNO_3$ solution (60, 80,100 mL) as the different volume ratios of 1:3, 1:4, 1:5 v/v in each conical flask under aseptic condition. The flask was heated and stirred with magnetic stirrer at different temperatures 40 °C, 50 °C, 60 °C and different stirring times (20 min, 40 min, 60 min). Then, it was placed in a dark place over night. A change in the colour was observed indicating the

formation of silver nanoparticles. The solution was centrifuged at 6000 rpm for 20 min to obtain silver nanoparticles and supernatant was discarded. Then, the obtained particles from *E.odoratum* (TBAgNPs) and *C. citratus* (LGAgNPS) were washed to purify and dried at 100°C in an oven for 24 h.

Characterization of the Prepared Silver Nanoparticles

The Field Emission Scanning Electron Microscopy (FESEM) technique was applied to analyze the surface morphology of fabricated TBAgNPs, LGAgNPs. The prepared AgNPs was characterized by XRD analysis and the particle size was calculated by using Debye- Scherrer equation. In addition, determination of particle size distribution of AgNPs was measured by using Zeta potential-DLS instruments at 25 °C with percent intensity. The localized surface plasmon resonance bands of AgNPs were studied by using Shimadzu UV-1800 spectrometer.

Screening of Antioxidant Activity of the Prepared Silver Nanoparticles

Antioxidant activity of the prepared AgNPs was determined UV-visible spectroscopic by using DPPH (1,1-diphenyl, 2-picryl, hydrazyl) radical scavenging assay (Halliwell, 2012).

Preparation of test sample solutions

4 mg of each of the prepared TBAGNPs and LGAgNCs was dissolved in 10 mL of 95 % ethanol and thoroughly mixed by vortex mixer. The mixture solution was filtered and filtrate was used as a stock solution, 4 μg/mL. Desired concentrations (400, 200, 100, 50, 25, 12.5, 6.25, 3.125 μg/mL) of sample solutions were prepared from the stock solution by serial dilution with appropriate amount of 95 % ethanol.

Preparation of 60 μM DPPH solution

To get a 60 μM DPPH solution, 2.364 mg of DPPH was thoroughly dissolved in 100 mL of 95 % ethanol. This solution was freshly prepared in the brown coloured flask and kept in refrigerator for no longer than 24 h.

Preparation of blank solution

Blank solution was prepared by mixing 1.5 mL each of the test sample solution with 1.5 mL of 95 % ethanol.

Procedure

The control solution was prepared by mixing of 1.5 mL of 60 μM DPPH solution and 1.5 mL of 95% ethanol using vortex mixer, (Halliwell, 2012). The sample solution was also prepared by mixing thoroughly 1.5 mL of 60 μM DPPH solution and 1.5 mL of test sample solution. The solution was allowed to stand at room temperature for 30 min. After 30 min, the absorbance of these solutions were measured at 517 nm by UV-visible spectrophotometer. Absorbance measurements were done in triplicate for each solution and the mean values so obtained were used to calculated the percent inhibition of oxidation, (Kahlonene, 1999).

$$\% \text{ oxidative inhibition of the sample} = \frac{A_{Control} - (A_{Sample} - A_{Blank})}{A_{Control}} \times 100$$

$A_{Control}$ = absorbance of DPPH in 95 % EtOH solution

A_{Sample} = absorbance of sample and DPPH solution

A_{Blank} = absorbance of sample and 95 % EtOH solution

$$\text{Average, } \bar{x} = \frac{x_1 + x_2 + x_3 + \ldots + x_n}{n}$$

$$\text{Standard derivation (SD)} = \sqrt{\frac{(\bar{x}-x_1)^2+(\bar{x}-x_2)^2+(\bar{x}-x_3)^2+\ldots+(\bar{x}-x_n)^2}{n-1}}$$

$\bar{x}$ = average % inhibition

$x_1+x_2+\ldots..+x_n$ = % inhibition of test sample solution

n = number of times

Then, IC_{50} (50 % oxidative inhibitory concentration) values were also calculated by linear regressive excel program.

Screening of Antimicrobial Activity of the Prepared AgNPs

Agar well diffusion method (Balouiri *et al.*, 2016) was employed for determining antimicrobial activity of the plant extracts and prepared AgNPs: TBAgNPs and LGAgNPs against six pathogenic microorganisms namely *Bacillus subtilis*, *Staphylococus aureus*, *Pesudomonas aeruginosa*, *Bacillus pumilus*, *Candida albicans* and *Escherichia coli*, (Cruickshank, 1960) at PRD, Pharmaceutical Research Department, Ministry of Industry, Yangon, Myanmar.

Results and Discussion

Sample Collection and Preparation of Reducing Agent

Currently, the leaves of *E.odoratum* and *C. citratus* were collected from Hmone Pya village, Daw Oo Khu Quarter, Loikaw Township, Kayah state, Myanmar. The dried leaves powdered was extracted with distilled water and it was used as reducing agent as well as capping agent in the preparation of AgNPs.

Synthesis of Silver Nanoparticles (AgNPs)

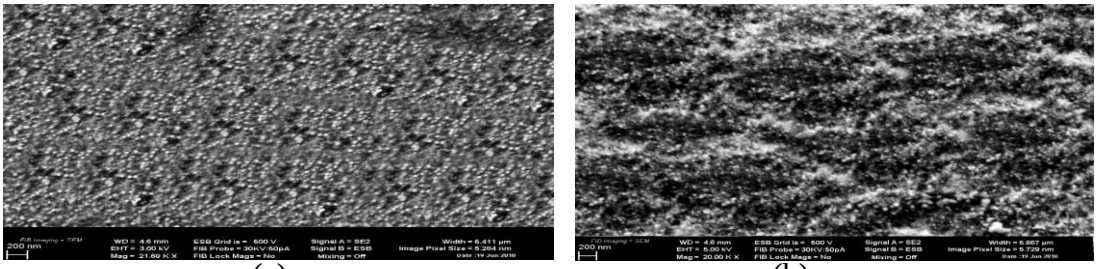
The solution of silver nitrate (0.001 M) was used as a metal precursor for this synthesis of silver nanoparticles. The fabricated silver nanoparticles (TBAgNPs, LGAgNPs) were observed under visual condition. AgNPs were formed with a colour change from yellow to brownish-black colour during the reaction period within 20 min. The colour change of brownish-black was observed in the formation of AgNPs and it was due to the effect of reducing agent as well as capping agent of *E. odoratum* and *C. citratus* leaves extracts of selected sample in this research. The extract (20 mL) from each of the *E. odoratum* and *C. citratus* leaves was added to different volumes 0.001 M of silver nitrate solution (60, 80,100 mL). The various ratios of leaves extracts and $AgNO_3$ (1:3, 1:4, 1:5 v/v) solutions were separately placed in a conical flask. The solution

was mixed on a magnetic stirrer while heating at a temperature of about 40 °C, 50 °C, 60 °C. After stirring time for 20, 40, 60 min, it was kept in the dark place. Then, this reaction process was carried out in dark to avoid unnecessary photochemical reactions (Harris and Bali, 2008). A change in the colour was observed and it indicated the formation of silver nanoparticles. The solution was centrifuged at 6000 rpm for 20 min to obtain silver nanoparticles and supernatant was discovered. Among them, 1:5 v/v of leaves extracts and $AgNO_3$ solution at 60 °C and stirring time 60 min was observed not in the colloidal state and can be filtered easily than other conditions. The particles obtained were washed and dried at 100 °C in an oven for 24 h. The dried particles were observed in 1.816 g.

Characterization of the Prepared Silver Nanoparticles

FESEM analysis

The AgNPs (TBAgNPs and LGAgNPs) prepared by using 1:5, v/v ratio of each plant extract and 0.001 M $AgNO_3$ solution at 60 °C with 20 min were chosen for studying their characteristics. The surface morphology of prepared TBAgNPs and LGAgNPs were studied by the field emission scanning electron microscopy (FESEM). From these observation, the surface morphology of each prepared silver nanoparticles TBAgNPs was observed very smoothly and in more spherical nature than LGAgNPs (Figure 2).

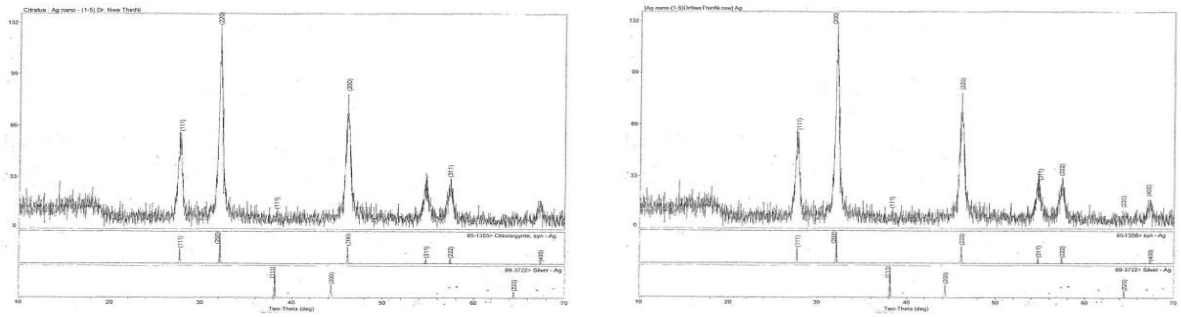


(a) (b)

Figure 2 FESM images of the prepared AgNps (a) TBAgNPs (b) LGAgNPs

XRD analysis

The average crystalline size of the prepared TBAgNP and LGAgNPs was determined by X Ray Diffratometer (XRD) analysis and calculated by using Debye-Scherrer's equation. From XRD diffractogram of TBAgNP, the four distinct diffraction peaks at 2θ values of 29.879, 30.128, 36.401 and 44.566 were respectively indexed to 111, 200, 220 and 311 reflection planes of face centered cubic structure of silver. In addition, the average particle size of LGAgNP was also calculated from three distant peaks of 111, 220, 200 with 2θ values of 35.138, 40.401, 37.495. The average particle size of TBAgNP (32 nm) occurred smaller than that of LGAgNPS (38 nm) (Figure 3).

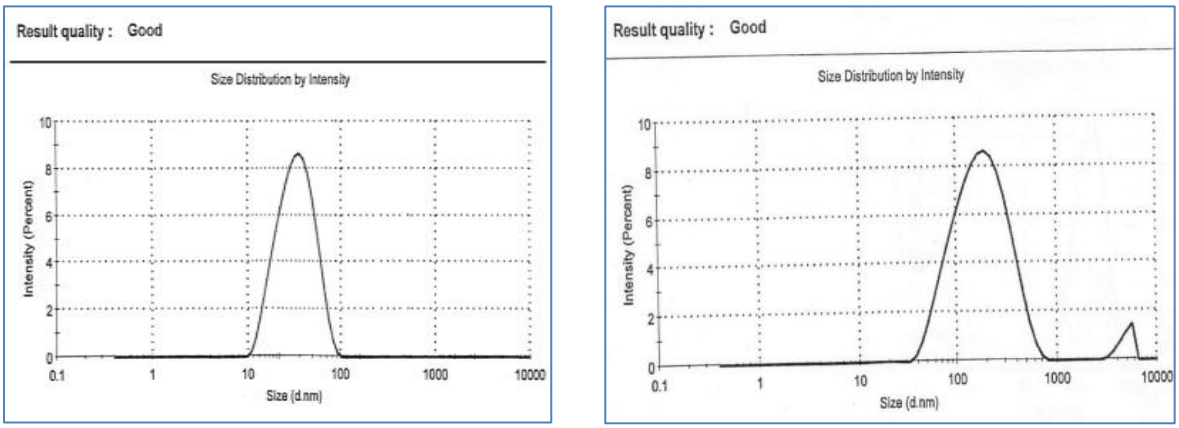


(a) (b)

Figure 3 XRD Diffractograms (a) TBAgNPs (b) LGAgNPs

Zeta potential-DLS analysis

The size distribution of prepared AgNPs was reported with intensity using zeta potential-DLS and shown in Figure 4. From this result, the size distribution of prepared TBAgNPs and LGAgNPs were observed between 1-100 nm range with peak intensity.

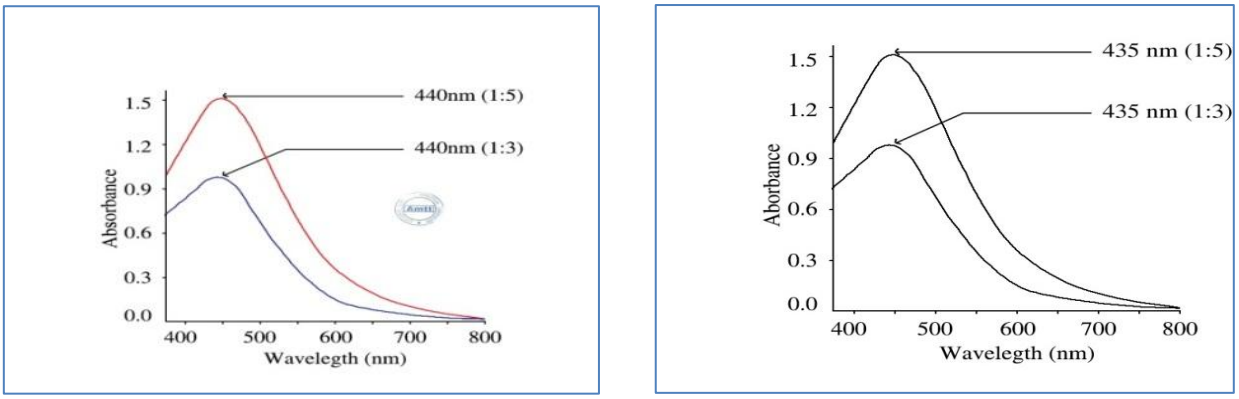


(a) (b)

Figure 4 The Zeta potential- DLS spectra of the prepared (a) TBAgNPs (b) LGAgNPs

UV-visible spectral analysis

The formation of stability of metal nanoparticles in aqueous solution was determined by using the UV-visible spectroscopy and it is an important technique to exhibit UV-visible absorption maximum in the range of 300-360 nm due to the excitation of surface plasmon vibration. The localized surface plasmon resonance bands were observed at 440 nm for TBAgNPs and 435 nm for LGAgNPs. The increase in the concentration of the silver nitrate increased the absorbance intensity but the wavelength was not changed (Figure 5). The absorbance intensity of the AgNPs prepared by using 1:5, v/v ratio of leaves extract and $AgNO_3$ solution was observed to be lighter than the AgNPs prepared by using 1:3 v/v ratio of leaves extracts and $AgNO_3$ solution.

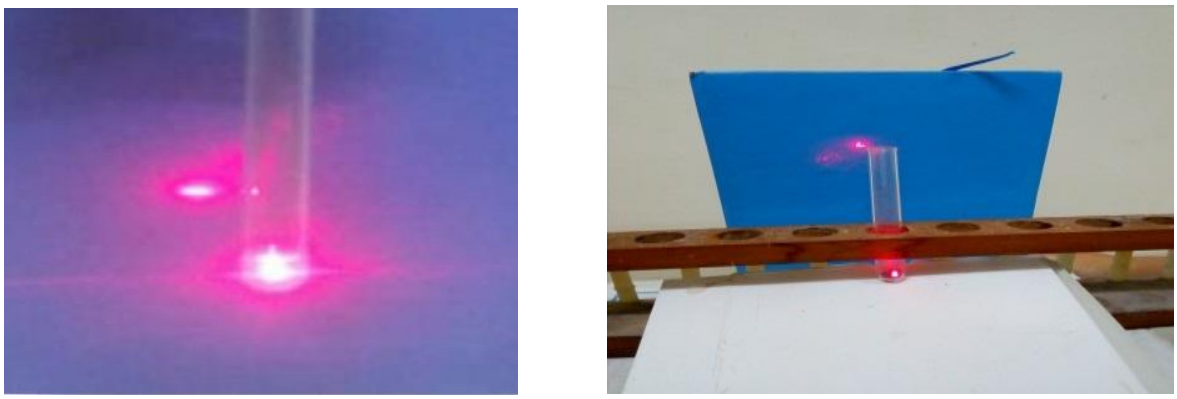


(a) (b)

Figure 5 UV visible absorption spectra of the prepared AgNPs (a) TBAgNPs (b) LGAgNPs

Tyndall effect

The Tyndall effect, also known as Willis-Tyndall scattering is light scattering by particles in a colloid or in a very fine suspension. The particle even large enough that they can be scattered light, the Tyndall effect occurred (Saxena, 2012). Since the presence of a colloidal suspension can be monitored by the reflection of a laser beam from the particles because a laser pointer emitted that the polarized light, and the pointer can also be oriented that the beam appear to disappear. If the colloidal particles are present, the laser beam passed and if the particles are absent, the beam did not pass through it. In these experiments, the laser beam was completely observed to pass through the both of the prepared nanoparticles of TBAgNPs and LGAgNPs, (Figure 6).



(a) (b)

Figure 6 Tyndall effect of light scattering the prepared AgNPs (a) TBAgNPs (b) LGAgNPs

Antioxidant Activity of the Prepared Silver Nanoparticles

The antioxidant activity was expressed as 50 % oxidative inhibitory concentration (IC_{50}). The lower the IC_{50} values, the higher the antioxidant activity of the sample. By using DPPH free radical scavenging assay, the IC_{50} values of the prepared TBAgNPs and LGAgNP were respectively observed to be 49.27 μg/mL and 94.32 μg/mL. The more potent antioxidant activity was observed in TBAgNPs than LGAgNPs, (Figure 7 and Table 1). Besides, the IC_{50} values of the standard ascorbic acid was found to be 7.28 μg/mL.

Antimicrobial Activity of Watery Extract of Plant Leaves and and Prepared Silver Nanoparticles

The antimicrobial activities of the plant leaves extracts and the prepared TBAgNPs and LGAgNPs were evaluated against six strains of microorganisms: *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus pumilus, Candida albicans, Escherichia coli* by using agar well diffusion method (Table 2). Therefore, nanoparticles were generally more active than reducing agent (plant extract) against the selected microorganism. It is reasonable to state that the binding of the particles to the bacteria depends on the surface area available for interaction. This is because, the silver nanoparticles may attach to the surface of the cell membrane and disturb its power function such as permeability and respiration, (Balouiri *et al.*, 2016). The two plant extracts and prepared AgNPs showed moderately antimicrobial activities. Among them, more potent antimicrobial activity was observed in TBAgNPs (20 mm) than LGAgNPs (18 mm) against *Bacillus pumilus*, and *Escherichia coli*.

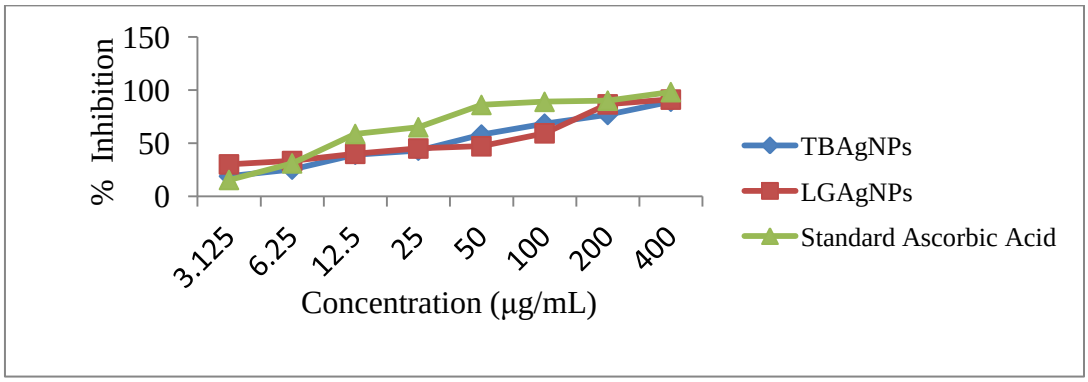


Figure 7 % inhibition of different concentrations of the prepared AgNPs and standard ascorbic acid

Table 1 Oxidative Percent Inhibitions and IC_{50} Values of Different Concentrations of Prepared Silver Nanoparticles and Standard Ascorbic Acid

Sample	% Inhibitions (Mean ± SD) in various Concentrations (µg/ml)								IC_{50} (µg/mL)
	3.125	**6.25**	**12.5**	**25**	**50**	**100**	**200**	**400**	
TBAgNP	19.134 ± 0.253	25.392 ± 0.267	39.421 ± 0.187	**43.239** ± 0.394	**58.344** ± 0.181	68.558 ± 0.258	76.788 ± 0.402	89.247 ± 0.597	49.27
LGAgNP	30.289 ± 0.503	33.421 ± 0.401	40.231 ± 1.502	45.238 ± 0.651	**47.342** ± 0.678	**59.518** ± 1.702	86.718 ± 0.501	91.238 ± 1.823	94.32
Standard Ascorbic acid	15.646 ± 0.542	**31.225** ± 0.472	**58.891** ± 0.723	65.221 ± 0.626	86.221 ± 0.792	89.128 ± 0.392	90.234 ± 1.143	98.245 ± 0.682	7.28

Table 2 Inhibition Zone Diameters of Watery Extract of Plant Leaves and AgNPs Against Six Microorganisms by Agar Well Diffusion Method

No.	Microorganisms	Inhibition zone diameter (mm)			
		Watery extract of Tawbizat	Watery extract of Lemongrass	Prepared TBAgNPs	Prepared LGAgNPs
1.	*Bacillus subtilis*	16 (++)	17 (++)	15 (++)	17 (++)
2.	*Staphylococcus aureus*	16 (++)	17 (++)	16 (++)	17 (++)
3.	*Pseudomonas aeruginosa*	15 (++)	16 (++)	17 (++)	15 (++)
4.	*Bacillus pumilus*	15 (++)	17 (++)	**20** (+++)	18 (+++)
5.	*Candida albicans*	16 (++)	16 (++)	18 (++)	16 (++)
6.	*Escherichia coli*	17 (++)	17 (++)	**20** (+++)	18 (+++)

(+) = low activity, (++) = medium activity, (+++) = high activity

Conclusion

From this research work, biosynthesis of silver nanoparticles is environmental friendly and non-toxic effect in environment. In the preparation of AgNPs (55.06 %) watery extracts of *E.odoratum* (Taw-Bizat) leaves and *C. citratus* (Lemon-Grass) were used as reducing agent as well as capping agent. The surface morphology of TBAgNPs was observed to show more spherical shape and to be more smooth than LGAgNPs according to result of FESEM. In addition, the particle size distribution of TBAgNPs and LGAgNPs showed with peak intensity in the range of (10-100) nm range under the zeta potential-DLS. The average particle size of TBAgNPs (32 nm) was slightly smaller than LGAgNPs (38 nm) determined by XRD analysis. The absorbance intensity of fabricated TBAgNPs (440 nm) and LGAgNPs (435 nm) were observed under the UV- visible spectrometer. In addition, if the nanoparticles were present, the light scattering passed through was observed. Therefore, Tyndall Effect of the laser beam passed through colloidal TBAgNPs and LGAgNPs. The antioxidant activity of prepared TBAgNPs (49.27μg/mL) was higher than that of LGAgNPs (76.78 μg/mL). Furthermore, the antimicrobial activity of prepared TBAgNPs (20 mm) was observed to be higher than that of leaves extracts of Taw-Bizat and Lemon-Grass and LGAgNPs against on *Escherichia coli* and *Bacillus pumilus.*

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INVESTIGATION OF TOTAL PHENOL CONTENT AND ANTIOXIDANT POTENCY OF TAMARIND LEAVES

Thin Thin Swe[1], Kaythi Min Ko[2]

Abstract

This research deals with the study of total phenol content and antioxidant potency of leaves of *Tamarindus indica* L. (Magyi). The phytochemical investigation of tamarind leaves indicated that alkaloids, α-amino acids, carbohydrates, flavonoids, glycosides, organic acids, phenolic compounds, reducing sugars, saponins, steroids, terpenoids and tannins were found whereas starch was not detected. Nutritional values of tamarind leaves determined by AOAC methods showed that tamarind leaves consist of 11.12 % of moisture, 3.56 % of fat, 18.67 % of fiber, 5.38 % of ash, 11.63 % of protein and 49.64 % of carbohydrate. The energy value of tamarind leaves was found to be 277.12 kcal/100 g. The extractable matter contents of tamarind leaves in petroleum ether, ethyl acetate, acetone, ethanol and water determined by WHO standard method. The total phenol contents of crude extracts were analyzed by UV-visible spectrophotometric method. The total phenol contents of watery and ethanol extracts of tamarind leaves were 24.70 ± 3.38 μg GAE/mg extract and 13.30 ± 3.30 μg GAE/mg extract. Antioxidant activity of tamarind leaves was also investigated by using DPPH assay. The IC_{50} values of watery and ethanol extracts of tamarind leaves were observed to be 57.13 μg/mL and 34.47 μg/mL, respectively. The ethanol extract is more effective than watery extracts in antioxidant activity. All extracts showed mild activity when compared to the standard ascorbic acid (7.97 μg/mL).

Keywords: *Tamarindus indica* L., nutritional values, extractable matter contents, total phenol content, antioxidant activity

Introduction

Tamarind is indigenous to tropical Central Africa and is a very adaptable species, drought hardy, preferring semi-arid areas and woody grasslands, tolerating salty conditions, coastal winds, and even monsoon climates.

Tamarind has been used for a large number of purposes e.g. animal fodder, food for people and medicinal uses. The first medicinal use of tamarind was reported from India. Tamarind products, leaves, fruits and seeds have been extensively used in traditional Indian and African medicines. Tamarind leaves are usually ground into powder and used in lotions or infusions (Bhadoriya *et al*., 2015).

The leaves, mixed with salt and water, are used to treat throat infection, coughs, fever, intestinal worms, urinary troubles and liver ailments. Leaf extracts also exhibit antioxidant activity in the liver. Young leaves are reported to cure other eye infections, sprains and wounds (El-Siddig *et al.,* 2000). Leaves are useful in fevers, scalding of urine, gastropadthy, helminthiases, wound, ulcers, jaundice, scabies, tumors, ringworm, boil, smallpox, otalgia and conjunctivitis (Tariq *et al.,* 2013). Leaves are used as astringent, as gargle, and also made into a poultice to treat inflammatory swellings (Shah, 2014).

Antioxidants are substances that may protect cells from the damage caused by unstable molecules known as free radicals. Antioxidants interact with and stabilize free radicals and may prevent some of the damage free radicals might otherwise cause. Antioxidants are also widely

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used us ingredients in dietary supplements to maintain health and preventing diseases such as cancer and coronary heart disease. Plant based antioxidants are preferred to the synthetic ones because of their multiple mechanisms of actions and non-toxic nature (Hamid *et al.*, 2010). The aim of this research was to investigate total phenol contents and antioxidant potency of tamarind leaves.

Materials and Methods

Sample Collection and Preparation

Tamarind leaves sample was collected from Kyaunggon Township. Tamarind leaves sample was washed with water and air dried at room temperature. This sample was ground into powder in an electric blender and stored in an airtight container to prevent the moisture and other contaminations.

Phytochemical Investigation on Tamarind Leaves Sample

In order to find out the types of organic constituents present in tamarind leaves sample, preliminary photochemical investigation was carried out by the test tube methods (Vogel, 1989).

Determination of Nutritional Values of Tamarind Leaves

In the present study, some nutritional values such as moisture, fat, fiber, ash, protein, carbohydrate and energy value of tamarind leaves were determined by AOAC method at Food Industries Development Supporting Laboratory (FIDSL), Yangon.

Determination of Extractable Matter Contents

This method determines the amount of active constituent extracted with different solvents from a given amount of medicinal plant material. The extractive values provide an indication of the extract of polar, moderately polar and non-polar components present in the medicinal plant material (WHO, 1998).

The powdered tamarind leaves (2 g) was placed in a conical flask. 50 mL of the solvent was macerated for the tamarind leaves sample concerned for 6 hours shaking frequently then allowed to stand for 18 hours. The extract was filtered rapidly taking care not to lose any solvent and transferred to a porcelain basin and evaporated to dryness on water bath. The dried filtrate was then placed in oven maintained until constant weight, at 105 ° C.

Determination of Total Phenol Content of Tamarind Crude Extracts by Folin-Ciocalteu Method

(a) Construction of gallic acid standard calibration curve

Firstly, 0.5 mL each of different concentrations of gallic acid solutions (100, 50, 25, 12.5, 6.25 and 3.125 mg/mL) was mixed with 5 mL of 10 % FC reagent in each test tube and incubated for 5 min. To each tube, 4 mL of 1 M Na_2CO_3 was added and the tubes were kept at room temperature for 15 min and absorbance of reaction mixture was read at 765 nm. A standard calibration curve was obtained by plotting the absorbance against concentration of standard gallic acid.

(b) Determination of gallic acid equivalent in crude extracts sample

The total phenol content in the watery and ethanol extracts of tamarind leaves was determined by Folin-Ciocalteu (FC) method according to the procedure described by Rekha *et al.* (Rekha *et al*., 2012). Each extract sample solution (0.5 mL) was added into 5 mL of 10 % FC reagent and incubated for 5 min. To each tube, 4 mL of 1 M Na_2CO_3 was added and the tubes were kept at room temperature for 15 min and the absorbance of reaction mixture was read at 765 nm. The blank solution was prepared as the above mentioned procedure by using distilled water instead of sample solution. Total phenol content was estimated as mg gallic acid equivalents per milligram (mg GAE/mg) of sample.

Screening of Antioxidant Activity by DPPH Assay Method

The antioxidant activity of 95 % EtOH and H_2O crude extracts are by studied by DPPH Assay Method (Marinova, 2011).

DPPH radical scavenging activity was determined by spectrophotometric method. The control solution was prepared by mixing 1.5 mL of 0.002 % (w/v) DPPH solution and 1.5 mL of 95 % ethanol with vortex mixer. The sample solution was also prepared by mixing thoroughly 1.5 mL of 0.002 % (w/v) DPPH solutions and 1.5 mL of test sample solution. The solutions were allowed to stand at room temperature for 30 min. After 30 min, measurement of absorbance at 517 nm was made by using UV-visible Spectrophotometer (UV-7504, KWF, China). Absorbance measurements were done in triplicate for each solution and the mean value was obtained, and then used to calculate % inhibition of oxidation by the following equation,

$$\%\,\text{oxidative inhibition of test sample} = \frac{A_c - (A - A_b)}{A_c} \times 100\,\%$$

A_c = absorbance of the control (DPPH + EtOH)
A_b = absorbance of the blank (EtOH + Test sample solution)
A = absorbance of test sample solution

Then, IC_{50} (inhibitory concentration) values were calculated by linear regressive excel program.

Results and Discussion

Phytochemical Investigation of Tamarind Leaves

Phytochemicals are biologically active, naturally occurring chemical compounds found in plants which provide health benefits for human further than those attributed to macronutrients and micronutrients. The phytochemicals present in the sample can be tested by test tube method (Saxena *et al*., 2013).

Phytochemical investigation was carried out to know the types of phytoorganic constituents present in the Tamarind (*Tamarindus indica* L.) leaves. According to these results alkaloids, α-amino acids, carbohydrates, flavonoids, glycosides, organic acids, phenolic compounds, reducing sugars, saponins, steroids, terpenoids and tannins were found to be present while starch was not present in tamarind.

Nutritional Values of Tamarind Leaves

The nutritional values such as moisture, protein, fat, ash, fiber, carbohydrate and energy contents of the leaves of tamarind were determined according to the WHO procedure. It was found that carbohydrate, protein, and fiber were present as major nutrients in the leaves of tamarind and the results are shown in Table 1.

Table 1 Nutritional Values of the Leaves of *Tamarindus indica* L.

No.	Parameters	Content (%)
1	Moisture	11.12
2	Fat	3.56
3	Fiber	18.67
4	Ash	5.38
5	Protein	11.63
6	Carbohydrate	49.64
7	Energy value (kcal/100 g)	277.12

Extractable Matter Contents of Tamarind Leaves

The extractable matter contents of leaves of Tamarind were determined by the WHO standard method (WHO, 1998). In this experiment, the solvents used were water, ethanol, acetone, ethyl acetate and petroleum ether. The extractable matter contents of Tamarind leaves are shown in Table 2.

The water, ethanol, acetone, ethylacetate and petroleum ether soluble matters were observed to be 68.05, 69.40, 42.85, 44.85 and 14.00 mg/g in tamarind leaves, respectively.

According to these results, it was observed that the ethanol crude extract of tamarind leaves (69.40 mg/g) was higher than the other crude extracts. Aqueous extract had maximum yield followed by ethanol extract. Generally the amount of polar constituents was higher than that of non polar constituents in the leaves of tamarind.

Table 2 Extractable Matter Contents of the Tamarind Leaves

No.	Solvents used	Extractable matter (mg/g)
1	Petroleum ether	14.00
2	Ethylacetate	44.85
3	Acetone	42.85
4	Ethanol	69.40
5	Water	68.05

Total Phenol Content of Tamarind Crude Extract by FC (Folin-Ciocalteu Reagent) Method

Total phenolic compounds prevent from damage of nutrients contain double bonds such as fatty acids, flavour compounds even proteins and amino acids and other compounds.

The antioxidant activity of phenolic compounds increases with increasing degree of hydroxylation, as is the case of the trihydroxylated gallic acid, which shows a high antioxidant activity. However, substitution of the hydroxyl groups at the 3- and 5- positions with methoxyl groups as in syringic acid reduces the activity. The antioxidant activity of phenolic compounds is

due to their ability to scavenge free radicals, donate hydrogen atoms or electrons, or chelate metal cations (Aberoumand, 2008).

Phenols react with an oxidizing agent phosphomolybdate in FC reagent under alkaline conditions and results in the formation of blue coloured complex, molybdenum blue which is measured at 765 nm colorimetrically. In the present study, the total phenolic content of tamarind extracts were estimated by Folin-Ciocalteu method according to the procedure described. Gallic acid (3,4,5- trihydroxybenzoic acid) was used to construct standard calibration curve. The standard calibration curve of gallic acid was prepared by varying the different concentrations of gallic acid (100, 50, 25, 12.5, 6.25, 3.125 µg/mL) (Figure 1). Total phenolic content was expressed as micro gram of gallic acid equivalent per milligram/milligram of extract (mg GAE/mg extract). Total phenol content of watery extract was found to be higher than that of ethanol extract. This means that phenolic compounds were more soluble in water. The result of phenolic content of tamarind extracts are presented in Table 4 and Figure 2.

Table 3 The Absorbance of Gallic Acid Standard Solution at λ_{max} 765 nm

Sr. No.	Concentration (µg/mL)	Absorbance at λ_{max} 765 nm
1	3.12	0.057
2	6.25	0.065
3	12.5	0.080
4	25	0.109
5	50	0.170
6	100	0.286

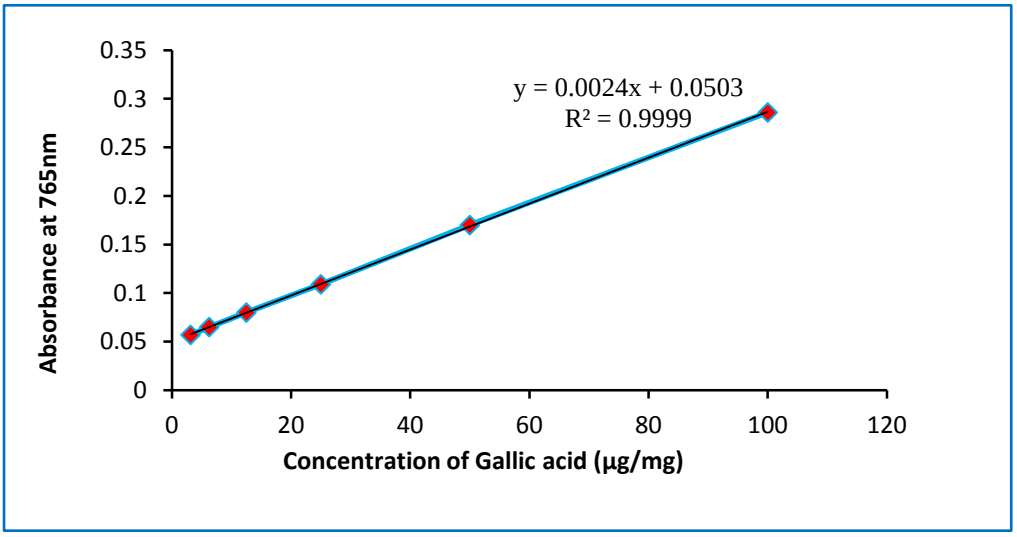

Figure 1 Plot of absorbance vs concentration of standard gallic acid

Table 4 Total Phenol Contents of Watery and Ethanol Extracts from the Tamarind Leaves

Sample	TPC (µg GAE/ mg ± SD)
Water extract	24.70 ± 3.38
Ethanol extract	13.30 ± 3.30

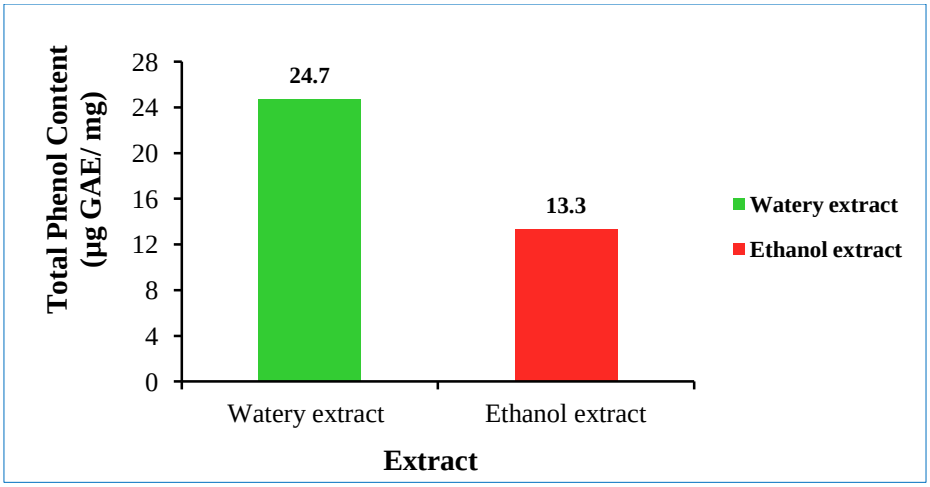

Figure 2 Total Phenol Contents of the Tamarind Leaves Crude Extracts

Antioxidant Activity of Tamarind Leaves by Radical Scavenging DPPH Assay

Antioxidants play an important role as health protecting factor. Most of the antioxidant compounds in a typical diet are derived from plant sources and belong to various classes of compound with a wide variety of physical and chemical properties.

A lot of different procedures have been developed to test the antioxidant activity of foodstuffs. A rapid simple and inexpensive method to measure antioxidant capacity of food involves the use of the free radical, 2,2- diphenyl-1- picrylhydrazyl (DPPH) which is widely used to test the ability of compound to act as free radical scavengers or hydrogen donor and to evaluate antioxidant activity. The DPPH assay method is based on the reduction of DPPH, a stable free radical. The free radical DPPH with an odd electron gives a maximum absorption at 517 nm (purple colour). When antioxidants react with DPPH, which is a stable free radical becomes paired off in the presence of a hydrogen donor (e.g., a free radical scavenging antioxidant) and is reduced to the DPPH and as consequence the absorbances decreased from the DPPH. Radical to the DPPH-H form, results in decolorization (yellow colour) with respect to the number of electrons captured. More the decolorization more is the reducing ability. IC_{50} (50 % inhibition concentration) values calculated after linear regression analysis of the observed inhibition percentages Vs concentration of sample, where lower IC_{50} values indicate higher antioxidant activity (Shekhar and Anju, 2014). The results are shown in Table 5 and Figure 3.

The IC_{50} values for crude extracts and standard ascorbic acid are tabulated in Table 3.9 and Figure 3.3. The IC_{50} values of watery extract, ethanol extract and standard ascorbic acid were observed 57.13, 34.47 and 7.97 µg/mL, respectively. Since the lower the IC_{50} values indicates the higher the antioxidative property, ethanol extract is more effective than water extract. All extracts showed mild antioxidant activity when compared to the standard ascorbic acid.

Table 5 Percent Oxidative Inhibition of Different Concentrations and IC_{50} Values of

Test samples	Percent Oxidative Inhibition of Different Concentrations (µg/mL)						IC_{50} (µg/mL)
	6.25	12.5	25	50	100	200	
Watery extract	18.52 ± 0.39	29.81 ± 1.24	37.12 ± 0.47	48.08 ± 2.49	61.54 ± 0.27	76.34 ± 1.25	57.13
EtOH extract	24.81 ± 1.36	38.46 ± 0.16	45.19 ± 0.16	57.88 ± 5.31	64.81 ± 2.31	71.15 ± 1.93	34.47
Standard ascorbic acid	49.06 ± 1.15	52.48 ± 0.52	53.91 ± 0.23	55.83 ± 0.55	59.73 ± 0.36	65.25 ± 0.33	7.97

Tamarind Leaves Extracts and Standard Ascorbic Acid

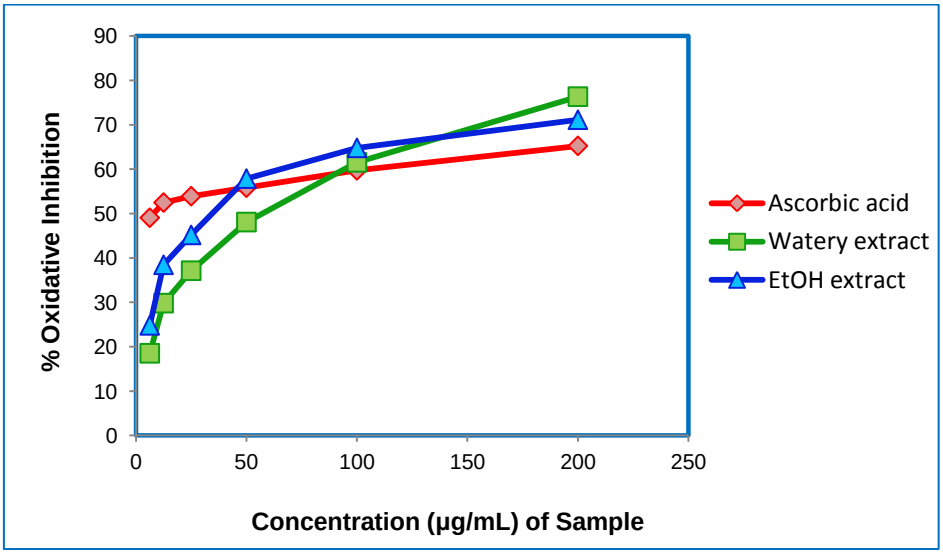

Figure 3 A plot of % oxidative inhibition vs concentrations of different tamarind leaves extracts and standard ascorbic acid

Conclusion

From the overall assessment of the present research work, the following conclusion can be drawn.

Preliminary phytochemical investigation of tamarind leaves sample indicated the presence of alkaloids, α-amino acids, carbohydrates, flavonoids, glycosides, organic acids, phenolic compounds, reducing sugars, saponins, steroids, terpenoids and tannins whereas starch was not found.

Nutritional values analyses showed that tamarind was found to contain 11.12 % of moisture, 3.56 % of fat, 18.67 % of fiber, 5.38 % of ash, 11.63 % of protein, 49.64 % of carbohydrate and 277.12 kcal/100 g of energy values, respectively.

The extractable matter contents were determined by WHO standard method using different polarity of solvents such as petroleum ether, ethyl acetate, acetone, ethanol and water. In the tamarind leaves, the value of extractable matter of ethanol extract is the highest among the extracts.

The total phenol content (TPC) in crude extracts was investigated by UV spectrophotometric method by using Folin – Ciocalteu reagent (FCR). In the determination of total phenol content, gallic acid (3,4,5-trihydroxy benzoic acid) was used for the preparation of standard curve. The TPC content of ethanol and watery extracts were respectively found to be 13.30 ± 3.30 µg GAE/ mg and 24.70 ± 3.38 µg GAE/mg.

The antioxidant activities of tamarind leaves extracts were investigated by using DPPH assay. In DPPH assay, 50 % inhibition concentration IC_{50} (µg/mL or mg) whereas the reducing power ability was presented as absorbance at 517 nm. The IC_{50} values of ethanol and watery extracts of tamarind leaves were to be 34.74 and 57.13 μgmL^{-1}, respectively. The ethanol extract is more effective than watery extract. All extracts showed mild activity when compared to the standard ascorbic acid (7.97 µg/mL).

According to these observations, tamarind leaves possess not only phytochemicals and phenolic compounds, but also antioxidant activity that have proven health benefits.

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ISOLATION AND FERMENTATION CONDITIONS OF SELECTED SOIL BACTERIUM (YN-51) FROM LAPUTTA TOWNSHIP AND THEIR ANTIMICROBIAL ACTIVITIES

Yoon Noe Aung[1], Ye Myint Aung[2], Zar Zar Yin[3]

Abstract

Soil samples from three different areas of Laputta Township, Ayeyawady Region were cultured on five kinds of media. A total of 79 bacterial colonies were isolated from these soil samples. The purpose of this study was to screen for antimicrobial activities and fermentation conditions for selected bacterium. In this study, antimicrobial activities of all isolated strains were examined by agar well diffusion assay with seven test organisms. Among them, ten strains namely YN-1, 6, 7, 35, 51, 55, 56, 57, 59, and 63 showed different levels of antimicrobial activities. Especially, YN-51 showed the highest activity on *Candida albicans.* The fermentation conditions of YN-51 were optimized by the studies of fermentation period, proper size, age, different carbon and nitrogen sources utilization, pH, temperature, static and shaking culture on antimicrobial metabolite production on *Candida albicans.* In the fermentation period, YN-51 showed the highest activity (22.31 mm) in 3 days followed by (21.82 mm) in 2 days against *Candida albicans.* In the investigation, YN-51 was found that 15% of size of inoculum and 72 h of age of old culture were suitable conditions. The addition of glucose as a carbon source resulted better growth of YN-51 and the inhibition zone reached 27.07 mm in sucrose. In the nitrogen source, the maximum growth of YN-51 was found in yeast extract and the highest antifungal activity (30.49 mm) was found in beef extract. And then, effects of pH range and temperature were also determined. According to the results, pH 7.5 and temperature 40 °C were found to be the best activities (29.64 mm and 27.29 mm) on *Candida albicans* respectively. In the comparison between shaking culture and static culture, the antifungal activity reached (25.61 mm) in shaking culture and (21.70 mm) in static culture respectively.

Keywords: antimicrobial activity, antifungal activity, soil bacterium, fermentation medium, *Candida albicans*

Introduction

Soil microorganism are the studies of organisms in soil, their functions and how they effect soil properties. It is believed that between two and four billion year ago, the first ancients bacteria and microorganisms came about in Earth's primitive seas. These bacteria could fix nitrogen, in time multiplied and as a result released oxygen into the atmosphere. This release of oxygen led to more advanced microorganisms. Microorganisms in soil are important because they affect the structure and fertility of different soils (Subba, 1999).

In their natural habitats, bacteria utilize the antibiotics they produce as protective substances by rendering the invasion of other bacterial species. Protection is not the only function of antibiotics. Hence, antibiotics also act as signaling molecules that bacteria use as a means of communication between cells (Linares *et al.,* 2006). The natural products that had been developed into drugs, many come from plant resources, but there had been a considerable number of important drugs harvested from microorganisms the production of antimicrobial substances depends upon the substrate medium for their optimal growth, temperature, pH and the concentration of nutrients in the medium (Leifert *et al*., 1995).

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In the presence of various carbon and nitrogen sources microorganisms have diverse levels of phosphate solubilization activity. Carbon source is an important parameter for active proliferation of organisms and production of organic acids and nitrogen source is important for the production of organic acids (Kucey, 1989). Carbon and nitrogen sources together with fermentation time have been reported to play significant roles in the determination of the final morphology of the culture. Carbon sources in the media play a very critical role in the production of antimicrobial substances by the bacteria (Papagianni, 2004). The aim of this research is to screen for antimicrobial activities and fermentation conditions for selected bacterium.

Materials and Methods

Collection of Soil Samples

Three different soil samples were collected from three different sites of Laputta Township, Ayeyawady Region in June, 2017. These samples were collected in depth 6 inches from soil surface. The samples were placed in plastic bag. The experiment was carried out at the laboratory of Biotechnology and Development Centre of Pathein University. Soil type, pH, moisture and location of these soil samples are shown in Table 1.

Table 1 Some Physiochemical Parameters of Three Different Soil Samples Collected from Laputta Township

Soil samples	Soil type*	Soil pH	Moisture %	Location
Aung-Taw-Mu (S-1)	Sandy Loam	6.83	1.77	N 16º 8.867" E 94º 45.658"
Tapin-Shwe-Tee (S-2)	Loam	6.06	3.02	N 16º 9.3" E 94º 48.359"
Laputalote (S-3)	Sandy Loam	6.83	0.93	N 16º 9.811" E 94º 42.604"

*Soil type was classified at Department of Agriculture, Insein Township, Yangon.

Isolation of Bacteria from Soil Samples

The soil bacteria were enumerated by serial dilution method on media such as CAS medium, Centenum medium, Nutrient Agar medium, Dextrose Casein Peptone Agar medium and Glucose Peptone Agar medium.

Serial dilutions method

Serial dilutions of fermented, plating and streaking techniques described by Salle (1948), Collins (1965) and Pelezar and Chan (1972) were used for the isolation of microorganisms from soil. An appropriate amount (1 g) of soil was introduced into a conical flask containing 99 mL of distilled water to make a soil-water dilution ratio of 1: 100. The flask was then shaken for about 30 min in order to make the soil particles free from each other. This solution was then serially diluted into 10^{-3} to 10^{-7} dilution in separate test tubes and 1 mL each of the above dilutions was separately transferred into sterile petridishes under aseptic condition. A sterile pipette was used for each transfer. The sterilized medium in conical flask was cooled down to about 45°C and

separately poured into each of the petridish containing the respective soil dilutions. The inoculated plates were shaken clock-wise and anti-clockwise direction for about 5 min so as to make uniform distribution of the bacterial inoculums. When the agar was solidified, the inoculated plates were inverted and incubated at 30°C for 24 h. Various types of colonies developed on the inoculated plates. They were separately streaked over another set of pertridishes containing the same sterile medium. Each of the discrete colonies visible in the second set of inoculated plates was separately transferred to sterile nutrient agar medium. The isolates were maintained in nutrient agar medium for further experiments.

Preliminary Study on Antimicrobial Activities of Isolated Bacteria

The isolated soil bacteria were inoculated into seed medium and incubated for 1 day at 27 ºC. After one day, the seed culture (1%) was transferred into the fermentation medium and carried out by static culture. Then, the fermented broth was used to check the antimicrobial activity by agar well method (Collins, 1965). Agar well having 8 mm in diameter were utilized for antimicrobial activity.

Screening of Antimicrobial Activity by Agar Well Diffusion Method

One day old culture test broth (0.2 mL) was added to 25 mL warm assay medium (glucose 1.0 g, yeast extract 0.3 g, peptone 0.2 g, agar 1.8 g, DW 100 mL) and thoroughly mixed and poured into plate. After solidification, the agar was left to set. Cork borer was used to make the wells (8 mm in diameter). And then, the fermented broth (20 µL) was carefully added into the well and incubated at room temperature for 24-48 h. The diameter of the zones of inhibition around each well measured and recorded after 24-48 h incubation.

Effect of Sizes and Ages of Inoculums for Fermentation

The cultivation times (24, 48, 72, 96, 120, 144 and 168 h) were employed for the production of antimicrobial metabolite. In the investigation of sizes of inoculums 5%, 10%, 15%, 20%, 25% and 30% were used for the antifungal activity of YN-51. Seed culture was inoculated in the 150 mL conical flask and incubated at room temperature. In the investigation of ages of inoculums, the incubation of seed culture times (24, 48, 72, 96, 120, 144 and 168 h) were used and transferred into the fermentation media. Fermentations were carried out for 7 days and antifungal activity was tested by agar well diffusion method.

Effects of Different Carbon Sources Utilization

Carbon sources (each 1.0 g) such as glucose, xylose, sucrose, mannitol, lactose, starch, fructose, maltose, glycerol, tapioca powder, molasses, soluble starch, potato, dextrose, corn, oat, carrot and rice were used. Fermentation media were incubated at 25 ºC for 2 days.

Effects of Different Nitrogen Sources Utilization

Nitrogen sources (each 1.0 g) such as ammonium chloride, ammonium nitrate, ammonium oxalate, asparagine, ammonium sulphate, beef extract, casein, fish cake, gelatin, meat extract, milk, malt extract, peanut, peptone, soy bean, sodium nitrate, potassium nitrate, urea and yeast extract were also used. Fermentation media were incubated at 25 ºC for 2 days.

Effect of Incubation pH and Temperature on the Selected Bacterium YN-51

Effects of different pH were used for antifungal activity of pH 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5 and 9. These different pH were adjusted by NaOH and HCl. The selected bacteria YN-51 was inoculated and incubated at six different temperatures 20 °C, 25 °C, 30 °C, 35 °C, 40 °C and 45 °C.

Antifungal Activity on Shaking and Static Culture of the Selected Bacterium YN-51

100 mL conical flask containing 50 mL of the best fermentation medium was incubated on the shaker (100 rpm) for 3 days. At the same time, another those fermentation medium was incubated under static condition without shaking. These shaking culture and static culture were compared by using ager well diffusion assay method.

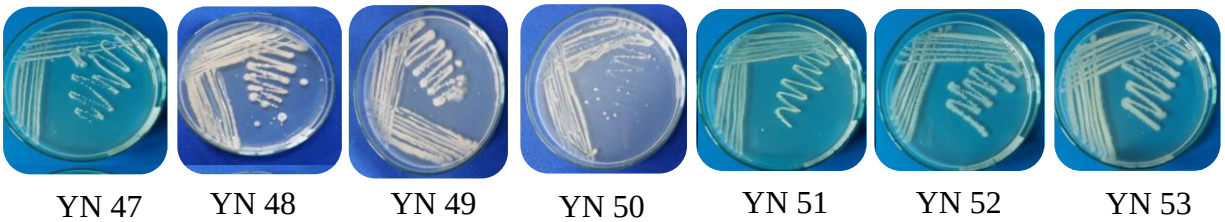
Results and Discussion

Isolation of Bacteria

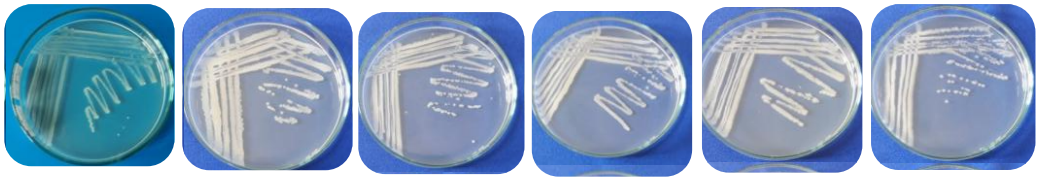
In the course of the isolation of bacteria, three different samples were collected from Laputta Township, Ayeyawady Region. A total of 79 strains were isolated from these soil samples. 27 isolates were obtained from CAS medium, 9 isolates were formed from Centenum medium, 10 isolates from Nutrient Agar medium, 13 isolates from Dextrose Casein Peptone Agar medium and 20 isolates from Glucose Peptone Agar medium. These results are shown in Table 2 and Figure 1.

Table 2 Isolated Bacteria from Soil Samples

Soil Sample No.	CAS medium	Centenum medium	Nutrient Agar	Dextrose Casein Peptone Agar medium	Glucose Peptone Agar medium	Total strains (Soil Samples)
S-1	YN 1-24= 24	YN28-35= 8	YN37-39= 3	YN47-51= 5	YN60-76= 17	57
S-2	YN 25-26= 2	-	YN40-46= 7	YN52 = 1	-	10
S-3	YN 27 = 1	YN36 =1	-	YN53-59= 7	YN77-79= 3	12



YN 47 YN 48 YN 49 YN 50 YN 51 YN 52 YN 53



YN 54 YN 55 YN 56 YN 57 YN 58 YN 59

Figure 1 Morphological characters of isolated bacteria (YN 47-59) on dextrose casein peptone agar medium

Antimicrobial Activities of Isolated Bacterial Strains

Preliminary studies of antimicrobial activity of isolated bacteria (YN 1-79) were tested by seven test organisms with agar well diffusion assay method. 57 strains showed different level of antimicrobial activity. Among them, ten strains showed the moderate antimicrobial activity on almost seven test organisms. YN-7 showed the antibacterial activity (15.58 mm) at 1 day followed by YN-1 (15.56 mm) at 2 days and YN-6 (13.90 mm) at 3 days against *Agrobacterium tumefaciens*.

Especially, YN-56 gave the respective antibacterial activity (26.16 mm, 23.23 mm and 21.63 mm) at 1 day against *Bacillus pumilus*, *Bacillus subtilis* and *Escherichia coli,* respectively. Moreover, YN-56 exhibited the antifungal activity (23.02 mm) at 1 day on *Candida albicans* followed by YN-51 (21.47 mm) and YN-7 (16.81 mm). And then, YN-51 and 59 gave the antibacterial activity (17.28 mm and 16.80 mm) against *Staphylococcus aureus* and *Pseudomonas fluorescens.* Thus, YN-51 was selected for further studies (Table 3 and Figure 2-5).

Table 3 Antimicrobial Activity of Selected Bacteria against Seven Test Organisms

No.	Test Organisms	Strains and Inhibition zone (mm)					
		YN-1	**YN-6**	**YN-7**	**YN-51**	**YN-56**	**YN-59**
1.	*Agrobacterium tumefaciens*	15.56	13.90	15.58			
2.	*Bacillus pumilus*			-	-	26.16	-
3.	*Bacillus subtilis*			-	-	23.23	-
4.	*Candida albicans*			16.81	21.47	23.02	-
5.	*Escherichia coil*			-	-	21.63	-
6.	*Pseudomonas fluorescens*			-	-	-	16.80
7.	*Staphylococcus aureus*			-	17.28	-	-

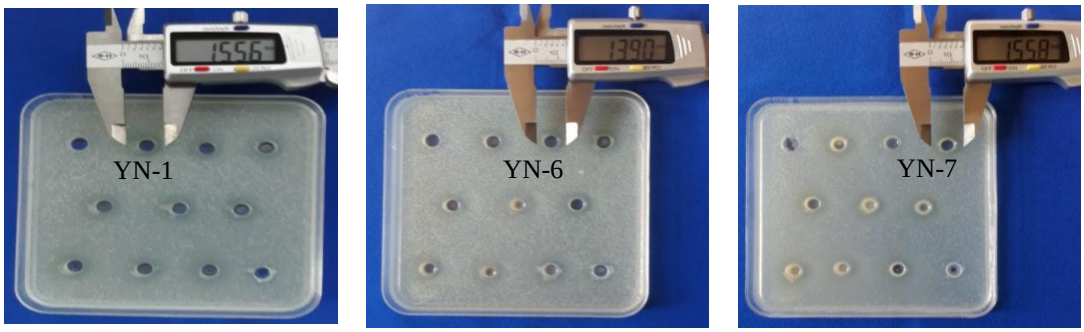


Figure 2 Antibacterial activity of selected bacteria against *Agrobacterium tumefaciens*

Figure 3 Antibacterial activity of selected bacteria against (a) *Bacillus pumilus* and (b) *Bacillus subtikis*

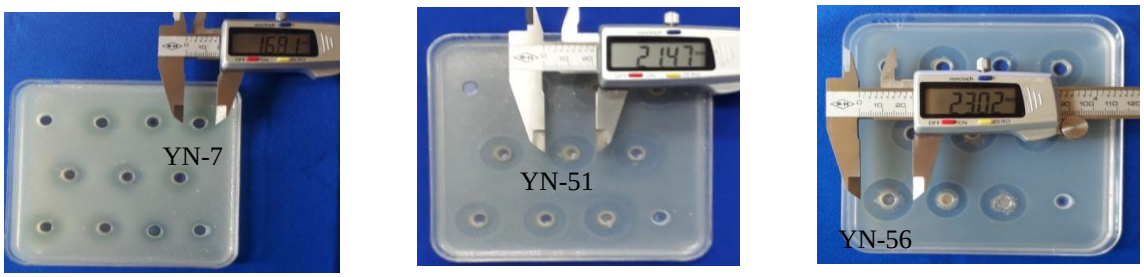


Figure 4 Antifungal activity of selected bacteria against *Candida albicans*

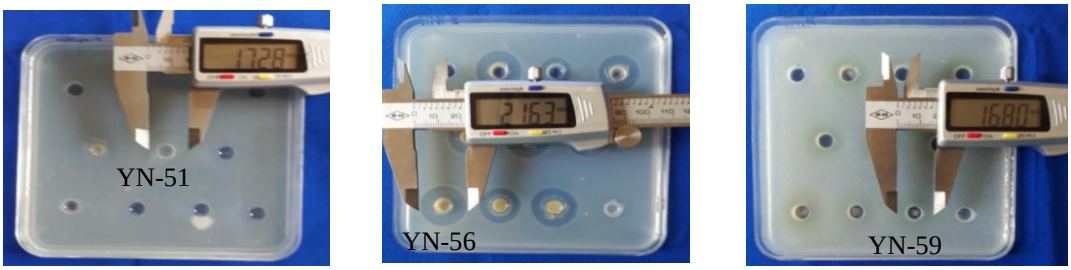


Figure 5 Antibacterial activity of selected bacteria against (a) *Staphylococcus aureus* (b) *Escherichia coil* and (c) *Pseudomonas fluorescens*

Effect of Fermentation Periods on Selected Strain YN-51

In the study of fermentation periods, the incubation zone indicated that if increased from 2 days to 3 days and the maximum highest activity showed in 3 days of fermentation period (Table 4 and Figure 6).

Table 4 Effect on Fermentation Periods of Selected Bacterium YN-51 Against *Candida albicans*

Fermentation period (Day)	Antifungal activity (mm)
1	20.42
2	21.82
3	**22.31**
4	19.58
5	18.79

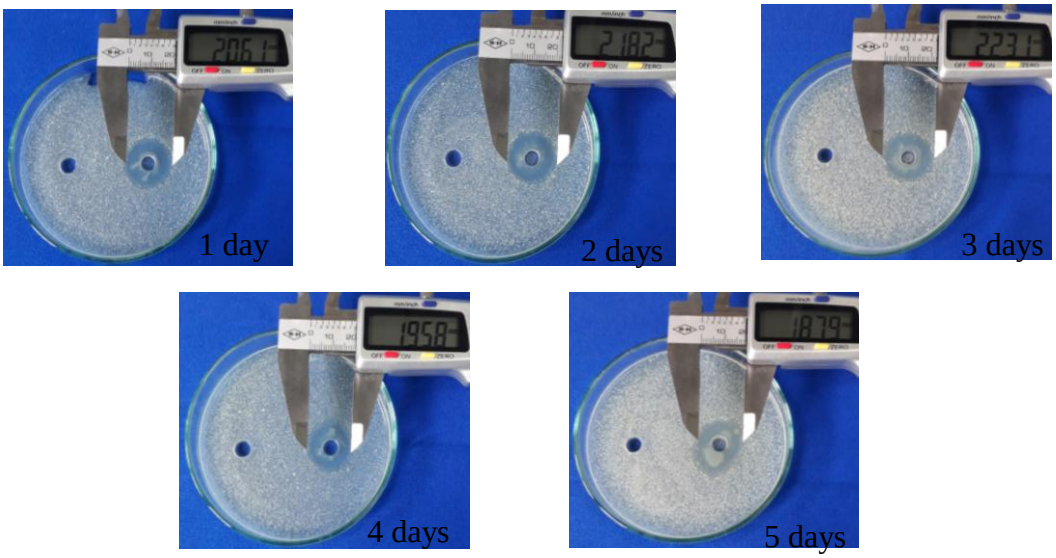


Figure 6 Effect on fermentation periods of selected bacterium YN-51 against *Candida albicans*

Effect of Sizes of Inoculums for YN-51

In this study, the effect of sizes of inoculums was studied by using 5%, 10%, 15%, 20%, 25% and 30% inoculums for the maximum production of antifungal activity. Using 15% inoculums exhibited significantly higher activity (23.17 mm), followed 25% (22,64 mm) and showed in 5%, !0%, 20%, 30% respectively. The result found that significant antifungal activity was increased at 15% for the optimal formation of antifungal activity (Table 5 and Figure 7).

Table 5 Effect of Sizes of Inoculums for YN-51

Sizes of Inoculums (%)	Antifungal activity (mm)
5	20.49
10	22.37
15	**23.17**
20	21.47
25	22.64
30	22.15

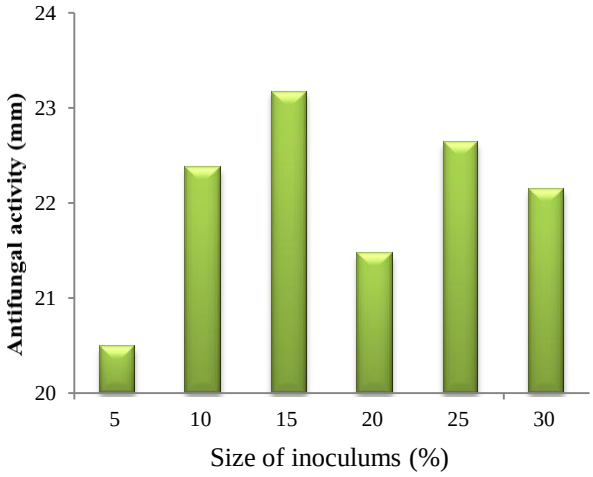


Figure 7 Effect of sizes of inoculums

Effect of Ages of Inoculums for YN-51

In the study of the effect of inoculums age, it was observed that from (27.20 mm) on 72 h, followed (26.76 mm) 96 h, (26.38 mm) 120 h, and showed in 144 h, 168 h, 48 h and 24 h respectively. The result indicate that antifungal activity of YN-51 reached the highest activities (27.20 mm) in 72 h age of inoculums on *Candida albicans* (Table 6 and Figure 8).

Table 6 Effect of Ages of Inoculums for YN-51

Ages of Inoculums (hrs)	Antifungal activity (mm)
24	20.92
48	26.24
72	**27.20**
96	26.76
120	26.38
144	22.29
168	20.37

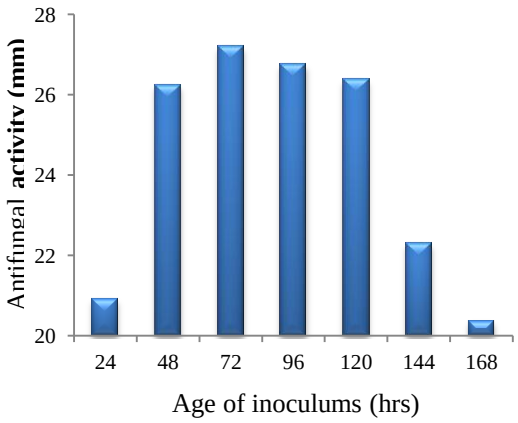


Figure 8 Effect of ages of inoculums

Investigation of Carbon and Nitrogen Sources Utilization

The effects of different carbon sources were observed for the growth rate. The addition of glucose, xylose, starch, corn and rice gave excellent growth, fructose, glycerol, tapioca powder, molasses and carrot were moderate growth and other sources showed good results. For the growth with nitrogen utilization, it was found that excellent growth of YN-51 on beef extract, milk, peanut and yeast, moderate growth on ammonium chloride, asparagine, casein, gelatin, meat extract, soy bean, sodium nitrate and urea and other nitrogen sources showed good results (Table 7).

Table 7 Growth of YN-51 on Carbon and Nitrogen Sources

Carbon sources	Growth (mm)	Nitrogen sources	Growth (mm)
Glucose	**excellent (10.43)**	Ammonium chloride	moderate (3.19)
Xylose	good (6.82)	Ammonium nitrate	good (6.81)
Sucrose	excellent (7.58)	Ammonium oxalate	good (5.38)
Mannitol	excellent (7.56)	Asparagine	moderate (4.12)
Lactose	good (6.66)	Ammonium sulphate	good (6.35)
Starch	excellent (7.25)	Beef extract	excellent (7.90)
Fructose	moderate (4.51)	Casein	moderate (4.47)
Maltose	good (5.60)	Fish cake	good (5.39)
Glycerol	moderate (4.51)	Gelatin	moderate (3.78)
Tapioca powder	moderate (3.78)	Meat extract	moderate (3.95)
Molasses	moderate (3.19)	Milk	excellent (7.94)
Soluble Starch	good (5.60)	Malt extract	good (5.60)
Potato	good (6.35)	Peanut	excellent (7.02)
Dextrose	good (6.79)	Peptone	good (6.49)
Corn	excellent (7.69)	Soy bean	moderate (4.64)
Oat	good (5.55)	Sodium nitrate	moderate (4.82)
Carrot	moderate (3.99)	Potassium nitrate	good (5.81)
Rice	excellent (8.26)	Urea	moderate (4.51)
-	-	**Yeast extract**	**excellent (9.83)**

1 – 2.9 mm = poor 3 – 4.9 mm = moderate
5 – 6.9 mm = good 7 & above = excellent

Effect of Different Carbon and Nitrogen Sources on the Antifungal Activity of YN-51 Against *Candida albicans*

The effects of different carbon sources were observed for the maximum antimicrobial metabolites production. The results indicated that sucrose showed the highest activity (27.07 mm), followed by molasses (27.07 mm) and potato (23.40 mm) in carbon sources (Table 8 and Figure 9)and Beef extract reaching the highest activity (30.49 mm), followed by malt extract (25.45 mm) and fish cake (24.34 mm) in nitrogen sources (Table 9 and Figure 10).

Table 8 Effect of Different Carbon Sources on the Antifungal Activity of YN-51 Against *Candida albicans*

No	Carbon sources	Inhibitory Zone (mm)	No	Carbon sources	Inhibitory Zone (mm)
1.	Glucose	17.16	10.	Tapioca powder	20.90
2.	Xylose	20.34	11.	Molasses	27.02
3.	**Sucrose**	**27.07**	12.	Soluble Starch	13.18
4.	Mannitol	23.39	13.	Potato	23.40
5.	Lactose	18.16	14.	Dextrose	19.44
6.	Starch	11.39	15.	Corn	16.57
7.	Fructose	19.22	16.	Oat	19.05
8.	Maltose	18.53	17.	Carrot	22.57
9.	Glycerol	24.84	18.	Rice	12.46

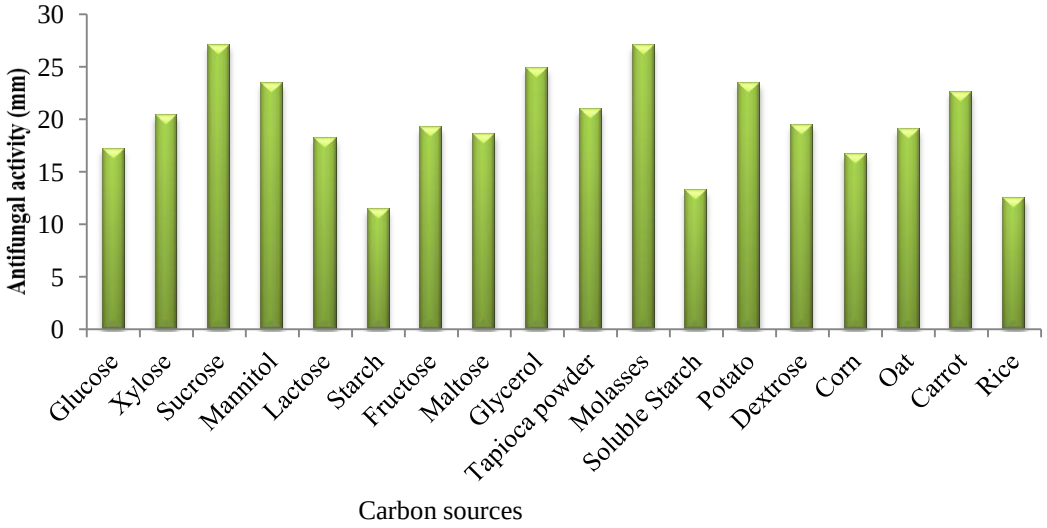


Figure 9 Effect of different carbon sources on antifungal activities of YN-51

Table 9 Effect of Different Nitrogen Sources on the Antifungal Activity of YN-51 Against *Candida albicans*

No	Nitrogen sources	Inhibitory Zone (mm)	No	Nitrogen sources	Inhibitory Zone (mm)
1.	Ammonium chloride	13.65	11.	Milk	19.53
2.	Ammonium nitrate	17.63	12.	Malt extract	25.45
3.	Ammonium oxalate	12.94	13.	Peanut	12.26
4.	Asparagine	23.51	14.	Peptone	17.46
5.	Ammonium sulphate	13.00	15.	Soy bean	18.45
6.	**Beef extract**	**30.49**	16.	Sodium nitrate	15.60
7.	Casein	-	17.	Potassium nitrate	23.86
8.	Fish cake	24.34	18.	Urea	18.86
9.	Gelatin	14.81	19.	Yeast extract	15.08

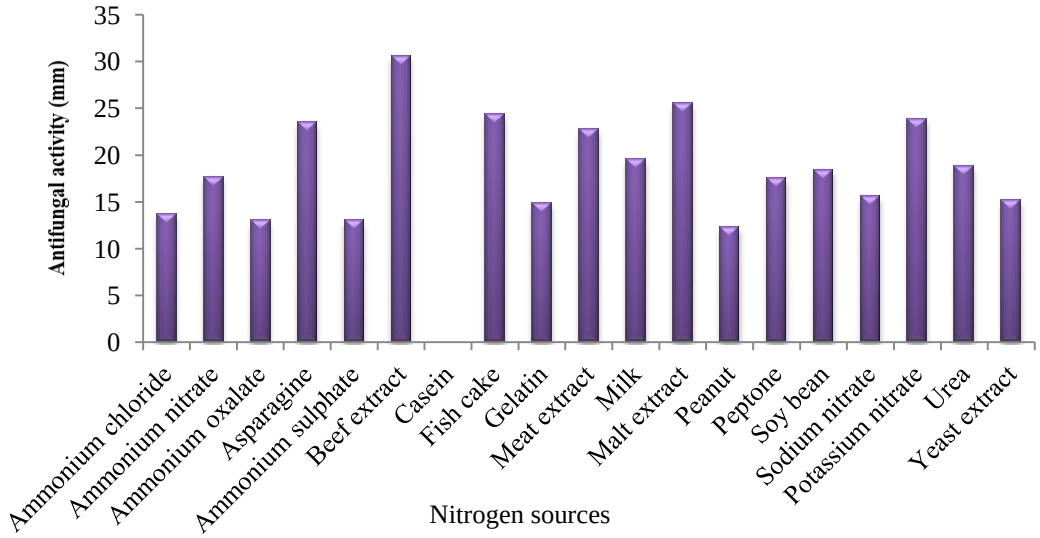


Figure 10 Effect of different nitrogen sources on antifungal activities of YN-51

Effects of pH and different temperature utilization of YN-51 against *Candida albicans*

The effect of pH and temperature were tested with pH levels (pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0) and different temperature ranges (20°C, 25°C, 30°C, 35°C, 40°C and 45°C). Maximum inhibitory zone was occurred in pH 7.5 (29.64 mm) and it was followed by pH 7 (29.06 mm) and pH 8 (28.52 mm) (Table 10 and Figure 11). Maximum antifungal activity was recorded at 40°C (27.29 mm), followed by 35°C (26.91 mm) (Table 11 and Figure 12).

Table 10 Effects of pH Utilization of YN-51 Against *Candida albicans*

pH range	Inhibitory zone (mm)
4.0	-
4.5	-
5.0	-
5.5	23.95
6.0	24.19
6.5	24.59
7.0	29.06
7.5	**29.64**
8.0	28.52
8.5	26.76
9.0	26.63

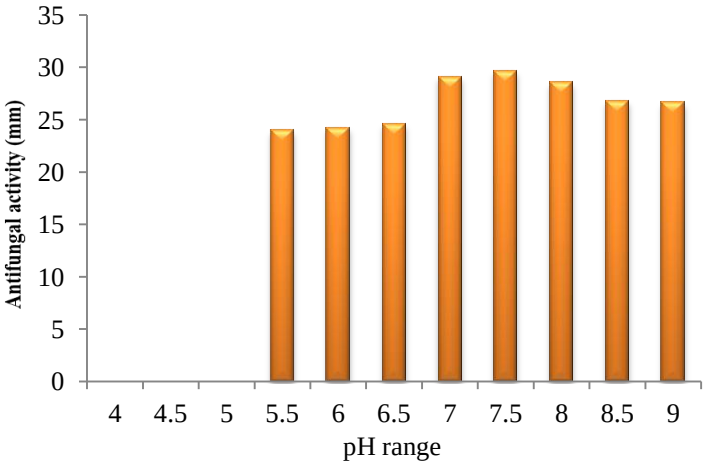


Figure 11 Effects of pH utilization of YN-51 against *Candida albicans*

Table 11 Effects of Different Temperature Utilization YN-51 Against *Candida albicans*

Temperature range (ºC)	Inhibitory zone (mm)
20	22.79
25	23.66
30	24.90
35	26.91
40	27.29
45	23.03

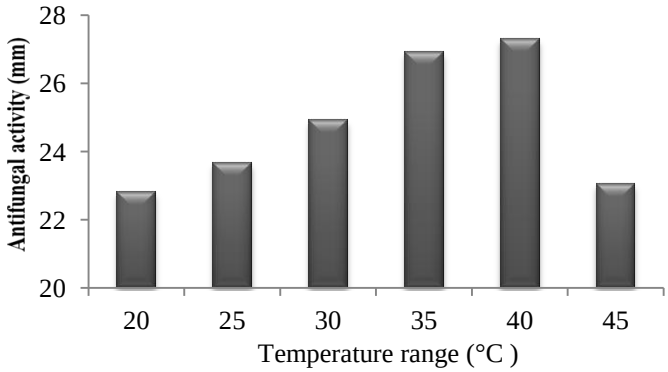
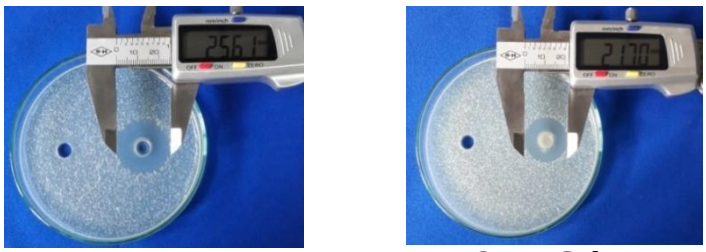


Figure 12 Effects of different temperature utilization YN-51 against *Candida albicans*

Comparison between Shaking Culture and Static Culture of YN-51

In this investigation, the comparison between shaking culture and static culture were observed. The shaking culture showed the inhibitory zone 25.61 mm and the static culture showed that 21.70 mm for 2 days fermentation (Figure 13).



Shaking Culture Static Culture

Figure 13 Comparison between shaking culture and static culture of YN-51

Conclusion

In the course of the isolation of bacteria, 79 strains were isolated from three different samples collected from Laputta Township, Ayeyawady Region. Five different media employed in the investigation of the isolation of bacteria, it was found that 27 isolates were obtained from CAS medium, 9 isolates were formed from Centenum medium, 10 isolates from Nutrient Agar medium, 13 isolates from Dextrose Casein Peptone Agar medium and 20 isolates from Glucose Peptone Agar medium. The isolated bacteria were designated as YN-1 to YN-79.

Some isolated bacterial strains were tested for antimicrobial activities by seven test organisms with agar well diffusion method and these strains showed different levels of antimicrobial activities. YN-7 showed the antibacterial activity (15.58 mm) at 1 day followed by YN-1 (15.56 mm) at 2 days and YN-6 (13.90 mm) at 3 days against *Agrobacterium tumefaciens*. Especially, YN-56 gave the respective antibacterial activity (26.16 mm, 23.23 mm and 21.63 mm) at 1 day against *Bacillus pumilus*, *Bacillus subtilis* and *Escherichia coli*, respectively. Moreover, YN-56 exhibited the antifungal activity (23.02 mm) at 1 day on *Candida albicans* followed by YN-51 (21.47 mm) and YN-7 (16.81 mm). And then, YN-51 and 59 gave the

antibacterial activity (17.28 mm and 16.80 mm) against *Pseudomonas fluorescens* and *Staphylococcus aureus*

In the investigation to optimize the fermentation, it was found that 72 h ages of inoculums and 15% of size of inoculum were suitable for fermentation. The effects of variation of carbon and nitrogen sources were observed for the growth and maximum metabolite production. The addition of glucose, xylose, starch, corn and rice as carbon sources were better growth and the maximum inhibition zone resulted in sucrose (27.07 mm) followed molasses (27.02 mm). Potato showed good growth but optimum inhibition zone was 23.40 mm.

In the utilization of nitrogen sources, the maximum production of antifungal metabolite of YN-51 was found in the present of beef extract (30.49 mm). The supplement of yeast extract and milk were excellent growth in the morphology but the optimum metabolite production were the former (15.08 mm) and the latter (19.53 mm) respectively. In the study of different pH and temperature utilization for the fermentation, the highest activity was found at pH 7.5 (29.64 mm) and the optimum temperature at 40°C (27.29 mm). Comparison between shaking culture and static culture showed shaking culture (25.61 mm) and static culture (21.70 mm). Hence, the antifungal activity of shaking culture was larger than the static culture in metabolite production.

The present study concluded that the optimal conditions required for the production of bioactive metabolites by selected bacterium YN-51 were determined and metabolites showed better antifungal activity against human pathogen, *Candida albicans*.

Acknowledgements

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PRELIMINARY PHYTOCHEMICAL INVESTIGATION AND SCREENING OF ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES FROM THE STEM BARK OF *CROTON OBLONGIFOLIUS* ROXB. (THAK-RING-KRI)

Moh Moh Aye[1], Kyi Kyi Khine[2]

Abstract

Croton oblongifolius Roxb., a middle size tree as medicinal plants, was chosen to investigate phytochemical constituents, nutritional values, screening of antimicrobial, total phenolic contents, total flavonoid contents and antioxidant activities. Preliminary phytochemical investigation of Thak-ring-kri bark revealed the presence of flavonoids, phenolic compounds, glycosides, saponin, terpenoids, steroids, carbohydrate, tannin, starch and α-amino acid. Alkaloids and cyanogenic glycoside were not detected in the sample. The AOAC method was used to determine the nutritional values such as moisture, ash, protein, crude fiber, fat, carbohydrate and energy value. The content of these values are 6 %, 10 %, 5.73 %, 11.45 %, 7.43 %, 59.4 % and 327 kcal/100g, respectively. The antimicrobial activity of pet-ether, dichloro methane, ethyl acetate, ethanol, methanol and watery extracts was determined by agar well diffusion method against six species of microorganisms such as *Bacillus substilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus pumilus*, *Candida albicans* and *E. coli*. It was found that all extracts showed antimicrobial activity against all tested microorganisms. Its watery extract showed less activity (12 mm) and pet-ether extract was observed the most pronounced antimicrobial activity (16~35 mm) against all microorganisms tested. Total phenolic contents was determined by spectrophotometric method using Folin-Ciocalteu reagent. Ethanol and watery extracts of the total phenolic contents were found to be (197.50 ± 0.01) µg GAE/mg and (41.42 ± 0.00) µg GAE/mg respectively. Total flavonoid contents was studied by spectrophotometric method. The ethanol extract contain flavonoid content (115.34 ± 0.01) µg QE/mg higher than watery extracts (70.00 ± 0.00) µg QE/mg. From the screening of free radical scavenging activity of Thak-ring-kri stem bark by DPPH assay, it was found that ethanol extract (IC_{50} = 5.9 µg/mL) showed the higher radical-scavenging activity than watery extract (IC_{50} = 18.5 µg/mL).

Keywords: *Croton oblongifolius* Roxb., phytochemical investigation, nutritional values, antimicrobial activity, free radical scavenging activity

Introduction

A medicinal plant is plant that has similar properties as conventional pharmaceutical drugs. Humans have used them throughout history to either cure or lessen symptoms from an illness. Nowadays plants are still important sources of medicines, especially in developing countries that still use plant-based traditional medicine for their healthcare. In 1985, it was estimated in the Bulletin of the World Health Organization (WHO) that around 80% of the world's population relied on medicinal plants as their primary healthcare source. *Croton oblongifolius* bark was selected for this research to investigate some biological activities. Scientifically which is known as *C. oblongifolius* and its plant family is Euphprbiaceae. Myanmar name is Thak-ring-kri. *C. oblongifolius* plant is a middle sized tree (about 8 m high) deciduous bark, brownish, branches lapidate. Leaves are alternate and 12.5-25 cm long, crowded towards the end of the branchlets, oblong-lanceolate, subacute and is found in many parts of

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Myanmar and in other Asian countries (Chuakul *et al*., 1997). *C.oblongifolius* plant has been reported to possess antioxidant property, antitumor, antibacterial, antileshmanial and anti-inflammatory activities. Chemical constituents present in Thak-ring-kri stem bark are steroids, monoterpenes, diterpenes, sesquiterpenes, phenyl propanoids, glycosides, mixture of steroid glycosides and flavonoids (Kumar *et al*., 1996). The present study deals with investigation of phytochemicals, antimicrobial activity, total phenolic content and antioxidant activity of the stem bark of *C. oblongifolius*.

Materials and Methods

Sample Collection

The samples was collected from Sittway Township, Rakhine State. After collection, the scientific name of *C.oblongifolius* was identified by authorized botanist at the Department of Botany, Sittway University. The collected sample was clean and dried. The air dried sample was made up to powder in electric grinder and stored in air-tight container to prevent moisture changes and other contamination.

Phytochemical Investigation of Thak-ring-kri Stem Bark

A phytochemical is a natural bioactive compound found in plant foods that works with nutrients and dietary fiber to protect against diseases. Phytochemical investigation was carried out to know the types of phytoorganic constituents present in Thak-ring-kri stem bark by test tube method (Trease and Evans, 1980; Robinson, 1983; M-Tin Wa, 1970; Vogel, 1996; Harborne, 1984; Marini-Bettolo, 1981).

Deterimnatoin of Nutritional Values

The nutritional values such as moisture, ash, protein, crude fiber, ether extract (crude fat) and carbohydrate of Thak-ring-kri bark were determined by AOAC methods at Myanmar Food Processors and Exporters Association (MFPEA) in Lanmadaw Township, Yangon, Myanmar (Mark and Stewart, 1975, Pearson, 1981, Joslyn, 1973, AOAC, 2000, Anderson, 1984).

Screening of Antimicrobial Activity

Antimicrobial activity of the Thak-ring-kri stem bark was studied by using agar well diffusion method in various solvents system on six microorganisms such as *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus pumilus*, *Candida albicans* and *Escherichia coli* at Fermentation Laboratory, Pharmaceutical Research Department, Ministry of Industry, Yangon (Finegold, 1978).

Determination of Total Phenolic Contents (TPC)

Total phenolic contents (TPC) was determined spectrophotometrically using Folin-Ciocalteu reagent (Rekha *et al*., 2012). Crude extract (0.01 g) was dissolved in 20 mL methanol to obtain concentration of 500 µg/mL. 5 mL of Folin-Ciocalteu reagent was added to 0.5 mL of the extract and incubated at room temperature for 30 min. Next, 4 mL of 1M Na_2CO_3 was added and kept at room temperature for 15 min and absorbance of reaction mixture was measured at 760 nm by a UV-visible spectrophometer. The samples were prepared in triplicate for each

analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of gallic acid and the calibration line was constructed. Gallic acid is used as the reference standard compound and total phenolic contents was estimated as microgram gallic acid equivalent per milligram (GAE).

Determination of Total Flavonoid Contents (TFC)

Aluminum chloride colorimetric method was used to determine the total flavonoid contents in the plant extracts. This method is based on the determination of the flavonoid-aluminum complex between flavonoid of the crude extract and aluminum chloride. Briefly, 1 mL of extract in methanol (6.25 - 50 µg/mL) was mixed with 1 mL aluminum chloride in ethanol (20 µg/mL) and a drop of acetic acid. The resulting mixture was then diluted with ethanol to 25 mL. The absorption at 415 nm was read after 40 min. A blank sample was prepared in similar fashion omitting the extract. The calibration curve of quercetin was plotted using the same procedure and the amount of total flavonoids was calculated from linear regression equation obtained from the curve $y = 0.0023x + 0.0879$, $R^2 = 0.9993$ and expressed as quercetin equivalents (QE) per gram of the plant extract.

Screening of Antioxidant Activity

Antioxidant activity of ethanol and watery extracts of Thak-ring-kri stem bark was carried out by determination of DPPH (1, 1-Diphenyl-2-picryl hydrazyl) free radical scavenging property using UV spectroscopic method (Marinova and Batchvarov, 2011).

2 mg of each test sample and 10 mL of ethanol was thoroughly mixed and the mixture solution was filtered and the filtrate was used as a stock solution. Desired concentrations (5, 2.5, 1.25 and 0.625 µg/mL) of sample solutions were prepared from this stock solution by dilution with appropriate amount of ethanol.

Sample solution was prepared by thoroughly mixing 1.5 mL of 0.002% DPPH solution and 1.5 mL of test sample solution in the brown bottle. The control solution was also prepared by mixing 1.5 mL of 0.002% DPPH solution and 1.5 mL of ethanol. The solutions were then allowed to stand at room temperature for 30 min. Ascorbic acid (Vitamin C) synthetic antioxidnat was used as a standard and ethanol without sample was employed as control. After that, absorbance of these solutions was measured at 517 nm by using UV spectrophotometer. Decrease in absorbance indicates increases in radical scavenging activity. Absorbance measurements were done in three times for each sample solution and the mean value so obtained were used to calculate % inhibition of oxidation by using the following equation. Then IC_{50} (50 % inhibition concentration) was determined by using linear regressive excel program.

$$\% \text{ Inhibition of oxidation} = \frac{A_{DPPH} - (A_{sample} - A_{Blank})}{A_{DPPH}} \times 100$$

A_{DPPH} = Absorbance of DPPH solution

A_{sample} = Absorbance of sample + DPPH solution

A_{blank} = Absorbance of solvent

Results and Discussion

Phytochemical Investigation

The phytochemical investigation of Thak-ring-kri stem bark was determined and the results are summarized in Table 1. According to these results, flavonoids, phenolic compounds, glycosides, saponins, terpenoids, steroids, carbohydrate, tannins, starch and α-amino acids were found to be present in the sample. Alkaloids and cyanogenic glycosides were absent in the sample.

Nutritional Values

The nutritional values of moisture, fiber, ash, protein, carbohydrate and energy content were found as shown in Table 2 and Figure 1. As a result, the nutritional parameter of carbohydrate was rich and fiber was present as major nutrient in Thak-ring-kri stem bark.

Table 1 Results of Phytochemical Test on Thak-ring-kri Stem Bark

No.	Test	Extract	Test reagent	Observation	Remark
1.	Alkaloids	1 % HCl	Wagner's reagent Dragendorff's	No white ppt No orange ppt	- -
2.	Flavonoids	EtOH	Conc.HCl & Mg turning	Red colour sol^n	+
3.	Glycosides	H_2O	10 % lead acetate	White ppt	+
4.	Phenolic compounds	H_2O	1 %$FeCl_3$ solution & $K_3Fe(CN)_6$ solution	Deep blue colour sol^n	+
5.	Saponins	H_2O	Distilled water	Frothing	+
6.	Terpenoids	$CHCl_3$	Acetic anhydride & conc. H_2SO_4	Pink colour sol^n	+
7.	Steroids	Pet-ether	Acetic anhydride & conc. H_2SO_4	Green colour sol^n	+
8.	Carbohydrate	H_2O	10% α-naphthol and conc. H_2SO_4	Red ring	+
9.	Tannins	H_2O	10% NaCl & 1% gelatin	White ppt	+
10.	Starch	H_2O	I_2 solution	Deep blue sol^n	+
11.	α-Amino acids	H_2O	Ninhydrin	Pink spot on TLC	+
12.	Cyanogenic glycosides	H_2O	Conc. H_2SO_4 & sodium picrate solution	No brick red ppt	–

(-) = absence (+) = presence

Table 2 Results of Nutritional Values from Thak-ring-kri Stem Bark

No.	Types of Nutrients	Observed values
1	Moisture (%)	6
2	Ash (%)	10
3	Protein (%)	5.73
4	Crude fiber (%)	11.45
5	Fat (%)	7.43
6	Carbohydrate (%)	59.4
7	Energy value (kcal/100g)	327

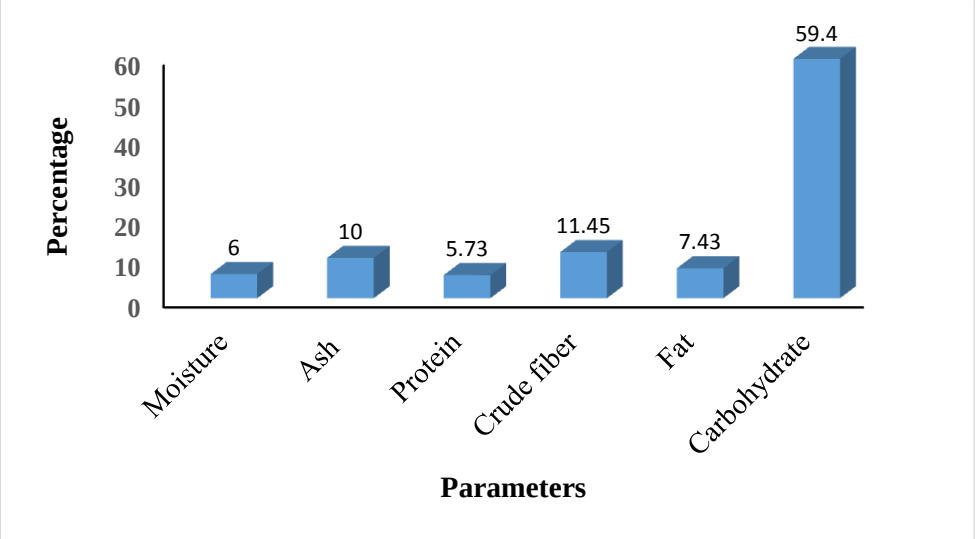

Figure 1 Histogram of nutritional values of Thak-ring-kri stem bark

Antimicrobial Activity

Screening of antimicrobial activity of various crude extracts such as pet-ether, dichloro methane, ethyl acetate, ethanol, methanol and watery extracts from Thak-ring-kri bark was investigated by employing agar well diffusion method. In this study, the samples were tested on six species of microorganisms such as *B. substilis*, *S. aureus*, *P. aeruginosa*, *B. pumilus*, *C. albicans* and *E. coli* species. The inhibition zone diameters of all crude extracts against all six microorganisms tested are shown in Table 3 and Figure 2.

From these results, it was found that all extracts exhibit antimicrobial activity against all tested microorganisms. Pet-ether, dichloro methane, ethyl acetate, ethanol, methanol and watery extracts from Thak-ring-kri stem bark exhibited inhibition zone diameters ranged in 16 ~ 35, 15 ~ 20, 14 ~ 16, 16 ~ 18, 17 ~ 19 and 12 mm, respectively, against all microorganisms tested. Thus, its watery extract showed less activty and pet-ether extract was observed to show the most pronounced antimicrobial activity against all microorganisms tested.

Table 3 The Results of Antimicrobial Activity of Various Crude Extracts from Thak-ring-kri Stem Bark

No.	Type of Organisms	Diameter of Inhibition Zone (mm)					
		PE	CH_2Cl_2	MeOH	EtOAc	EtOH	H_2O
1	*B. subtilis*	19	17	18	15	18	12
2	*S. aureus*	20	18	18	16	17	12
3	*P. aeruginosa*	16	15	18	-	17	12
4	*B. pumilus*	35	20	17	14	16	12
5	*C. albicans*	25	18	19	16	18	12
6	*E.coli*	21	18	18	14	17	12

Agar well diameter = 10 mm, 10 mm ~14 mm = (+) (low activity),
15 mm ~ 19 mm = (++) (medium activity),
20 mm ~ above = (+++) (high activity),
Not detected = (-)

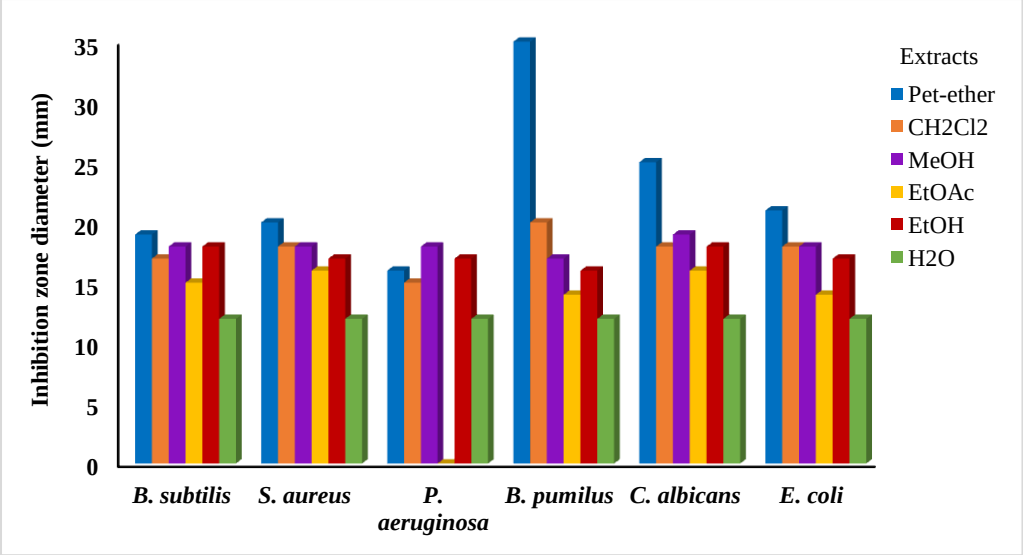

Figure 2 Histogram of antimicrobial activity of different extracts from Thak-ring-kri stem bark on six microorganisms by agar well diffusion method

Total Phenolic Contents

Total phenolic contents in the Thak-ring-kri stem bark extracts using the Folin-Ciocalteu's reagent is expressed as microgram of gallic acid equivalent per milligram of crude extract (µg GAE/mg). Standard calibration curve of gallic acid was prepared by dilution of the stock solution (1000 µg/mL) to obtain various concentrations (100, 50, 25, 12.50, 6.25, 3.125) µg/mL. Gallic acid standard curve gave a straight line. The total phenolic contents of ethanol and watery extracts were found to be (197.50 ± 0.01) µg GAE/mg and (41.42 ± 0.00) µg GAE/mg, respectively. Thus, ethanol extract was more effective than watery extract. The results are recorded in Tables 4, 5 and Figures 3, 4.

Table 4 Total Phenolic Contents (TPC) of Thak-ring-kri Stem Bark Extracts by Folin-Ciocalteu Method

Sample (500 µg/mL)	TPC (µg GAE/mg ± SD)
Ethanol extract	197.50 ± 0.01
Watery extract	41.42 ± 0.00

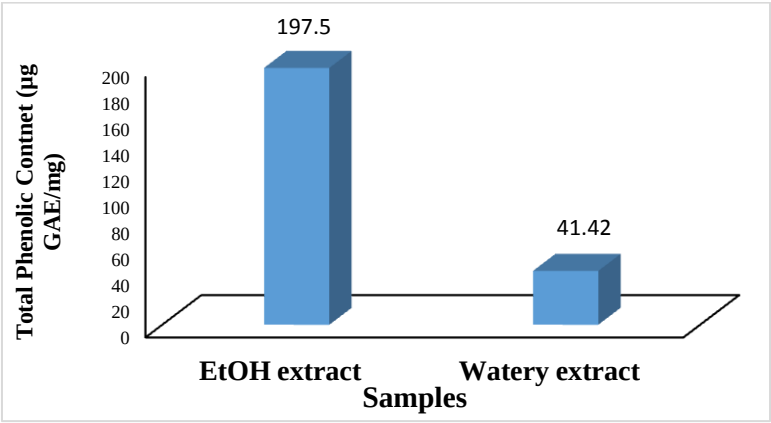

Figure 3 Total phenolic contents of ethanol and watery extracts

Table 5 Absorbance of Various Concentrations of Standard Gallic Acid

Concentration of Gallic Acid (µg/mL)	Absorbance at 760 nm
3.125	0.201
6.25	0.221
12.50	0.237
25.00	0.284
50.00	0.402
100.00	0.598

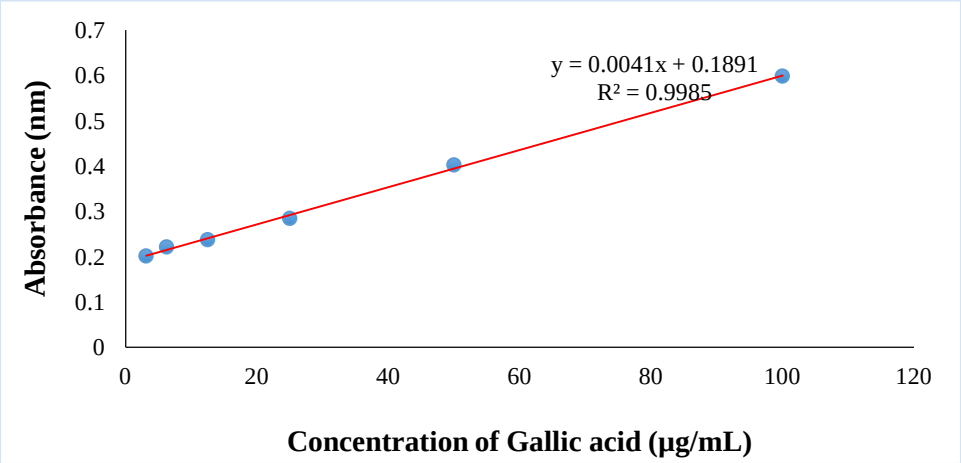

Figure 4 Standard calibration curve of gallic acid concentration vs absorbance at λ_{max} 760 nm

Total Flavonoid Contents (TFC)

Spectrophotometric method using Aluminum Chloride Colorimetric (ACC) method was used to determine the total flavonoid contents in the selected plant extracts. This method is based on the determination of the flavonoid-aluminum complex between flavonoid of the crude extract and aluminum chloride. The standard quercetin solution was prepared by dilution with the different concentration of 100, 50, 25, 12.5 and 6.25 µg/mL. The standard quercetin gave a straight line. The amount of total flavonoid contents was calculated from linear regression equation obtained from the curve y = 0.0023x + 0.0879, R^2 = 0.9993 and expressed as quercetin equivalents (QE) per gram of the plant extract. The total flavonoids contents of ethanol and watery extracts were found to be (115.34 ± 0.01) µg QE/mg and (70.00 ± 0.00) µg QE/mg, respectively. Thus, ethanol extract was more effective than watery extract. The results are recorded in Tables 6, 7 and Figures 5, 6.

Table 6 Total Flavonoid Contents (TFC) of Ethanol and Watery Extracts

No.	Extracts (300 µg/mL)	Absorbance	TFC (µgQE/mg ± SD)
1	Ethanol	0.168	115.34 ± 0.01
2	Watery	0.137	70.00 ± 0.00

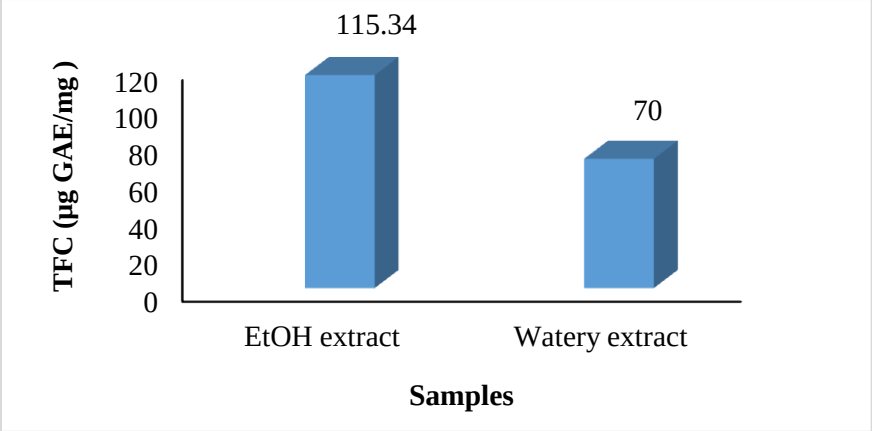

Figure 5 A bar graph of total flavonoid contents of ethanol and watery extracts

Table 7 Absorbance of Standard Compound Quercetin at λ_{max} 415 nm

No.	Concentration of Quercetin (µg/mL)	Absorbance at 415 nm
1	3.125	0.092
2	6.25	0.104
3	12.50	0.118
4	25.00	0.145
5	50.00	0.198
6	100.00	0.314

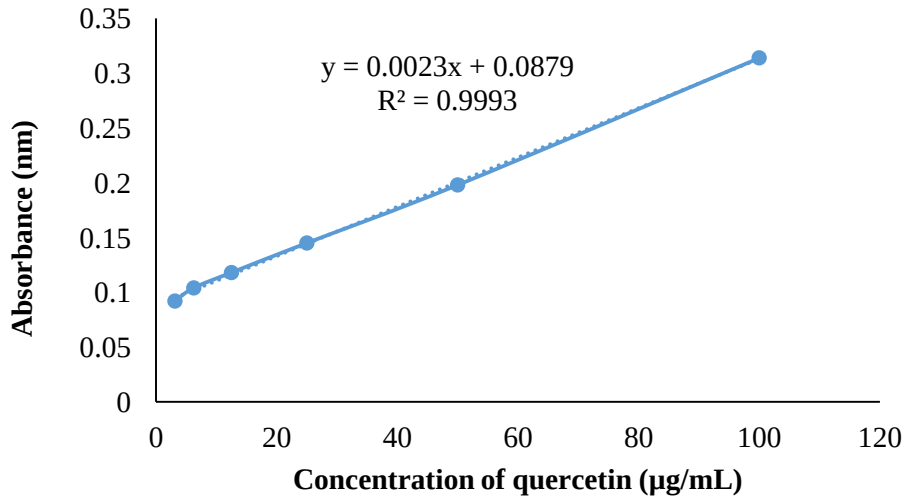

Figure 6 Standard calibration curve of quercetin concentration vs absorbance at λ_{max} 415 nm

Antioxdant Activity

The antioxidant activity of ethanol and watery extracts of Thak-ring-kri stem bark were studied by DPPH free radical scavenging assay. The resultant average % inhibition property values in different concentrations (50, 25, 12.5, 6.25 and 3.125 µg/mL) for all samples is

tabulated in Table 8 and Figures 7 and 8. From these figures, it can be seen that as the concentration of the samples increased, the respective % inhibition also increased.

The antioxidative potential of sample can be determined by IC_{50} (50% inhibition concentration). These IC_{50} value for each extract was determined by linear regressive excel program and can also be obtained from the plot of % inhibition *vs* concentration of the samples. The IC_{50} values were found to be 5.94 µg/mL for ethanol extract and 18.5 µg/mL for watery extract. Since the lower the IC_{50} values, the higher the free radical scavenging activity, i.e., the higher the antioxidative property. Watery extract has the higher IC_{50} than that of ethanol extract. Therefore, the antioxidant potential of ethanol extract was found to be higher than that of watery extract. In addition, it was found that all of these extracts have the lower antioxidant activity than standard ascorbic acid (IC_{50} = 1.9 µg/mL).

Table 8 Average % Inhibition of Oxidation and Values of Watery and Ethanol Extracts and Standard Ascorbic Acid

Samples	Average % inhibition in various concentrations (µg/mL)					IC_{50} (µg/mL)
	3.125	6.25	12.5	25	50	
watery extract	28.23	28.45	42.96	57.60	68.80	18.5
ethanol extract	28.42	52.37	71.22	73.09	76.41	5.9

Samples	Average % inhibition in various concentrations (µg/mL)					IC_{50} (µg/mL)
	0.16	0.8	4.0	20.0	100	
Ascorbic acid	16.34	39.20	70.52	88.09	95.95	1.9

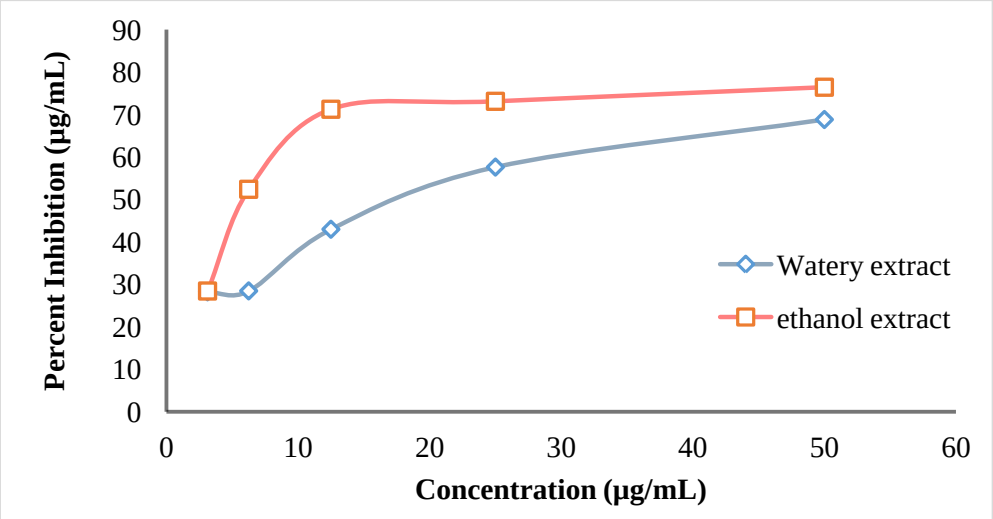

Figure 7 Percent inhibition of oxidation *vs* concentration (µg/mL) of ethanol and watery extracts from Thak-ring-kri stem bark

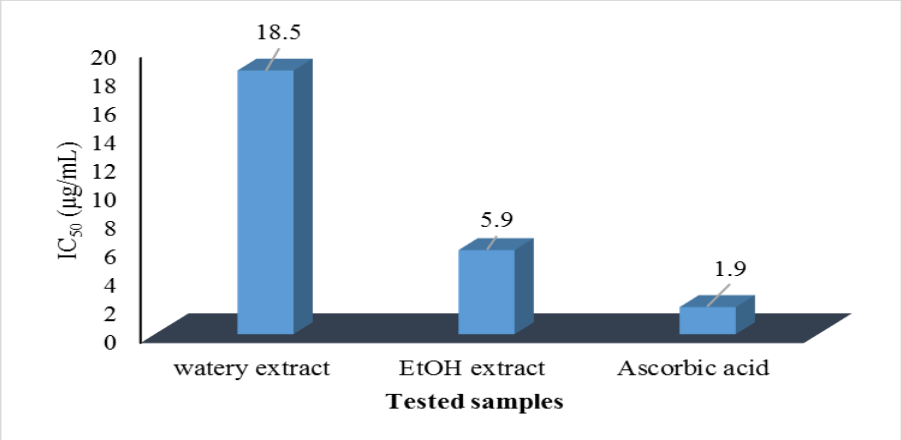

Figure 8 Bar graph of IC_{50} values of ethanol and watery extracts from Thak-ring-kri stem bark compared with standard ascorbic acid

Conclusion

Thak-ring-kri stem bark revealed the presence of flavonoids, phenolic compounds, glycosides, saponin, terpenoids, steroids, carbohydrate, tannin, starch and α-amino acid, and absence of alkaloids and cyanogenic glycosides. Nutritional values were found to be 6% of moisture, 10 % of ash, 5.73 % of protein, 11.45 % of crude fiber, 7.43 % of fat, 59.4 % of carbohydrate and 327 kcal/100 g of energy, based of dried sample.

Antimicrobial activity of six crude extracts such as pet-ether, dichloro methane, ethyl acetate, ethanol, methanol and watery extracts from selected sample was investigated by employing agar well diffusion method aganist *B. substilis*, *S. aeruginosa*, *B. pumilus*, *C. albicans* and *E. coli* species. According to the results obtained, all extracts showed the antimicrobial activity against all tested microorganisms. Pet-ether extract was observed the most pronounced antimicrobial activity and watery extract was the lowest activity agaisnt all tested microorganisms. The total phenolic contents of ethanol and watery extracts were found to be (197.5 ± 0.01) µg GAE/mg and (41.42 ± 0.00) µg GAE/mg respectively. Thus, ethanol extract was more effective than watery extract. The total flavonoids contents of ethanol and watery extracts were observed to be (115.34 ± 0.01) µg QE/mg and (70.00 ± 0.00) µg QE/mg respectively. Thus, ethanol extract was more effective than watery extract. From the screening of free radical scavenging activity by DPPH assay, it was found that ethanol extract (IC_{50} = 5.9 µg/mL) showed the higher antioxidant activity than watery extract (IC_{50} = 18.5 µg/mL). These two extracts showed less activity by comparing with the standard ascorbic acid (IC_{50} = 1.9 µg/mL). The present research is therefore, ethanol and watery extracts of Thak-ring-kri stem bark may be useful for the cure of bacterial infections and oxidative stress related diseases.

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STUDY ON *IN SITU* REMEDIATION OF CONTAMINATED SEDIMENT FROM FLOODED AREA OF MINBYA TOWNSHIP, RAKHINE STATE

Khin Myint[1], Shwe Sin[2] and Aye Aye Lwin[3]

Abstract

This research is concerned with the remediation of contaminated sediments from the flooded areas, Middle Lake and Small Lake of Minbya Township in Rakhine State.The two sediment samples were collected in September, 2018. The investigative analyses were done in terms of distribution of some heavy metals in the sediment samples and microbiological properties of sediments. Relative abundances of elements in the sediment samples were determined by EDXRF. The quantitative elemental analysis of the sediment samples were determined by AAS. Cadmium concentrations of the both sampling sites were not detected and those of arsenic , iron and lead were (0. 14 , 0.15) ppm, (24.17 , 24.57) ppm and (0.09, 0.23) ppm, respectively. Microbiological properties of sediments were examined by using 3M Petri Films. Total Coliform could be observed as (3000) and (9000) cfu g^{-1} in these study areas. However, *E.coli* could not be detected in both sites. Overall values of study areas indicated that the iron was in high level in the contaminated samples. The in-Situ treatment system for contaminated sediment was treated by using lime amendment and sand-cap. The cost effective in-situ treatment system has shown the significant removal percent of iron (50.29 %) to (50 %) compared with control (without amendment) system. This remediation technique code inferred the very beneficial for rural area as in cost effective and locally available technique.

Keywords : sediment, flooded areas, microbiological properties , in-situ treatment system

Introduction

Widespread accumulations of flood sediments were left behind in Minbya Township after the flood. The main purpose of this paper is to provide a review on the in-situ remediation technology of heavy metal in sediment (Peng *et al* ., 2009). The contamination of aquatic sediments with metals is a widespread environmental and human health problem (Akcil *et al*., 2015).

Heavy metals from various sources can enter a natural aquatic system after flood waters. Heavy metal contamination has become a worldwide problem through disturbing the normal functions of river and lakes). Heavy metals usually possess significant toxicity to aquatic organisms and then effect human health from food chain (Peng *et al* ., 2009).

Sediment, as the largest storage and resources of heavy metal, plays a rather important role in metal transformations . Heavy metals cannot fix in sediment forever. With the variation of the physical-chemical characteristics of water conditions, part of these fixed metals will re-enter the overlying water (Loser *et al*.,2007) .

Conventional remediation strategies include in-place sediment remediation strategies such as In Situ- capping and relocation actions are still widely applied (Renholds,2015). Normally, decreasing the direct contact area between water and the contaminated sediment

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is a good choice for lowering the release content of heavy metal . Therefore , capping the contaminated sediment with sandy materials becomes an effective remediation technique. This remediation technique can only reduce the transfer rate of metal in sediment, while their immobilization effect for heavy metal is small. Therefore, for enhancing their immobilization capacity , some amendments such as apatite or zeolite can also be added into the sand cap.

Materials and Methods

Collection for Flooded Sediment Samples

The study area is located at Minbya Township in Rakhine State. Its geographical coordination is 20°21' 45.78" North Latitude and 93°16' 02.87" East Longitude. This study was conducted in September, 2018. The main annual rainfall is about nearly 150 mm while the average temperature ranged between 21°C to 34°C in the prescribed zone.

The sediment samples were collected from two sampling sites, Middle Lake and Small Lake, at Minbya Township in Rakhine State as shown in Figures 1 and 2.Both Lakes are located about North Latitude 20°21' and about East Longitude 93º16'. These sediment samples were taken from the depth of 15 cm of sediment surface in a zigzag manner by using GPS (Global Positioning System). Then the sediment sample from each site was mixed thoroughly to homogenize and put it into the plastic bottle . Sediment samples were carried to the laboratory and kept in cold and dark place.

Figure 1 Photograph of Middle Lake

Figure 2 Photograph of Small Lake

Determination of Trace Elements in Sediment Samples by Atomic Absorption Spectrometry

0.1 g of air-dried sediment sample was placed in an Erlenmeyer flask. 20 mL of extracting solution (0.05 M HCl + 0.0125 M H_2SO_4) was added in the flask and shaken for 15 min. Then the solution was filtered into a 50 mL volumetric flask through filter paper and diluted to 50 mL with extracting solution.

The standard solution having the varying strength was sprayed into the gas flame of Atomic Absorption Spectrometer.

At least three known concentration of each standard metal solution were selected. These should be one concentration greatest and one less than that expected ppm range in

the sample. Each standard solution was aspirated in turn into the gas flame and recorded the absorbance from the display. By using the calibration curve of the three standards, the concentrations of sample were read out. The results are presented in Table 2.

Determination of Total Coliform and *E.coli* in Sediment Sample Using 3M Petri Film

Petri Film *E.coli* count plate was placed on a flat surface. The top film was lifted and dispensed 1 mL of sample, treated with potassium dihydrogen phosphate buffer solution, onto the center of the bottom film. The top film was slowly rowed down onto the sample to prevent the entrapment of air. The sample was distributed evenly within the circular well using gentle downward pressure on the center of the plastic spreader (flat side down). The spreader was not slide across the film. The spreader was removed and left plate undisturbed for 1 min to permit solidification of the gel. The plate were left in a horizontal position with the clear side up in stacks not exceeding 20 plates. The plate were incubated for 24 ± 2h and examined for coliform and *E.coli* growth. Some *E.coli* colonies require additional time to form blue precipitate. The plates were re-incubated for an additional 24 ± 2h to detect any additional *E.coli* growth. According to AOAC method (991.14), Petri Film plates were incubated at 35 ± 1 ˚C.

Experimental Design for In-situ Remediation

In order to determine the removal efficiency of amendment, eight beakers were prepared. The experiments were completely arranged in 8 different series (I, II, III, IV, V, VI, VII, and VIII) and three replications for each treatment. Each vessel was filled by sediment sample (50 g) at the bottom. The collected water sample from respective sampling site was added into all vessels. Lime powder (10 g) each was added to the four vessels (I, II, III, IV) and marked as tested sample. The other four vessels (V, VI, VII and VIII) were not added with lime and designated as control sample. All the samples were kept in cold and dark place for one week.

The sand sample, lower the release content of heavy metal, was collected from Sittway Beach near Sittway Hotel in Rakhine State. The collected sand was washed with distilled water (three times), 1 % sulphuric acid, and dried under the sunlight. The obtained dry sand (10 g) was filled to cover the tested sample (I, II, III , IV) and control sample (V, VI, VII, VIII). All the samples were caped in similar manner that was described above for one week.

After two weeks, the sediment sample was collected to determine the removal percentage of Fe metal using AAS .

Results and Discussion

Qualitative Elemental Analysis of Sediment Samples by EDXRF

Relative abundances of elements in sediment samples were determined by EDXRF spectrometer. The EDXRF spectra of sediment samples are shown in Figures 3 and 4. The results are recorded in Table 1. It can be observed that the sediment samples of Middle Lake contained Si (47.633 %), Fe (36.596 %), K(8.290 %), Ca (3.492 %) and Ti (3.022 %), respectively. 45.072 %, 41.929 %, 7.073 %, 2.739 % and 2.371 % of Si, Fe, K, Ca and Ti, respectively, were present in the Small Lake. A small amount of other elements such as, Zr , Sr , Cr, Zn, Cu, As, Mn, and Y were found in the Middle Lake and Zr, Mn, Cu, Zn, Sr, Cr and Y were found in the Small Lake.

Table 1 Relative Abundance of Elements in Sediment Samples by EDXRF

No.	Element	Relative Abundance (%)	
		Middle Lake	Small Lake
1	Si	47.633	45.072
2	Fe	36.596	41.929
3	K	8.290	7.073
4	Ca	3.492	2.739
5	Ti	3.022	2.371
6	Zr	0.341	0.236
7	Sr	0.130	0.096
8	Cr	0.112	0.090
9	Zn	0.100	0.098
10	Cu	0.082	0.107
11	As	0.072	ND
12	Mn	0.067	0.137
13	Y	0.061	0.052

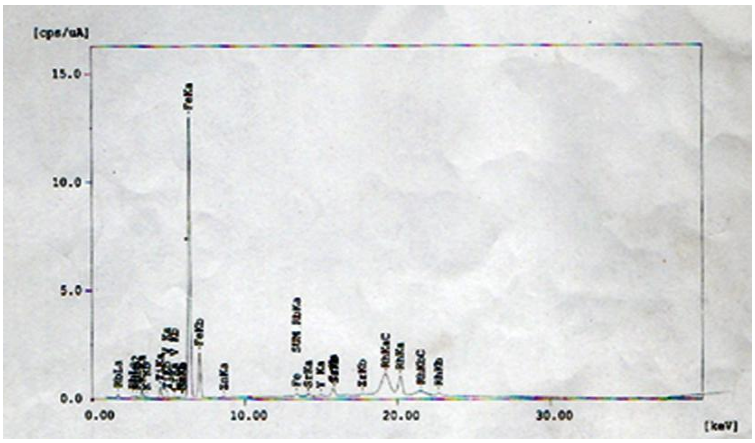

Figure 3 EDXRF spectrum of sediment sample from Middle Lake

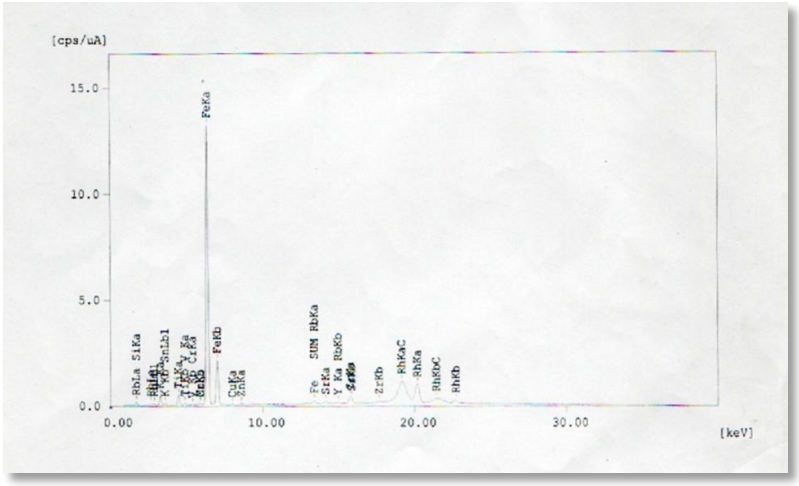

Figure 4 EDXRF spectrum of sediment sample from Small Lake

Determination of Heavy Metals in the Sediment Samples by Atomic Absorption Spectrophotometry

In this research, atomic absorption spectrophotometer (Perkin-Elmer) was employed for the determination of heavy metals in the collected sediment samples.

The contents of heavy metals in the collected sediment samples are shown in Table 2 and Figure 5. Iron concentrations of the two sampling sites were 24.17 and 24.57 ppm, arsenic concentrations were 0.14 and 0.15 ppm and lead concentrations were 0.09 and 0.23 ppm, respectively. Cadmium concentration of those samples were not detected in both sites. The level of Iron in these study areas were above the permissible limit set by the World Health Organization (1-3) ppm .

Table 2 Some Heavy Metal Contents of the Collected Sediment Samples by AAS

No.	Heavy Metals	Concentration (ppm)	
		Middle Lake	Small Lake
1	Fe	24.17	24.57
2	As	0.14	0.15
3	Pb	0.09	0.23
4	Cd	ND	ND

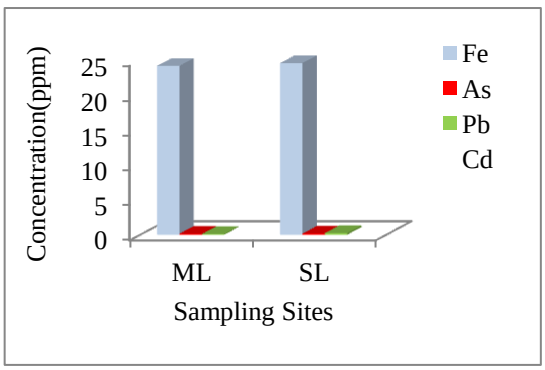

Figure 5 Heavy metal concentration of collected sediment samples

Total Coliform and *E.coli* in the Sediment Samples

In this research, 3 M Petri Film was used for the microbiological test of the collected sediment samples.

Total Coliform and *E.coli* contents of the collected sediment samples are shown in Table 3 and Figures 6 (a) and (b). Total Coliforms of Middle Lake and Small Lake were 3000 and 9000 cfu g^{-1}, respectively. Total Coliforms of these two Lakes were below the permissible limit of AOAC standard. However, *E.coli* was not detected in both sites.

Table 3 Coliforms and *E.coli* Contents of the Collected Sediment Samples

No.	Sediment Sample	Coliform (cfu/g)	*E.coli* (cfu/g)
1	Middle Lake	3000	ND
2	Small Lake	9000	ND

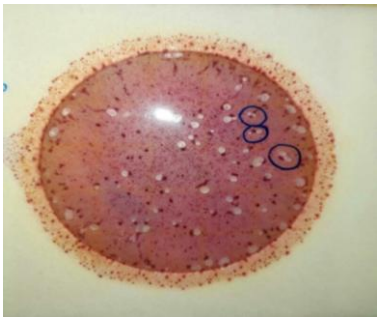

Figure 6 (a) Photograph of Coliform and *E.coli*

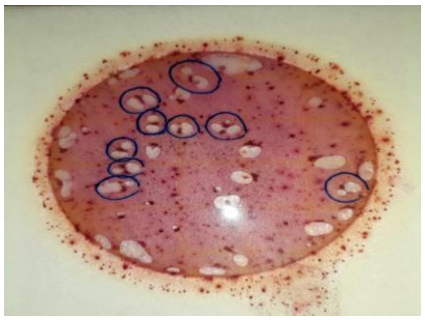

Figure 6 (b) Photograph of Coliform and *E.coli* colle in the Middle Lake in the Small Lake

Removal Percent of Iron from the Collected Sediment Samples by using In-situ Capping Method

In-situ capping method was used for the determination of removal percent of Iron in Middle Lake and Small Lake.

Removal Iron percents of the two study areas are shown in Table 4 and Figure 7. Removal Iron percent of Middle Lake and Small Lake were 50.29 % and 50.00 %.

Table 4 Removal Iron Percent of the Collected Sediment Samples by using In-situ Capping Method

No.	Sediment Sample	Removal Percent of Iron (%)
1	Middle Lake	50.29
2	Small Lake	50.00

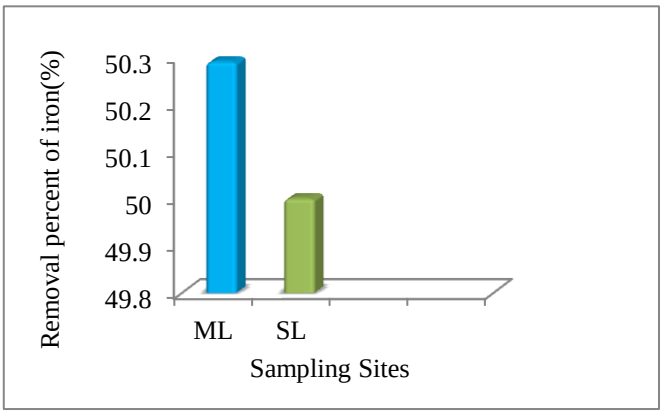

Figure 7 Removal percent of iron from the collected sediment samples

Conclusion

Nowadays, heavy metals pollution in river and lake sediments have gradually become a major concern worldwide. The remediation of contaminated sediment is necessary.

In this research, two lakes of flooded areas in Minbya Township were used as the sampling sites. EDXRF spectra indicated that the higher percents of Fe(36.596 %) in Middle Lake and Fe (41.929 %) in Small Lake . A small amount of other elements, Zr, Sr, Cr, Zn, Cu, As, Mn, and Y were found in the Middle Lake and Zr, Mn, Cu, Zn, Sr, Cr and Y were found in the Small Lake.

From the quantitative analysis of the sediment samples, it was found that iron concentrations of the two sampling sites were 24.17 and 24.57 ppm, arsenic concentrations were 0.14 and 0.15 ppm and lead concentrations were 0.09 and 0.23 ppm, respectively. Cadmium concentrations of those samples were not detected in both sites.

From the investigation of microbiological test using 3M Petri Film 3000 and 9000 cfu g^{-1} of total Coliforms were observed in Middle Lake and Small Lake, respectively. However, *E.coli* would not be detected in both sites.

According to the results of In Situ capping method to the contaminated sediments, the concentration of Irons (50.29 % and 50 % for Middle Lake and Small Lake) could be reduced significantly using lime amendment and sand-cap. This remediation technique code inferred the very beneficial for rural area due to their easy operation, low costs and fast remediation effect.

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ISOLATION OF BIOACTIVE COMPOUNDS FROM BARK OF *MIMUSOPS ELENGI* ROXB. (KHA-YAY) USED IN THE TREATMENT OF HYPERGLYCEMIA

Nyein Nyein Htwe[1], Mon Mon Thu[2], Win Naing[3]

Abstract

The present research focused on the investigation of antihyperglycemic activities of two crude extracts (70 % ethanol and water) and isolated organic compounds from bark of *Mimusops elengi* Roxb. (Kha-yay) by using adrenaline induced hyperglycemic rats. The optimum antihyperglycemic activities of two extracts showed 70 % ethanol extract (30.11 % reduction, $p < 0.01$, 1 h and 35.25 % reduction, $p < 0.01$, 2 h) and watery extract (19.94 % reduction, $p < 0.01$, 1 h and 21.61 % reduction, $p < 0.01$, 2 h) after treating with 2 g/kg body weight dose. Lupeol (0.0037 %, m.p- 215-217 °C) and spinasterol (0.0048 %, m.p- 167-169 °C) were isolated from pet-ether soluble portion of 70 % ethanol crude extract by column chromatographic method using pet-ether and ethyl acetate with increasing polarity ratio as eluent. Both compounds were identified by modern spectroscopic methods (UV, FT IR and ^{1}H NMR) and confirmed by comparison with the data reported in literature. Reduction percent of blood glucose levels of lupeol and spinasterol with a dosage of 2 mg/kg body weight showed 19.44 % ($p < 0.01$) and 22.01 % ($p < 0.01$) at 2 h respectively.

Keywords: *Mimusops elengi* Roxb., antihyperglycemic activities, lupeol, spinasterol

Introduction

Diabetes mellitus (DM) is a clinical syndrome characterized by hyperglycemia (high blood sugar level), due to deficiency or diminished effectiveness of insulin. It is the most frequently encountered metabolic disease in our societies today (Cho *et al*., 2018). Its prevalence has increased dramatically in recent years and it considered by WHO as a serious public health problem. According to IDF estimation, nearly 451 million people were suffering from diabetes in 2017 worldwide. This number is projected to reach 693 million by 2045 if current growth rate continues. It is a serious disease that can have a significant impact on the health, quality of life, and life expectancy of individuals, as well as on the health care system (Steven and Robert, 2001). The chronic hyperglycemia of diabetes is associated with long term damage, dysfunction and failure of various organs especially the eyes, kidneys, nerves, heart and blood vessels. Prevention and treatment involve maintaining a healthy diet, regular physical exercise, a normal body weight and avoiding use of tobacco. Control of blood pressure and maintaining proper food care are important for people with the disease.

Medicinal plants have been discovered and used in traditional medicine practices since prehistoric times. Plants synthesis hundreds of chemical compounds for functions including defense against insects, fungi, diseases, and herbivorous mammals (Ahn, 2017). Kha-yay is one of Myanmar medicinal plants. Botanical name is *Mimusops elengi* Roxb. (Sapotaceae family). English common names include as Spanish cherry, medlar, star flower and bullet wood. It has fragrant flowers and is planted as an ornamental and shade tree in gardens and along roads. Kha-yay contains variety of active phytoconstituents and thus possesses various kinds of biological

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and pharmacological activities. It possesses activities like antibacterial, antihemorrhoidal, antifungal, anticarciogenic, antioxidant, antineoplastic, antihyperglycemic, gastroprotective, antinociceptive , and diuretic effect, cognitive enhancing activity and cytotoxic activities (Gami, 2007). The bark can be used for cooling, cardiotonic, alexipharmic, stomachic, anthelmintic, astringent gargle and diarrhoea disease.

Materials and Methods

Sample Collection and Preparation

Bark of Kha-yay was collected from Yangon Region. The plant sample was identified at Department of Botany, Yangon University. The sample was thoroughly washed with water and cut into small parts. After being dried at room temperature for one week, the sample was made into powder and stored in air-tight container.

Preliminary Phytochemical Test

A few grams of dried powdered sample were subjected to the tests of steroids, terpenoids, flavonoids, glycosides, phenolic compounds, α-amino acids, carbohydrates, reducing sugars, saponins, tannins and alkaloids according to the standard procedures (Marini-Bettolo *et al*., 1981).

Preparation of Crude Extracts for Bioactivities

The dried powdered sample was percolated in 70 % ethanol and distilled water respectively. After one week, they were filtered and filtrates were concentrated to obtain 70 % ethanol and watery crude extracts.

Screening of Antihyperglycemic Activities of Crude Extracts

Healthy Wistar strain rats in male sex (250-300 g) were used in this experiment. Before the experiment, animals were kept fasting overnight for 18-20 h but were allowed free access to distilled water. The animals were divided into two groups (groups I and II) of five animals each and 70 % ethanol extract for group I and watery extract for group II were determined. All the rats were made diabetic by injecting them subcutaneously with standard dose 0.2 mg/kg body weight of adrenaline tartrate in distilled water using the method of Gupta *et al*. (1967). The blood glucose levels after injection of adrenaline were monitored in 1 h interval up to 4 h and the results were recorded. After one week, the blood glucose levels were again measured and the rats were made diabetic. Then, the blood glucose levels of each group were recorded hourly after administration of tested doses (2 g/kg body weight) from each extract by using superglucocard II and measured by blood glucose test meter (GT-1640).

The blood glucose levels were reported as mean ± Standard Error Mean (S.E.M). All results were evaluated statistically using student's t test by SPSS software. Probably level of less than 0.05 was considered significant.

Investigation of Acute Toxicity

In the present study, acute toxicity of different doses (2 g/kg, 4 g/kg and 8 g/kg body weight) of 70 % ethanol and watery extracts was evaluated by the method of Lorke (1983). The albino mice of both sexes (20-35 g) were assigned to seven groups with ten animals in each group. The animals were housed in standard cages with food and water at air conditioned room of 20 ± 5 ºC temperature with artificial light. Group I was treated with normal food and water and considered as control. Group II to IV mice were treated with 70 % ethanol extract and group V to VII mice were treated with aqueous extract orally. After oral administration of extracts, each group of mice was housed separately in a cage with free access to food and water. Observation and survivors were also observed for a total of 7 days.

Separation and Isolation of Organic Compounds

80 g of dried powdered bark was extracted with 70 % ethanol by Soxhlet extraction method for 15 h. This procedure was repeated for nine times. The combined solvents were evaporated under reduced pressure by means of a rotary evaporator and obtained ethanol extract. The ethanol extract was suspended in water and partitioned with pet-ether. Removal of the solvent from combined pet-ether layer provided pet-ether extract (6.33 g). 5 g of pet-ether extract was separated by column chromatography over silica gel (40 g) using pet-ether and ethyl acetate with increasing polarity ratio as eluent to yield 59 fractions. Each fraction obtained by column chromatography was checked by TLC. From the inspection of TLC chromatogram, the fractions of the same R_f values were combined to give 5 fractions. The fraction 2 was further purified by pet-ether and ethyl acetate (1:1 V/V) providing lupeol (26.26 mg, 0.0037 %). The fraction 5 was purified by pet-ether to provide spinasterol (34.51 mg, 0.0048 %). The structures of isolated compounds were identified by melting point determination and chemical methods such as behaviour on TLC using appropriate solvent systems and spraying reagents. In addition, spectroscopic methods such as ultraviolet, infrared and ^{1}H NMR were also employed. Furthermore, in some cases, identification was confirmed by co-TLC with authentic sample in various solvent systems and comparing with ACD Labs software.

Screening of Antihyperglycemic Activities of Isolated Compounds

Antihyperglycemic activities of isolated compounds were also investigated with a dosage of 2 mg/kg body weight according to the procedure mentioned in the previous section. Two groups (III and IV) of five animals each were divided and lupeol for group III and spinasterol for group IV were used in this experiment.

Results and Discussion

The phytochemical investigation showed the presence of steroids, terpenoids, flavonoids, glycosides, phenolic compounds, α-amino acids, carbohydrates, reducing sugars, saponins, tannins and alkaloids in bark of plant sample.

For the study on antihyperglycemic activities, 70 % ethanol crude extract produced its antihyperglycemic effect at 1 h (30.11 %, $p < 0.01$) and 2 h (35.25 %, $p < 0.01$) (Table 1 and Figure 1 a). The percent inhibitions of blood glucose levels were 30.11 %, 35.25 %, 6.69 % and 4.84 % at one hour interval. The blood glucose levels of watery crude extract were significantly decreased at 1 h (19.94 %, $p < 0.01$) and 2 h (21.61 %, $p < 0.01$) compared with that of control

rats (Table 2 and Figure 1 b). The percent inhibitions of blood glucose levels were 19.94 %, 21.61 %, 15.30 % and 6.02 %. From these results, it may be deduced that 70 % ethanol extract exhibits more antihyperglycemic effect than watery extract. In the case of isolated compounds, the blood glucose levels were significantly reduced after 2 h oral administration of each compound where 19.44 % reduction ($P < 0.01$) for lupeol and 22.01 % reduction ($P < 0.01$) for spinasterol (Tables 3, 4 and Figures 2 a, 2 b). The percent reductions of blood glucose levels were 16.18 %, 19.44 %, 14.16 % and 11.74 % for lupeol and 13.45 %, 22.01 %, 18.52 % and 10.22 % for spinasterol. From this data, it was found that the isolated compounds from bark of selected plant possessed the antihyperglycemic potency.

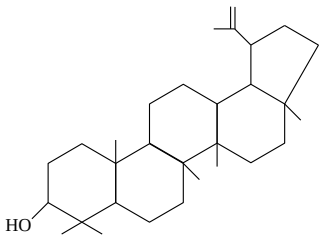
The acute toxicity screening for plant crude extracts (70 % ethanol and water) was done with the dosage of 2 g, 4 g and 8 g/kg body weight in mice. The condition of mice was denoted after one week treatment. All the animals remained alive and did not show any visible symptoms of toxicity like restlessness, respiratory disorders, convulsion, aggressive activities, coma and death at the tested dosage. So, the median lethal dose (LD_{50}) was more than 8 g/kg body weight. From these results, it was found that both plant extracts were free from acute toxic or harmful effects under experimental condition (Table 5).

Identification of Isolated Organic Compounds

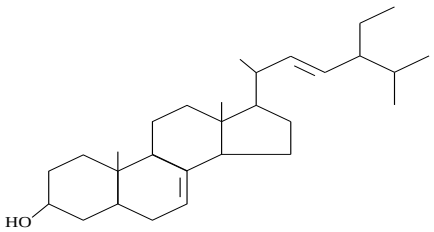
Lupeol ($C_{30}H_{50}O$) : colourless needle shape crystals, (R_f = 0.47, PE : EtOAC – 9:1 V/V), m.p. 215-217 °C (Lit. - 217°C, Merck Index, 2001). It was UV inactive. FT IR (KBr) ν_{max} (cm^{-1}): 3454 (O-H), 3070 (C=CH_2),2936, 2869 (C-H),1642, 1633 (C=C), 1461 (C-H), 1382 (CH_3), 1037 (CH-OH), 998 (C-H) : ^{1}H NMR (400 MHz,$CDCl_3$) (δ_H/ppm): 0.75-2.2 (47H,m,CH_3, CH_2, CH), 3.21 (1H,dd,-CH-OH), 5.11 (1H,d,=CH_2),5.51(1H,d,=CH_2) (Figures 3 and 4)

Spinasterol ($C_{29}H_{48}O$) : white amorphous powder (R_f = 0.46, PE : EtOAC – 2:1 V/V), m.p. 167-169°C (Lit. - 168-169°C, Merck Index, 2001). It was UV inactive. FT IR (KBr) ν_{max} (cm^{-1}): 3447 (O-H), 2940, 2867 (C-H), 1686, 1638 (C=C), 1451 (C-H), 1376 (CH_3), 1042 (C-OH), 882 (C-H) : ^{1}H NMR (400 MHz,$CDCl_3$) (δ_H/ppm): 0.65-2.5 (44H, m, CH_3, CH_2, CH), 3.20 (1H, m, CH-OH), 4.51 (1H, m, C=CH), 4.74 (1H, m, C=CH), 5.30 (1H, m, C=CH)

^{1}H NMR (ACD Labs software) (δ_H/ppm): 0.66-2.7 (44H, m, CH_3, CH_2, CH), 3.40 (1H, m, CH-OH), 5.01 (1H, m, C=CH), 5.17 (1H, m, C=CH), 5.48 (1H, m, C=CH) (Figures 5 and 6)



Lupeol



Spinasterol

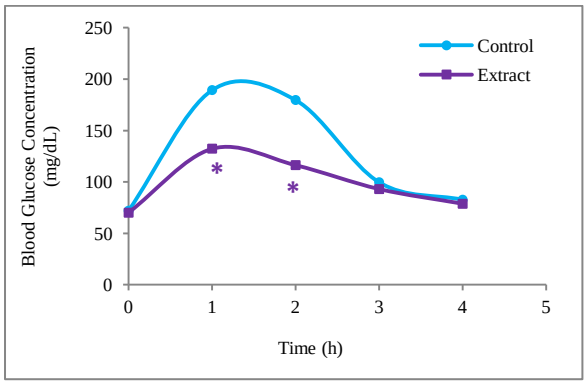
Table 1 Mean Blood Glucose Levels (± SEM) in Adrenaline Induced Hyperglycemic Rats with 70 % Ethanol Extract (2 g/kg Body Weight Dose)

Test Group	Blood Glucose Concentration (mg/dL)				
	0 h	**1 h**	**2 h**	**3 h**	**4 h**
Group I (Control) (n=5)	72.0 ± 1.0	189.33 ± 12.6	179.67 ± 0.6	99.67 ± 5.5	82.67 ± 14.6
Group I (Extract) (n=5)	70.0 ± 2.0	132.33 ± 22.7 (30.11 %R)*	116.33 ± 13.7 (35.25 %R)*	93.00 ± 7.2 (6.69 %R)	78.67 ± 12.2 (4.84 %R)

* = $p < 0.01$ R = Reduction in hyperglycemia

Table 2 Mean Blood Glucose Levels (±SEM) in Adrenaline Induced Hyperglycemic Rats with Watery Extract (2 g/kg Body Weight Dose)

Test Group	Blood Glucose Concentration (mg/dL)				
	0 h	**1 h**	**2 h**	**3 h**	**4 h**
Group II (Control) (n=5)	70.0 ± 2.0	210.67 ± 18.5	186.67 ± 11.7	126.33± 4.9	83.0 ± 13.9
Group II (Extract) (n=5)	68.0 ± 2.6	168.67 ± 6.0 (19.94 %R)*	146.33 ± 4.7 (21.61 %R)*	107.0 ± 10.6 (15.30 %R)	78.0 ± 10.1 (6.02 %R)



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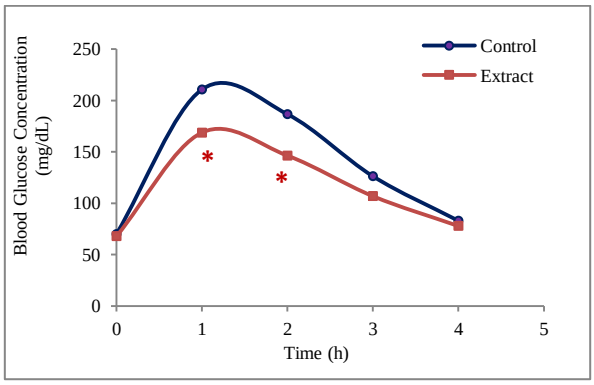


* = $p < 0.01$ (b)

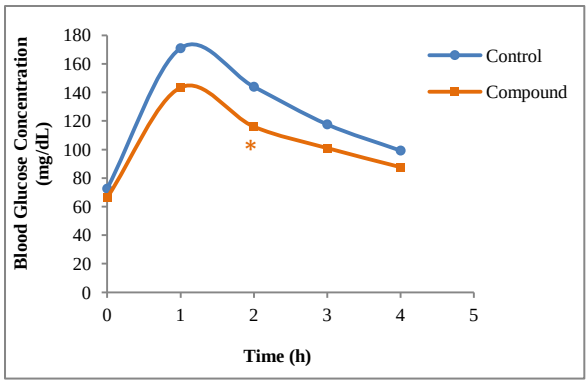
Figure 1 Time course effect of (a) 70 % ethanol extract and (b) watery extract on adrenaline induced hyperglycemic rats

Table 3 Mean Blood Glucose Levels (± SEM) in Adrenaline Induced Hyperglycemic Rats with Isolated Compound (Lupeol) (2 mg/kg Body Weight Dose)

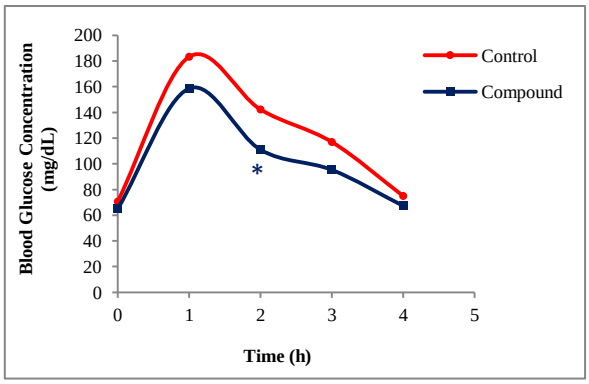
Test Group	Blood Glucose Concentration (mg/dL)				
	0 h	**1 h**	**2 h**	**3 h**	**4 h**
Group III (Control) (n=5)	72.67 ± 6.5	171.00 ± 22.6	144.00 ± 14.1	117.67 ± 3.5	99.33 ± 4.2
Group III (Lupeol) (n=5)	66.33 ± 5.1	143.33 ± 9.1 (16.18 %R)	116.00 ± 6.2 (19.44 %R)*	101.00 ± 4.4 (14.16 %R)	87.67 ± 4.2 (11.74 %R)

Table 4 Mean Blood Glucose Levels (± SEM) in Adrenaline Induced Hyperglycemic Rats with Isolated Compound (Spinasterol) (2 mg/kg Body Weight Dose)

Test Group	Blood Glucose Concentration (mg/dL)				
	0 h	1 h	2 h	3 h	4 h
Group IV (Control) (n=5)	70.67 ± 2.5	183.33 ± 12.5	142.33 ± 16.3	117.00 ± 13.7	75.00 ± 15.4
Group IV (Spinasterol) (n=5)	65.00 ± 3.0	158.67 ± 19.5 (13.45 %R)	111.00 ± 4.0 (22.01 % R)*	95.33 ± 4.2 (18.52 %R)	67.33 ± 3.1 (10.22 %R)



(a)



(b)

* = p < 0.01

* = p < 0.01

Figure 2 Time course effect of (a) isolated compound (Lupeol) and (b) isolated compound (Spinasterol) on adrenaline induced hyperglycemic rats

Table 5 Results of Acute Toxicity Test of 70 % Ethanol and Watery Extracts

Group No.	Doses (g/kg)	Ratio of Dead & Tested Mice No.	Observed % Dead	Expected % Dead	Observed-Expected
I (Control)	0	0:10	0	0	0
II & V (Ethanol and Water)	2	0 :10	0	0	0
III & VI (Ethanol and Water)	4	0 :10	0	0	0
IV & VII (Ethanol and Water)	8	0 :10	0	0	0

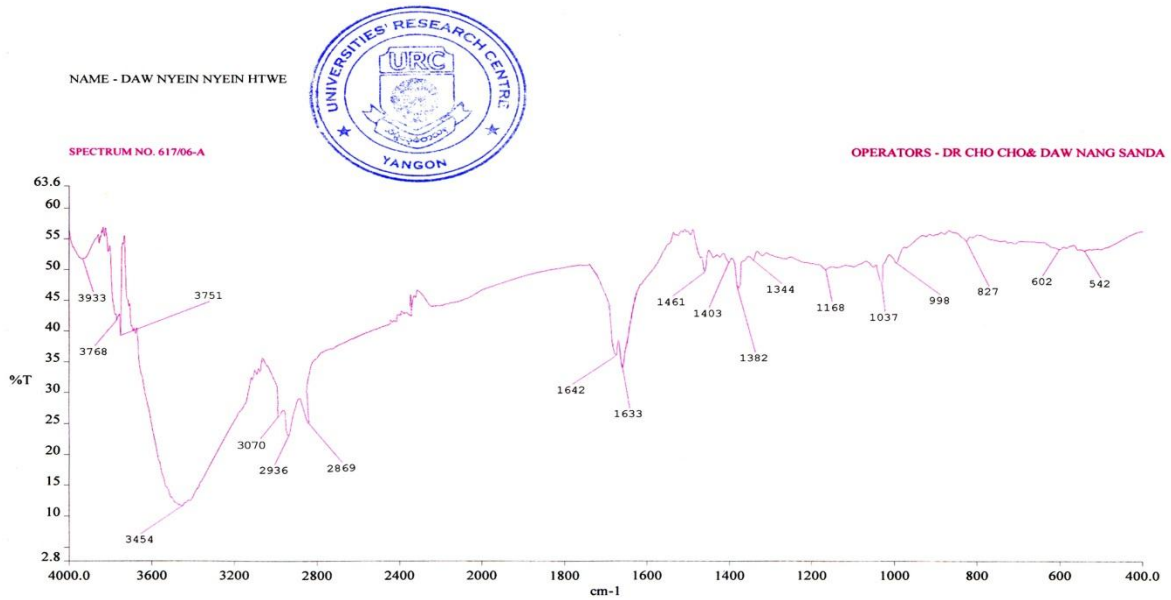


Figure 3 FT IR spectrum of isolated compound (Lupeol) (KBr)

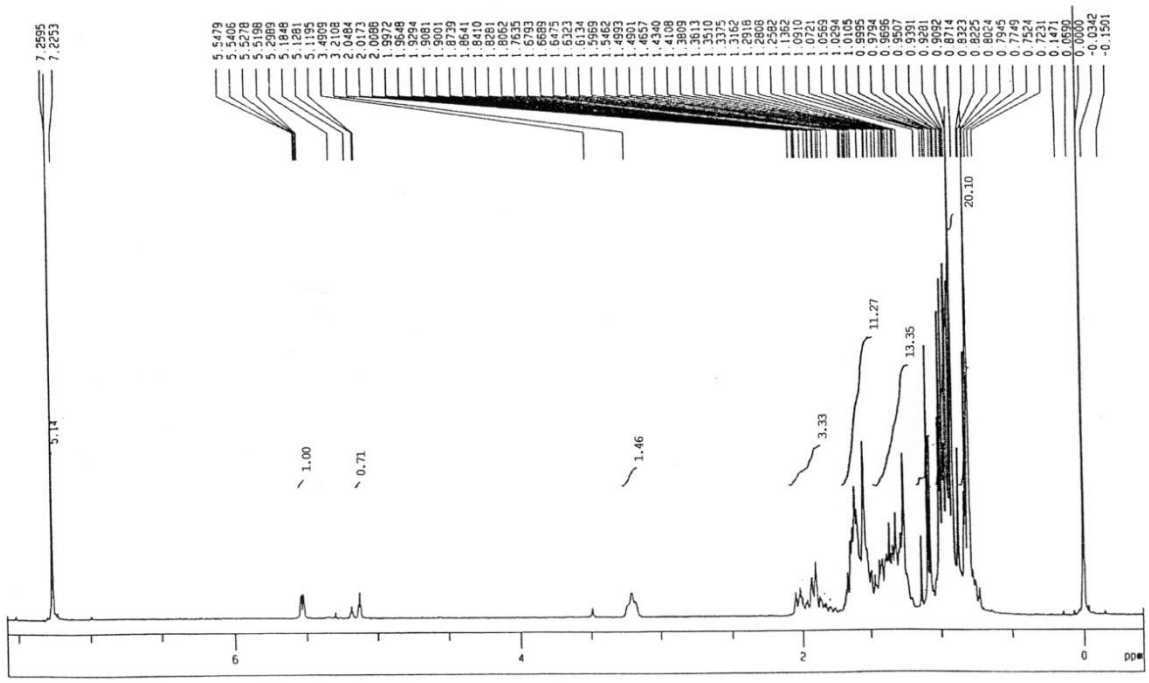


Figure 4 ^{1}H NMR (400 MHz in $CDCl_3$) spectrum of isolated compound (Lupeol)

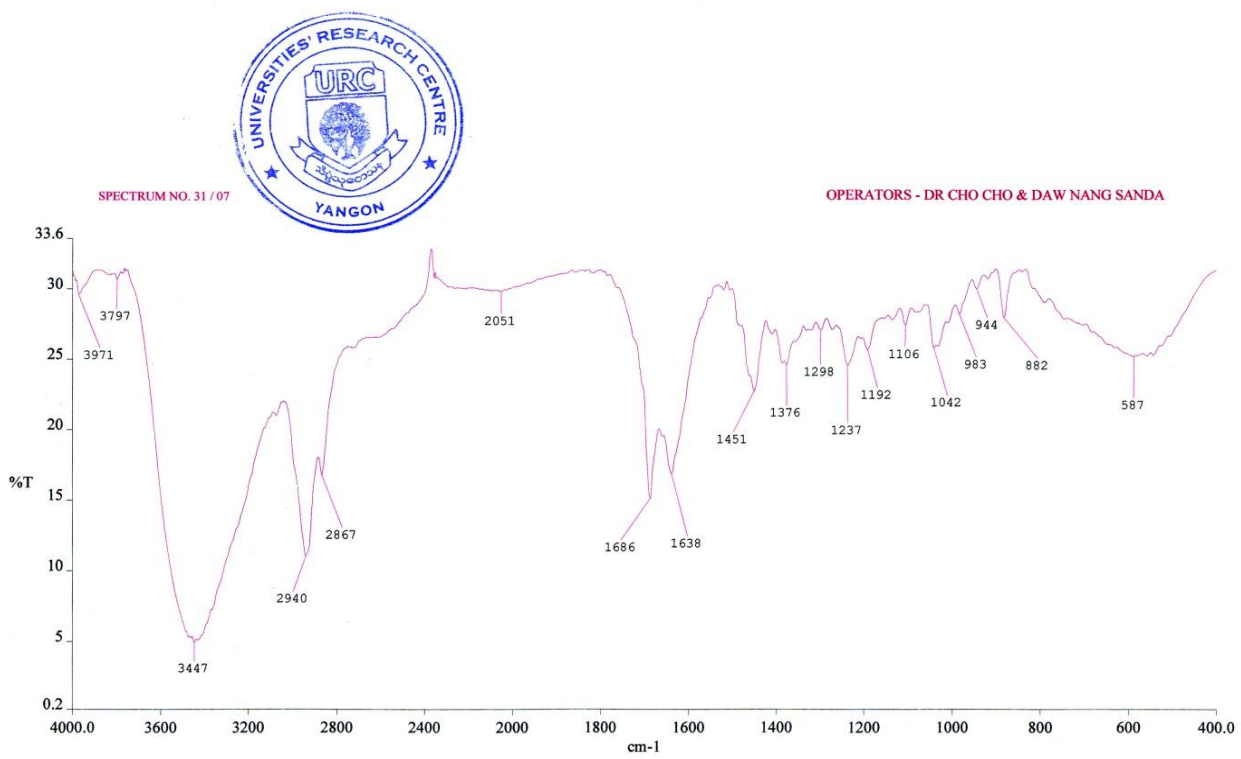


Figure 5 FT IR spectrum of isolated compound (Spinasterol) (KBr)

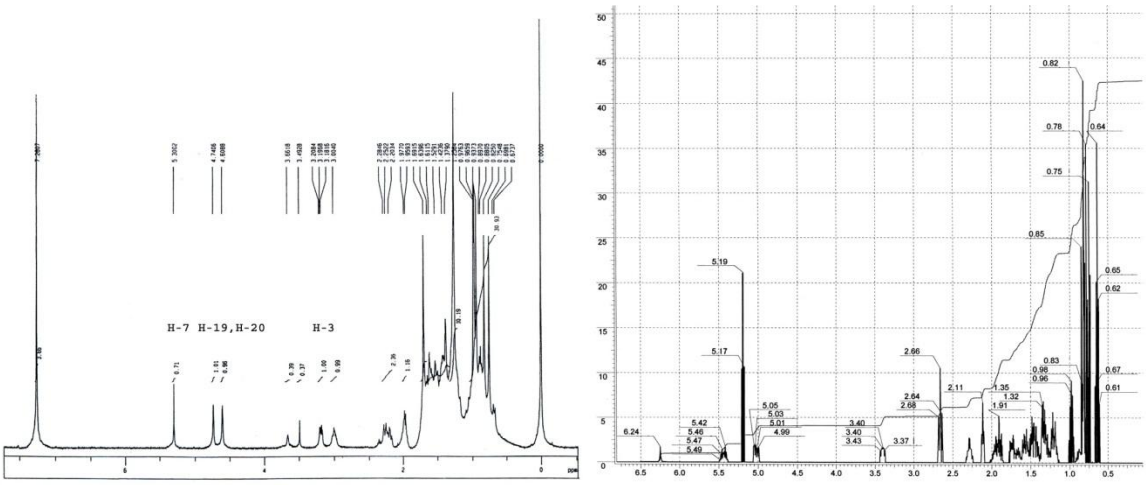


Figure 6 ^{1}H NMR (400 MHz in $CDCl_3$) spectrum of Spinasterol compared with predicted spectrum of ACD Labs software

Conclusion

Two crude extracts and two isolated compounds from the bark of Kha-yay were investigated the antihyperglycemic activities by using adrenaline induced hyperglycemic rats. The effective doses were observed 2 g/kg body weight for each extract and 2 mg/kg body weight for isolated compounds. 70 % ethanol extract showed the optimum antihyperglycemic effect at 1 h (30.11 % reduction, $p < 0.01$) and 2 h (35.25 % reduction, $p < 0.01$), and watery extract decreased blood glucose level at 1 h (19.94 % reduction, $p < 0.01$) and 2 h (21.61 % reduction, $p < 0.01$) compared with control group. From these results, 70 % ethanol extract was found to be more potent than watery extract in antihyperglycemic activity. The effective reduction percent of blood glucose levels of isolated compounds after 2 h showed 19.44 % ($p < 0.01$) for lupeol and 22.01 % ($p < 0.01$) for spinasterol. So, Antihyperglycemic activities of two isolated compounds are not quite different.

In acute toxicity test, the maximum dose for tested crude extracts (70 % ethanol and water) was found to be 8 g/kg body weight. From the result, LD_{50} was more than 8 g/kg body weight and it was inferred that both extracts were free from acute toxic or harmful effects.

According to this scientific investigation, it may be inferred that two crude extracts (70 % ethanol and water) and two isolated compounds (lupeol and spinasterol) of Kha-yay bark possessed antihyperglycemic activities.

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TOTAL PHENOLIC CONTENT, TOTAL FLAVONOID CONTENT AND ANTIOXIDANT ACTIVITY OF *CLITORIA TERNATEA* L. (AUNG-ME-NYO) FLOWERS

Khin Myo Myint Tun[1], Zaw Zaw Myint[2]

Abstract

In the present research, the flower of *Clitoria ternatea* L. (Aung-me-nyo) that is one of the most popular Myanmar medicinal plants was selected to study total phenolic content, total flavonoid content and antioxidant activity. Preliminary phytochemical analyses indicated the presence of alkaloids, glycosides, carbohydrates, phenolic compounds, saponins, tannins, α-amino acids, reducing sugars, steroids, flavonoids, anthocyanins, starch and organic acids. Total phenolic contents of various extracts were investigated by Folin-Ciocalteu reagent and total flavonoid contents were determined by aluminium chloride colourimetric method. According to the results, total phenolic contents of petroleum ether, ethyl acetate, methanol and aqueous extracts were 16.36, 33.16, 25.12 and 11.22 µg GAE/mg of extract, respectively. Total flavonoid contents of petroleum ether, ethyl acetate, methanol and aqueous extracts are 132.17, 86.52, 73.48 and 23.48 µg QE/mg of extract, respectively. Antioxidant activity of the extracts of *Clitoria ternatea* flowers was screened by DPPH assay method and powerful antioxidant activity of the flower extracts was observed.

Keywords: *Clitoria ternatea* L., total phenolic content, total flavonoid content, antioxidant activity

Introduction

The present work is directed towards the extensive study of widespread applications of *Clitoria ternatea* L. (Aung-me-nyo) flowers in the treatment of various life threatening diseases and disorders. In Myanmar, Aung-me-nyo plant has been of keen interest due to its wide spectrum of medicinal uses and biological activities. In the present work, phytochemical analyses of the flower of *C. ternatea* (Aung-me-nyo) were carried out. Moreover, *C. ternatea* was selected for the study on its antioxidant activity of various extracts.

Scientific Classification

Family	:	Fabaceae
Genus	:	*Clitoria*
Species	:	*C. ternatea*
Botanical name	:	*Clitoria ternatea* L.
Common name	:	Butterfly pea
Myanmar name	:	Aung-me-nyo (Kress *et al*., 2003)

The photographs of *C. ternatea* (Aung-me-nyo) plants and flowers are shown in Figure 1.

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Figure 1 *Clitoria ternatea* L. (Aung-me-nyo) plants, leaves and flowers

Medicinal Values of *C. ternatea*

The plant *C. ternatea* is traditionally used for food colouring, stress, infertility and gonorrhea. The plant has been widely used in Ayurveda. The juice of flowers is reported to be used in insect bites and skin diseases (Agrawal *et al*., 2007). The roots are useful in asthma, burning sensation, ascites, inflammation, leucoderma, leprosy, hemicranias, amentia, pulmonary tuberculosis, ophthalmology and reported as bitter refrigerant, ophthalmic, laxative, diuretic, cathartic, aphrodisiac, tonic (Nadkarni, 1976). The root, steam and flower are recommended for the treatment of snakebite and scorpion sting (Kazuma *et al*., 2003). Fresh flowers of *C. ternatea* showed hypoglycemic and hypolipidemic effect (Abhishek *et al*., 2013).

Chemical Constituents of *C. ternatea*

The flowers of *C ternatea* contain flavonol glycosides, 3-O-(2"-O-α-rhamnosyl-6"-O-malonyl)-β-glucoside, 3-O-(6"-O-α-rhamnosyl-6"-O-malonyl)-β-glucoside and 3-O-(2", 6"-di-O-α-rhamnosyl)-β-glucoside of kaempferol, quercetin and myricetin. Delphinidin glycosides, 3-O-β-glucoside, 3-O-(2"-O-α-rhamnosyl)-β-glucoside, 3-O-(2"-O-α-rhamnosyl-6"-O-malonyl)-β-glucoside of delphinidin, and eight anthocyanins (ternatins C1, C2, C3, C4, C5 and preternatins A3 and C4) are also present in the flowers of *C. ternatea* (Kogawa *et al*., 2006; Terahara *et al*., 1996; Terahara *et al*., 1998). Three flavonol glycosides, kaempferol 3-O-(2"-O-α-rhamnosyl-6" -malonyl)-β-glucoside, quercetin 3-O-(2"-O-α-rhamnosyl-6"-malonyl)-β-glucoside and myricetin 3-O-(2", 6"-di-O-α-rhamnosyl)-β-glucoside are also isolated from the petals of *C ternatea* (Kazuma *et al*., 2003).

The aim of the present research is to study the phytochemical constituents, total phenolic content, total flavonoid content and antioxidant activity of *C. ternatea* (Aung-me-nyo) flowers.

Materials and Methods

Collection of Plant Sample

The selected medicinal plant to study in the present research is *C. ternatea* (Aung-me-nyo) flowers. The plant sample was collected from Dawei Township, Taninthayi Region, Myanmar in December 2017. The plant and flower were identified by the Botanists of the Department of Botany, West Yangon University.

Preliminary Phytochemical Investigation

The flower of *C. ternatea* (Aung-me-nyo) was screened for the presence of various bioactive principles. Dried flower sample and crude extracts (petroleum ether, ethyl acetate, methanol and water) were conducted to study the presence of phytochemicals. The analyses were performed according to standard methods for testing different chemical constituents such as alkaloids, glycosides, carbohydrates, phenolic compounds, saponins, tannins, α-amino acids, reducing sugars, steroids, flavonoids, anthocyanins, starch, cyanogenic glycosides and organic acids. After treating the test solution with specific reagents, the tests were detected by virtual observation of colour change or precipitate formation (Harborne, 1998).

Determination of Total Phenolic Content

The total phenolic contents (TPC) in the petroleum ether, ethyl acetate, methanol and watery extracts of *C. ternatea* flowers were estimated by Folin-Ciocalteu Reagent method according to the procedure described by Sahu and Saxena (2013). First, 0.5 mL of prepared extract solution (500 µg/mL in methanol) was mixed with 0.5 mL of methanol. Then, 5 mL of FCR reagent (1:10 v/v) was added to the mixture and incubated for 30 min at 37 ˚C. 4 mL of 1 M sodium carbonate solution was added to each test tube and the test tubes were kept at room temperature for 15 min and the UV absorbance of reaction mixture was read at 760 nm. Gallic acid was used as a standard and calibration curve is shown in Figure 2. The total phenolic contents of the extracts were expressed as µg/mg gallic acid equivalents (GAE).

Determination of Total Flavonoid Content

The total flavonoid contents of *C. ternatea* flower extracts (petroleum ether, ethyl acetate, methanol and water) were determined according to the aluminium chloride colourimetric method (Lin and Tang, 2007). The assay was determined using 0.5 mL of each extract solution (200 µg/mL in methanol) in test tube. To each test tube 1.5 mL methanol, 0.1 mL aluminium chloride, 0.1 mL of potassium acetate solution and 2.8 mL of distilled water were added and mixed well. Sample blank for extracts were prepared in similar manner by replacing aluminium chloride solution with distilled water. After the 40 min incubation at the room temperature, absorbances of all the prepared solutions were measured at 415 nm with UV-visible spectrophotometer. Quercetin was used as standard compound and the amount of total flavonoids content were estimated from a quercetin standand curve shown in Figure 4.

Antioxidant Activity Assay

Screening of antioxidant activity of the crude extracts (petroleum ether, ethyl acetate, methanol and aqueous) of *C. ternatea* flowers were carried out by DPPH free radical scavenging assay using UV spectroscopic method (Brand-Williams *et al*., 1995). Absorbance measurements were done in triplicate for each sample solution. Absorbance values obtained were used to calculate % inhibition, 50 % inhibitory concentration (IC_{50}) and standard deviation. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity.

Results and Discussion

Phytochemicals Present in *C. ternatea* Flowers

Chemical investigations were carried out on the petroleum ether, ethyl acetate, methanol and water extracts of *C. ternatea* (Aung-me-nyo) flowers sample using standard procedures to identify the constituents. The phytochemical results are described in Table 1.

Table 1 Phytochemical Constituents of *C. ternatea* Flowers

Sr No.	Phytochemicals	Dried sample	PE extract	EtOAc extract	MeOH extract	H_2O extract
1	Alkaloids	+	-	+	+	+
2	Glycosides	+	+	+	+	+
3	Carbohydrates	+	+	+	+	+
4	Phenolic compounds	+	+	+	+	+
5	Saponins	+	-	+	-	+
6	Tannins	+	-	+	+	+
7	α-amino acids	+	+	+	+	+
8	Reducing sugars	+	-	+	+	-
9	Steroids	-	+	+	-	-
10	Flavonoids	+	+	+	+	+
11	Anthocyanins	+	-	-	+	+
12	Starch	+	-	+	+	+
13	Cyanogenic glycosides	-	-	-	-	-
14	Organic acids	+	+	-	+	+

(+) = presence (-) = not detected

Total Phenolic Contents of *C. ternatea* Flowers

Phenolic compounds make up one of the major families of secondary metabolites in plants, and they represent a diverse group of compounds. They possess especially antioxidant properties. Phenolics have been suggested to play a preventive role in development of cancer and heart disease (Marja *et al*., 1999).

In the present study, the total phenolic content for petroleum ether, ethyl acetate, methanol and water extracts of *C. ternatea* flowers were estimated by Folin-Ciocalteu Reagent method using gallic acid as standard. Calibration curve of standard gallic acid is shown in Figure 2. The total Phenolic contents in the *C. ternatea* flower extracts are shown in Table 2 and histogram is shown in Figure 3. It appears that ethyl acetate extract had the highest content of phenolic compounds (33.16 μg GAE/mg of extract) and watery extract had the lowest content (11.22 μg GAE/mg of extract). Methanol extract showed intermediate phenolic content followed by petroleum ether extract with 25.12 and 16.36 μg GAE/mg of extract, respectively. Phenolic extracts have been reported to retard lipid oxidation in oils and fatty foods (Rumbaoa *et al.*, 2009), decrease the risk of heart diseases by inhibiting the oxidation of low-density lipoproteins. They are also known to possess antibacterial, antiviral, antimutagenic and anticarcinogenic properties (Moure *et al*., 2001; Manach *et al*., 2004).

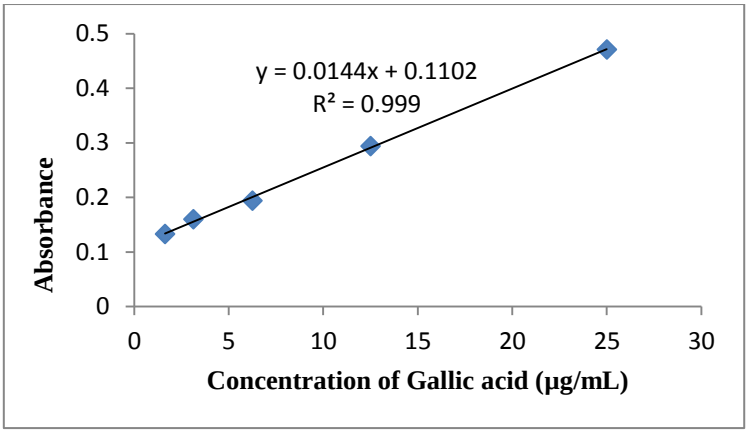

Figure 2 Standard calibration curve of gallic acid

Table 2 Total Phenolic Contents of *C. ternatea* L. Flowers

Sample (500 µg mL^{-1})	Total Phenolic Content (µg GAE/mg of extract)
PE Extract	16.36
EtOAc Extract	33.16
MeOH Extract	25.12
H_2O Extract	11.22

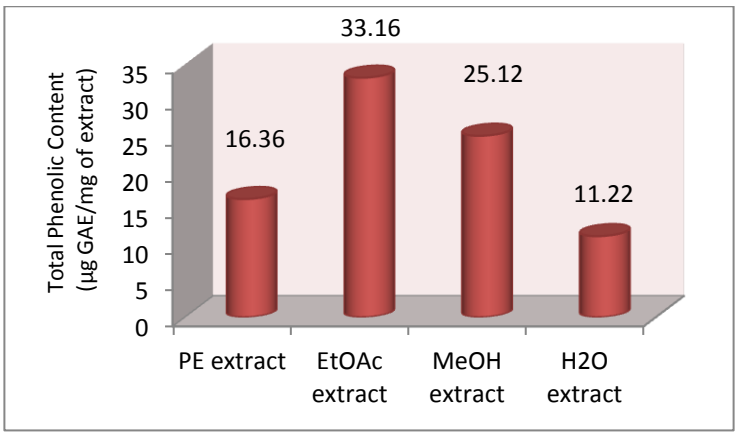

Figure 3 Histogram of total phenolic contents of *C. ternatea* flowers

Total Flavonoid Contents of *C. ternatea* Flowers

Flavonoids are widely distributed in plants, fulfilling many functions. Flavonoids are most important plant pigments for flower colouration, producing yellow, red or blue pigmentation in petals. The total flavonoid contents of *C. ternatea* flower extracts were determined according to the aluminium chloride colourimetric assay method adapted from Lin and Tang (2007). Quercetin was used as a standard and a calibration curve (Figure 4) was constructed from absorbances at 415 nm. Total flavonoid contents of the extracts were expressed as µg QE/mg of extract and given in Table 3 and histogram is shown in Figure 5. Among the four extracts, petroleum ether extract contained highest (132.17 µg QE/mg of extract) amount of total flavonoid content followed by ethyl acetate extract (86.52 µg QE/mg of extract), methanol extract (73.48 µg QE/mg of extract) and then water extract (23.48 µg QE/mg of extract).

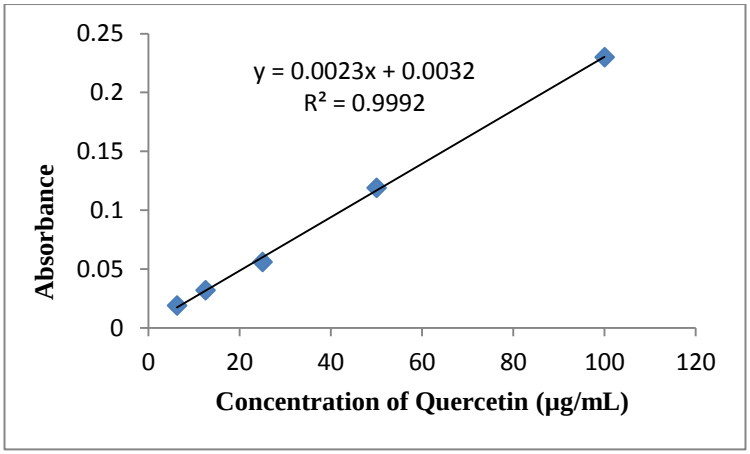

Figure 4 Standatd calibration curve of quercetin

Table 3 Total Flavonoid Content of *C. ternatea* Flowers

Sample (200 µg mL^{-1})	Total Flavonoid Content (µg QE/ mg of extract)
PE Extract	132.17
EtOAc Extract	86.52
MeOH Extract	73.48
H_2O Extract	23.48

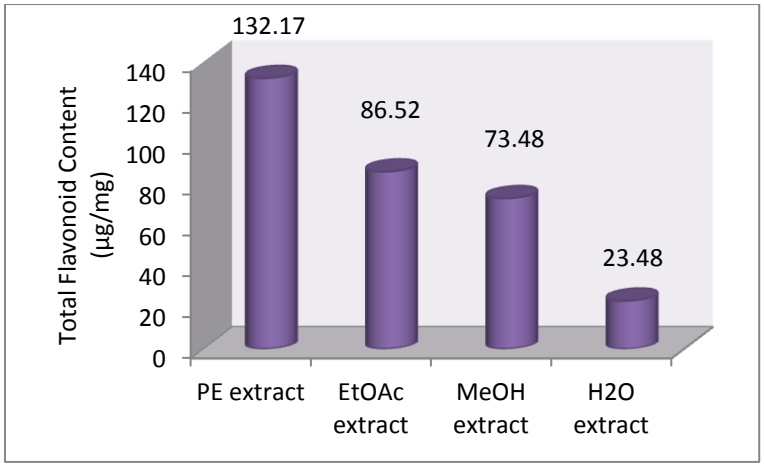

Figure 5 Histogram of total flavonoid contents of *C. ternatea* flowers

Antioxidant Activity of Extracts of *C. ternatea* Flowers

In the present study, the DPPH free radical scavenging activity of petroleum ether, ethyl acetate, methanol and aqueous extracts of *C. ternatea* flower extracts were determined according to the method reported by Brand-Williams *et al*. in 1995. The radical scavenging activity (% RSA) of extracts of *C. ternatea* flower are shown in Table 4 and Figure 6. All four extracts showed a similar increasing trend in antioxidant activity with an increase in their concentration. IC_{50} is used to express the amount or concentration of extracts needed to scavenge 50 % of the

free radicals. The IC_{50} value is inversely proportional to the scavenging activity of the extracts of the *C. ternatea* flower. IC_{50} values of extracts are indicated in Table 5.

IC_{50} value of ascorbic acid was 0.09 µg mL^{-1}. Among the four extracts ethyl acetate extract showed the highest scavenging activity with the IC_{50} value of 9.63 µg mL^{-1} while the petroleum ether extract had the lowest scavenging activity (IC_{50} = 190.77 mL^{-1}). The descending order of radical scavenging activities in the *C. ternatea* flower extracts are as follows: Ethyl acetate extract (IC_{50} = 9.63 µg mL^{-1}) > methanol extract (IC_{50} = 51.72 µg mL^{-1}) > aqueous extract (IC_{50} = 113.89 µg mL^{-1}) > petroleum ether extract (IC_{50} = 190.77 µg mL^{-1}). This means that phytochemicals present in ethyl acetate and methanol extracts possess a stronger potential to scavenge DPPH free radicals.

Table 4 Radical Scavenging Activity (% RSA) of Extracts of *C. ternatea* Flowers

Concentration (µg mL^{-1})	% RSA			
	PE extract	EtOAc extract	MeOH extract	H_2O extract
6.25	13.45	36.36	22.73	27.27
12.5	25.34	61.59	25.45	31.59
25	32.67	77.27	36.59	37.05
50	38.24	90.68	49.32	41.59
100	43.12	96.14	69.09	48.64
200	50.70	96.82	74.23	58.43

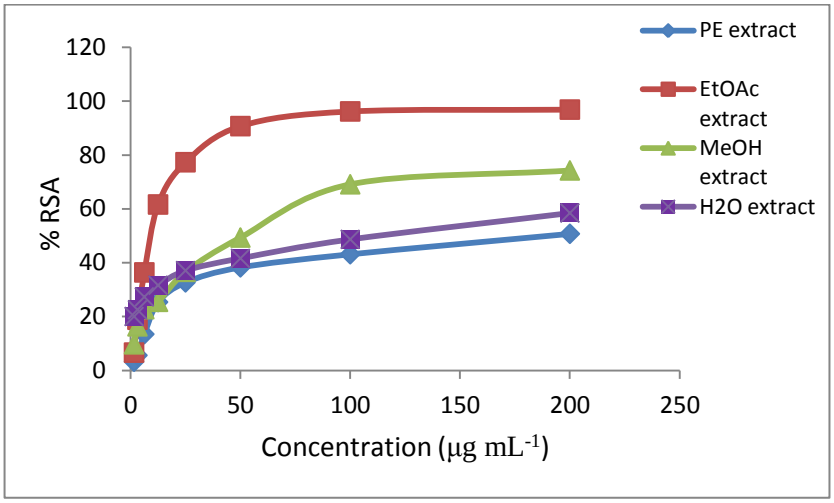

Figure 6 DPPH free radical scavenging activity of *C. ternatea* flowers

Table 5 IC_{50} Values of Ascorbic Acid and Extracts of *C. ternatea* Flowers

Sample	IC_{50} (µg mL^{-1})
PE extract	190.77
EtOAc extract	9.63
MeOH extract	51.72
H_2O extract	113.89
Ascorbic acid	0.09

Conclusion

In this study, the total phenolic contents and antioxidant activity of ethyl acetate extract are likely to show good relationship. Ethyl acetate extract of *C. ternatea* flower showed the highest level of total phenolic content and DPPH radical scavenging activity with the IC_{50} value of 9.63 μg mL^{-1} compared to other extracts and thus potential source for antioxidants. Hence, the *C. ternatea* (Aung-me-nyo) flowers used in this study can be suggested as a suitable source of natural antioxidants.

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The authors would like to thank the Department of Higher Education, Ministry of Education, Yangon, Myanmar, for the permission of doing this research. Special thanks are extended to Professor and Head, Dr Hlaing Hlaing Oo and Professor Dr Sanda Khar, Department of Chemistry, West Yangon University for their valuable advice and kind encouragement and for providing research facilities.

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PREPARATION AND IDENTIFICATION OF ESSENTIAL OIL FROM LEMON BASIL AND ITS ANTIMICROBIAL ACTIVITY

Myo Min[1], Aung Zaw Myo Thant[2]

Abstract

In Myanmar, lemon basil is commonly used as a fragrant spice in cooking. Volatile oils present in lemon basil, such as linalool, nerol and citral, which possess antibacterial and antimicrobial properties. Therefore, the essential oil of lemon basil was prepared by hydrodistillation. Then, the essential oil was analyzed by GC-MS method. In GC-MS analysis, GC oven temperatures were assigned by four levels in the range of 80^{o} to 250 ^{o}C. The increasing temperature rates were controlled by 10^{o} to 15 ^{o}C/min; carrier gas, helium at a constant flow 1.0 mL/min. The injector temperature and mass transfer line temperature were fixed at 270^{o} and 280 ^{o}C, respectively. The molecular masses are arranged in 15 to 250 amu (m/z) and assigned retention time (min) in the range of 3 to 11. According to the GC-MS analysis, the essential oil could contain linalool, terpinen-4-ol, *cis*-geranirol, *cis*-verbenol, citral, 3,3,7-trimethl-oct-6-enoic acid methyl ester, 1-methoxy-3,7-dimethyl-2,6-octadiene, caryophyllene, germacrene and 4-[(1E), 1,5-dimethyl-1, 4-hexadiene-1-yl]-1-methyl cyclohexene. The antimicrobial screening of the prepared essential oil from lemon basil was carried out by agar well diffusion method. In the screening, *B. subtilis*, *S. aureus*, *P. aeruginosa*, *B. pumilus*, *C. albicans* and *E. coli* species were used. From the screening, the antimicrobial activities of essential oil of lemon basil showed the high activities against all microorganisms.

Keywords: Antimicrobial activity, essential oil, GC-MS method, hydrodistillation, lemon basil

Introduction

Plant Description

Lemon basil (*Ocimum africanum*) (Figure 1) is a hybrid between basil (*Ocimum basilicum*) and American basil (*Ocimum americanum*). In Myanmar, it is commonly used as a fragrant spice in cooking. Lemon basil stems can grow to 20–40 cm (8–20 in) tall. It has white flowers in late summer to early fall. The leaves are similar to basil leaves, but tend to be narrower with slightly serrated edges. Seeds form on the plant after flowering and dry on the plant (Culloty, 2010).

Figure 1 Lemon basil plant

Scientific Classification

Kingdom	: Plantae
Order	: Lamiales
Family	: Lamiaceae
Genus	: *Ocimum*
Species	: *O. africanum*
Botanical name	: *Ocimum africanum* Lour.
English name	: Lemon basil
Myanmar name	: Pin-sein

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[2] Assistant Lecturer, Major, Department of Chemistry, Defence Services Academy

Health Benefits of Lemon Basil

Basil is used as in cooking and may have some health benefits. Basil's proposed benefits include reducing inflammation, and it is said to have anti-aging and antibacterial properties. Basil is used in traditional Tamil and Ayurvedic medicine, which is a form of traditional medicine popular on the Indian subcontinent. Alternatively, lime and lemon basil have a strong citrus scent due to their high concentration of limonene *(Baliga et al., 2013).*

The basil contains a wide range of essential oils, rich in phenolic compounds, and a wide array of other natural products including polyphenols such as flavonoids and anthocyanins. The basil possesses the potential for holy basil to prevent cancer and it contains, including eugenol, rosmarinic acid, apigenin, myretenal, luteolin, β-sitosterol, and carnosic acid, it may help prevent certain types of skin, liver, oral, and lung cancers. The beta-caryophyllene from basil has prominent anti-arthritic activity which may be attributed to its anti-inflammatory activity." Basil restricts the growth of numerous bacteria, including *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli* O157:H7, *Yersinia enterocolitica*, and *Pseudomonas aeruginosa (Baliga et al., 2013).*

A decoction of basil leaves is used against hepatic and gastritis disorders. Basil leaf juice is used to treat dysentery, night blind-ness and conjunctivitis. The essential oils of basil have larvicidal properties. Basil has excellent antimalarial properties and eugenol is the main constituent responsible for its mosquito-repellent properties. Basil leaf paste is effective against ringworm infection and to clear marks on the face. The occurrence of urosolic acid in the leaves helps to remove wrinkles and returns skin elasticity. Basil is highly beneficial in healing wounds, cuts and ulcers, and in removing parasites and worms (Bansod and Rai, 2008).

Lemon Basil Oil

Basil oil is obtained through hydro distillation from fresh plant material. The Basil oil is thin transparent fluid with light yellow to yellow-green colour with aroma characteristic of Basil. A native of Africa and Seychelles, it is grown as popular culinary herb and has small white flowers. The essence is distilled from leaves and has light greenish yellow with sweet green overtones. The Basil oil derived is known for intensely fresh, sweet-spicy and vibrant aroma. Lemon Basil Essential Oil has a sweet lemon aroma with the characteristic basil herbal notes but the body notes linger with a complex lemon-clean scent. The citral content is a natural occurrence in this subspecies of Basil (Texas Natural Supply, 2019).

Basil oil is known to have strong antioxidant properties. Research has shown the oil contains potent anticancer, antiviral and antimicrobial properties. Antioxidants are an important part of maintaining a healthy and balanced lifestyle, and basil maybe a very important source of these essential compounds (Tilebeni, 2011). However, despite these reputed properties, it is important to be aware that basil contains estragole, which may be carcinogenic. In Germany, for example, basil is not considered safe for pregnant women or children (Meyers, 2003).

AIM

To identify the chemical compounds of essential oil from lemon basil which possesses antimicrobial activity

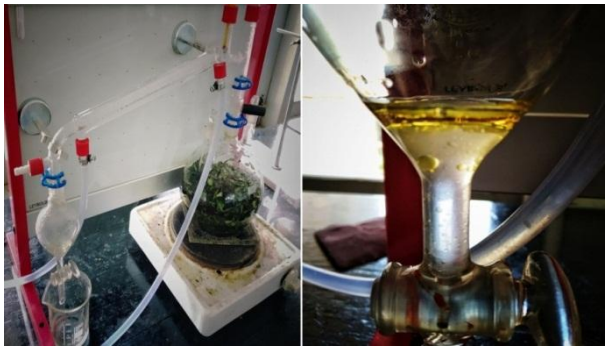
Materials and Methods

The lemon basil samples were collected from Hlaing Thar Yar Township, Yangon Region. Firstly, the essential oil from fresh sample was extracted by hydrodistillation method. Then, the basil oil was analyzed by GC-MS Autosampler (Model: Trace 1300, ISQ-QD, Germany). The antimicrobial activity of basil oil was examined by agar well diffusion method (Mar Mar Nyein *et al*., 1991).

Results and Discussion

The Basil Essential Oil

The collected fresh sample was used to prepare the essential oil by hydrodistillation method. 0.26 mL of basil oil was obtained from 100 g of fresh sample. The apparatus is shown in Figure 2.



(a) (b)

Figure 2 (a) The preparation and (b) separation of basil essential oil by hydrodistillation

GC-MS Analysis of the Lemon Basil Essential Oil

The prepared essential oil of basil was analyzed by GC-MS Autosampler (Trace 1300, ISQ-QD, Germany). In GC-MS analysis, GC oven temperatures were assigned by four levels in the range of 80 to 250 ^{o}C. The increasing temperature rates were controlled by 10 to 15 ^{o}C/min; carrier gas, helium at a constant flow rate 1.0 mL/min. The injector temperature and mass transfer line temperature were fixed at 270 and 280 ^{o}C, respectively. The molecular masses (mass fragmentations) are arranged in 15 to 250 amu (m/z) and assigned retention time in the range of 3 to 11 min. From the GC-MS analysis, the peaks were observed at 4.8, 6.0, 6.7, 6.9, 7.4, 7.9, 8.2, 9.6, 9.8 and 10.4 min of different retention times in GC chromatogram. The mass fragmentation patterns (m/z values) of each compound were matched with that of reference compounds from GC-MS data library. By using GC-MS, each mass spectrum at different retention times could be deduced for linalool (4.8 min), terpinen-4-ol (6.0 min), *cis*-geranirol (6.7 min), *cis*-verbenol (6.9 min), citral (7.4 min), 3,3,7-trimethl-oct-6-enoic acid methyl ester (7.9 min), 1-methoxy-3,7-dimethyl-2,6-octadiene (8.2 min), caryophyllene (9.6 min), germacrene (9.8 min) and 4-[(1E), 1,5-dimethyl-1,4-hexadiene-1-yl]-1-methyl- cyclohexene (10.4 min).

According to GC-MS analysis, citral was observed as the highest relative abundance of the essential oil from lemon basil. Other compounds such as linalool, verbenol, methyl ester compounds and caryophyllene were present in considerable compositions in the lemon basil oil. GC-MS analyzed data are shown in Figures 3 – 13.

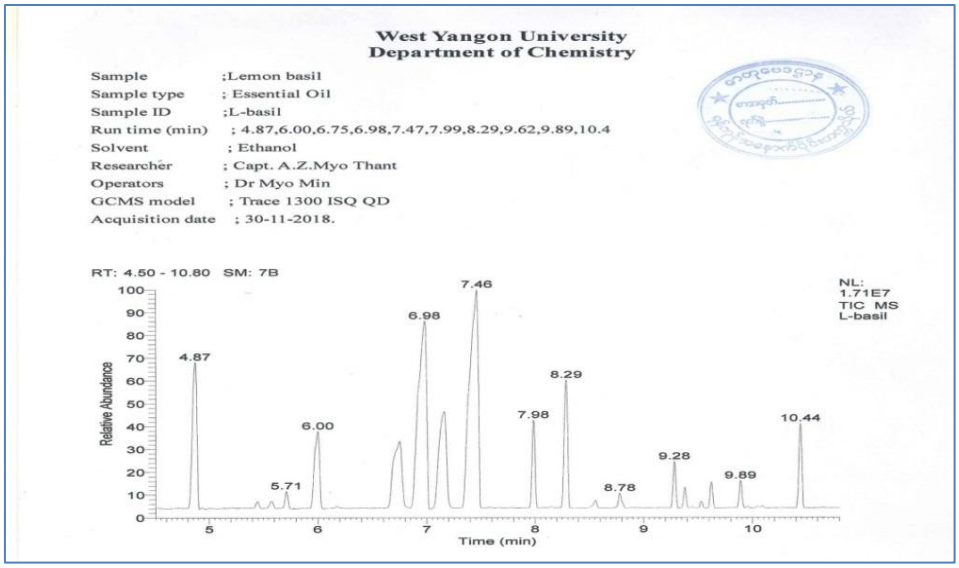


Figure 3 Gas chromatogram of the essential oils from lemon basil leaves

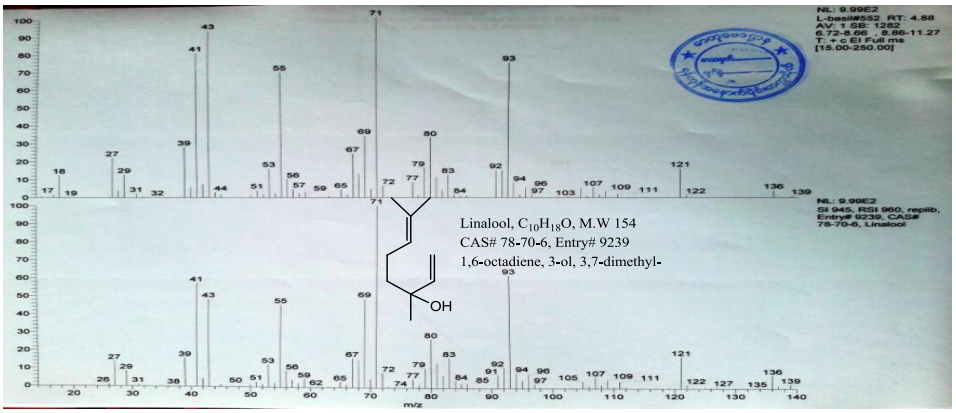


Figure 4 Mass spectra of linalool from the prepared essential oil (4.8 min) by replib library

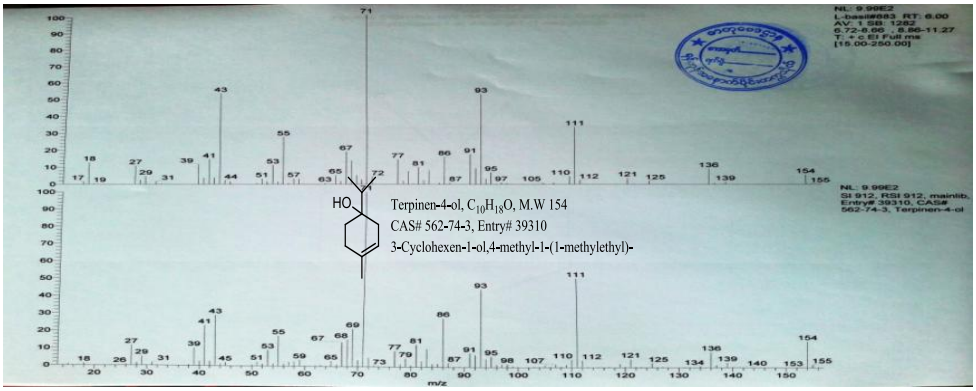


Figure 5 Mass spectra of terpinen-1-ol from the prepared essential oil (6.0 min) by replib library

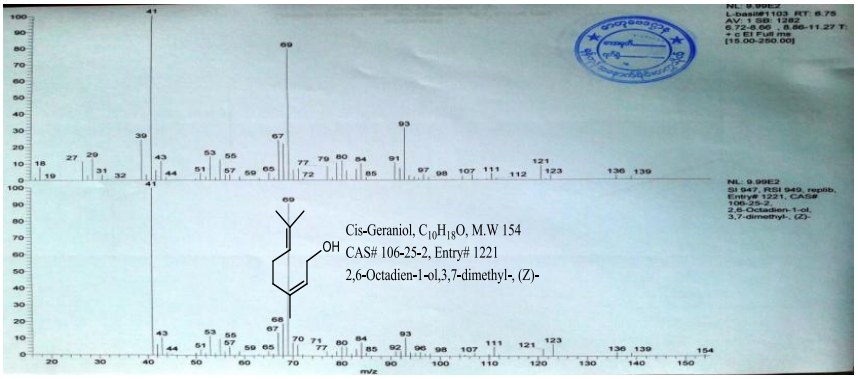


Figure 6 Mass spectra of *cis*- geraniol from the prepared essential oil (6.7 min) by replib library

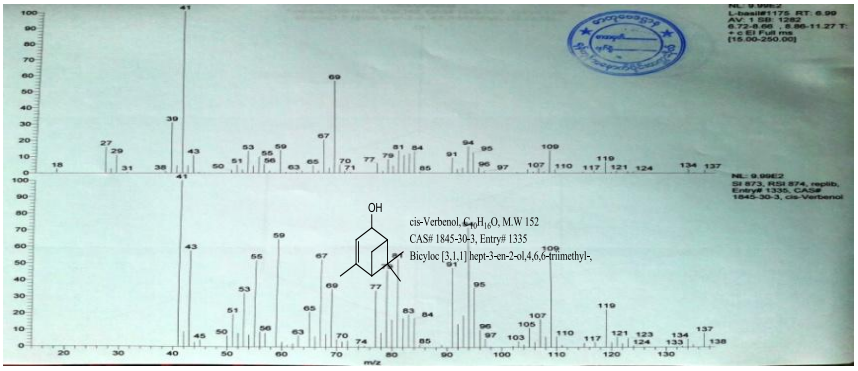


Figure 7 Mass spectra of *cis*-verbenol from the prepared essential oil (6.9 min) by replib library

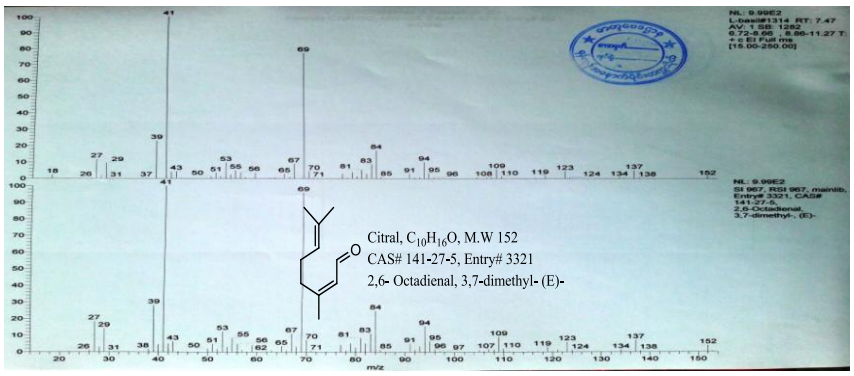


Figure 8 Mass spectra of citral from the prepared essential oil (7.4 min) by replib library

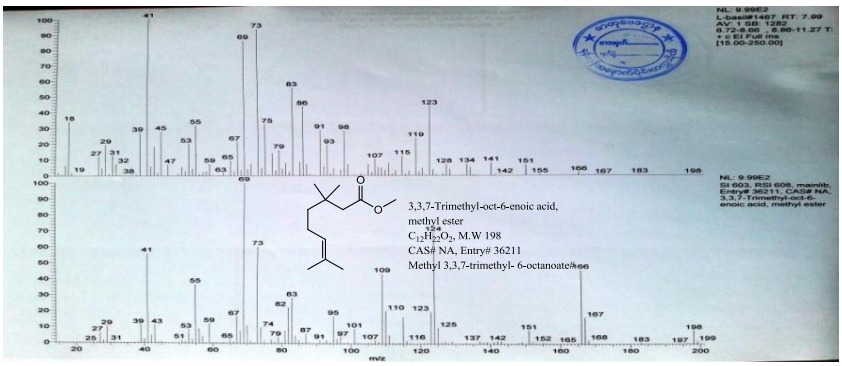


Figure 9 Mass spectra of 3,3,7-trimethyl-oct-6-enoic acid, methyl ester from the prepared essential oil (7.9 min) by replib library

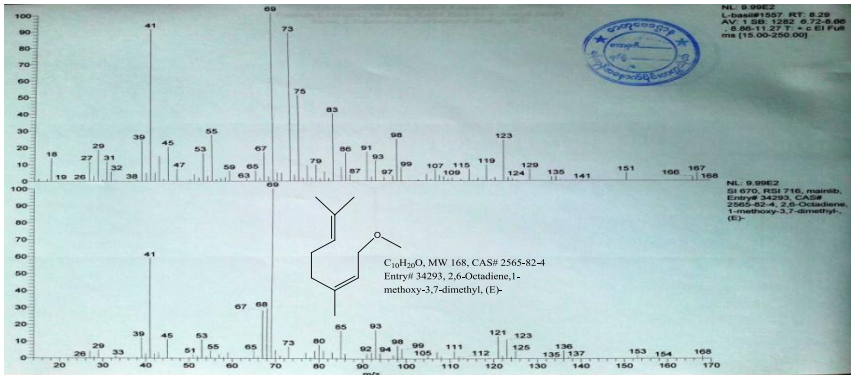


Figure 10 Mass spectra of 1-methoxy-3,7-dimethyl-2,6-octadiene from the prepared essential oil (8.2 min) by replib library

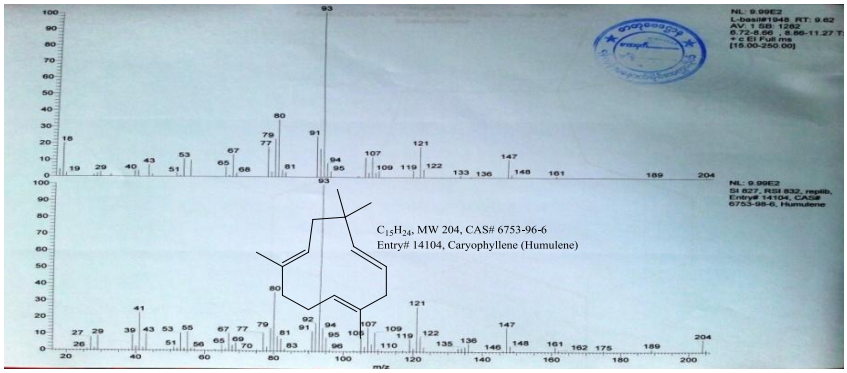


Figure 11 Mass spectra of caryophyllene from the prepared essential oil (9.6 min) by replib library

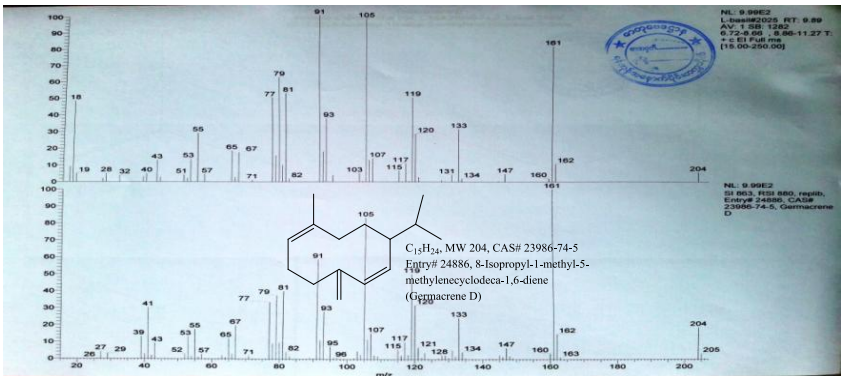


Figure 12 Mass spectra of 8-isopropyl-1-methyl-5-methylenecyclodeca-1,6-diene (germacrene D) from the prepared essential oil (9.8 min) by replib library

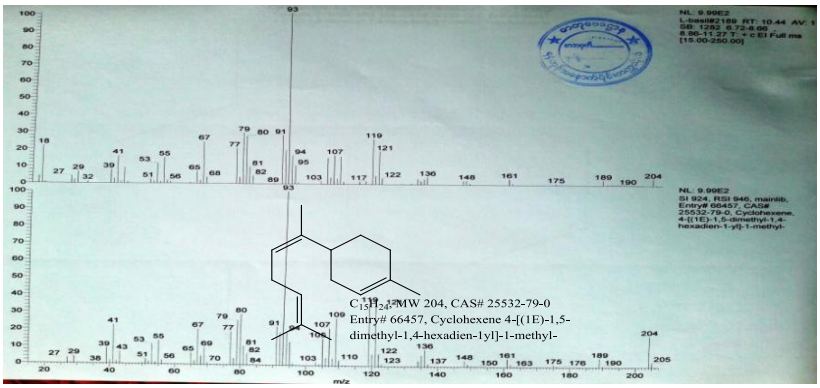
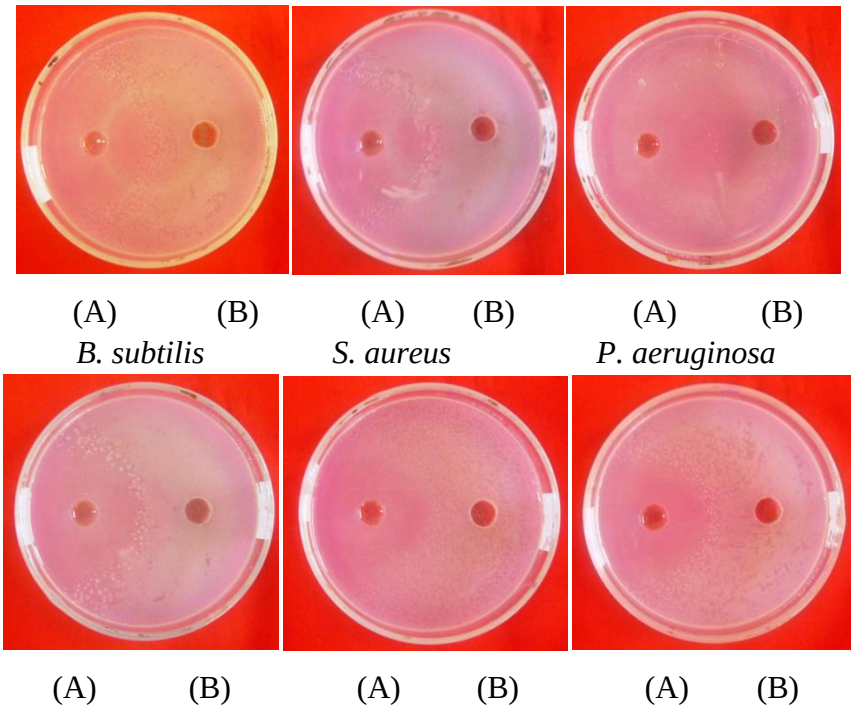


Figure 13 Mass spectra of 4-[(1E)-1,5-dimethyl-1,4-hexadien-1-yl]-1-methyl cyclohexene from the prepared essential oil (10.4 min) by replib library

Screening of Antimicrobial Activity

Antimicrobial activities of the essential oil from lemon basil were screened by agar well diffusion method and the results are shown in Figure 14 and Table 1. In this screening, the basil oil was tested on six species of microorganisms: *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus pumilus, Candida albicans* and *E.coli* species.

In the screening, the antimicrobial activities on all of the tested microorganisms are considerably high in the range of inhibition zone diameter 35 – 40 mm. The highest antimicrobial activities were observed at 40 mm with *B. pumilus* and *C. albicans* followed by *S. aureus, P. aeruginosa, B. subtilis* and *E. coli*. According to the antimicrobial screening, the basil oil may be used for the medicinal formulation of antimicrobial drugs.



(A = Basil essential oil, B = Control (n-hexane))

Figure 14 Screening of antimicrobial activities of basil essential oil

Table 1 Results of Antimicrobial Activity Screening of Basil Essential Oil

Sample	Inhibition zone diameter (mm) against different microorganisms					
	B. subtilis	***S. aureus***	***P. aeruginosa***	***B. pumilus***	***C. albicans***	***E. coli***
Essential oil	35 (+++)	38 (+++)	38 (+++)	40 (+++)	40 (+++)	35 (+++)
Control (n-hexane)	-	-	-	-	-	-

Agar well diameter: (10 mm) 10 mm - 14 mm (+) - mild activity
15 mm - 19 mm (++) - medium activity 20 mm - above (+++) - high activity

Conclusion

This research concerns with the GC-MS analysis and antimicrobial screening of the prepared essential oils from lemon basil leaves. Firstly, the preparation of essential oil from basil sample was carried out by hydrodistillation method. Then, the prepared essential oil was analyzed by GC-MS method. From the analysis, the essential oil was found to contain linalool (4.8 min), terpinen-4-ol (6.0 min), *cis*-geranirol (6.7 min), *cis*-verbenol (6.9 min), citral (7.4 min), 3,3,7-trimethl-oct-6-enoic acid methyl ester (7.9 min), 1-methoxy-3,7-dimethyl-2, 6-octadiene (8.2 min), caryophyllene (9.6 min), germacrene (9.8 min) and 4-[(1E), 1,5-dimethyl-1,4-hexadiene-1-yl]-1-methyl cyclohexene (10.4 min). According to GC-MS analysis, citral was observed as the highest relative abundance of the essential oil from lemon basil. Other compounds such as linalool, *cis*-verbenol, methyl ester compounds and caryophyllene were present in considerable compositions from the lemon basil oil.

In the antimicrobial screening of basil oil, the activities on all of the tested microorganisms are considerably high in the range of inhibition zone diameter 35 – 40 mm. The highest antimicrobial activities were observed at 40 mm with *B. pumilus* and *C. albicans* followed by *S. aureus, P. aeruginosa, B. subtilis* and *E. coli*. Therefore, from these results, lemon basil oil has therapeutic potential on diseases related to microbial infections. It can be suggested that lemon basil leaves could be used as good sources of natural antimicrobial agents with possible application in food industry, cosmetics or medicine.

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OBSERVATION ON PRESERVATIVE ACTION OF BETEL LEAVES ESSENTIAL OIL IN PEEL-OFF FACIAL MASK*

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Abstract

Natural preservative effect of betel leaves essential oil on the shelf-life of the peel-off facial mask was studied in the present research. Different concentrations (0.1 %v/w, 0.15 %v/w, 0.2 %v/w, 0.25 %v/w, 0.3 %v/w) of betel leaves essential oil were added into previously formulated peel-off facial mask for consumer like cosmetic product. 0.25 % v/w betel leaves essential oil was found to be suitable for the consumer like peel-off facial mask based on sensory evaluation and skin irritation test. Its preservative effect on the shelf-life of the finished product was observed monthly by changes in pH and viscosity. Microbiological examination such as total plate count (TPC) and total count of yeasts and molds was conducted monthly. Slightly changes in pH and viscosity were observed after six months storage at the room temperature (27-30 ºC). Bacterial constituents in terms of total plate count in peel-off facial mask were under acceptable limit of up to 10^3 cfu/g until six-month storage. The growth of yeasts and molds was found in six- month aged peel-off facial mask.

Keywords: Essential oil, betel leaves, peel-off facial mask, shelf-life

Introduction

Preservatives are natural or synthetic substances that are added to increase their shelf-life without affecting the properties of the products itself. Preservative systems in cosmetic products contain various combinations of chemical compounds that can inhibit the growth of microorganisms and prevent contamination by consumers while in use (Siti Zuliakha *et al*., 2015). Most of the consumers have noticed the safety of chemical preservatives on their health such as allergy reactions (e.g. asthma attack and migraine), skin irritation, causing cancer, etc. Therefore, the cosmetic products with natural preservative or preservative free cosmetics are being greatly demanded to avoid the side effects of chemical preservatives. One of the ideas of this problem emerges the replacement of chemical preservatives with the essential oil possessing antimicrobial properties (Dreger & Wielgus, 2013).

Current interest for the development of facial mask formulation for skin care is attributed to their tightening, and cleansing effects. Peel-off facial masks are a viable alternative to promote the incorporation of active compounds into a plastic film-forming formulation that is designed to allow complete residue removal (Zague *et al*., 2007). The ingredients in skin care products are used to directly contact and penetrate the skin for the purpose of repairing the damage and inhibiting the ageing of it. This is why the use of preservatives in peel-off facial mask are taken as the prime factor into consideration (Emerald *et al.*, 2016).

Essential oil from betel leaves consists of beneficial chemical compounds which shows antimicrobial activity. Betel vine (scientific name: *Piper betle* L.) is known as Kun in Myanmar (Dassanayake, 1996). The essence of betel leaf is owing to the presence of the essential oil which contain terpene and phenol compounds. The previous studies on betel leaves, root and whole

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extract (mixture of volatile and non-volatile) showed a very strong antimicrobial activity due to the presence of the large numbers of bioactive compounds (Sugumaran *et al*., 2011). Therefore, it has a great potential to develop commercial products with betel extract.

In the present study, the essential oil extracted from air-dried betel leaves by steam distillation were utilized as natural preservative in peel-off facial mask. The effects of various amounts of extracted essential oil on consumers' acceptance, skin irritation of the samples, the physicochemical properties and microbiological contamination in preserved peel-off facial mask were investigated.

Materials and Methods

Materials

For the extraction of essential oil from betel leaves, the sound and mature betel leaves (*Piper betle* L.) were collected from Kawhmu Township, Yangon Region. Air-dried leaves were used as raw materials in this research work. For the formulation of peel-off facial mask, cucumbers were purchased from Mingalardon Township, Yangon Region.

Extraction of Essential Oil from Betel Leaves by Steam Distillation

Before the extraction was carried out, the collected sample was washed with water to remove the dirt and the excess water on the surfaces was absorbed by paper towel or dry cloth. The sample was air-dried for 3 days in a clean and dry place. After that, the air-dried sample was pulverized into powder form for extraction. (100) g of betel leaves was used in each extraction. Each extraction was conducted for 4 hours. The resultant essential oil was collected from the Clevenger apparatus and was isolated using petroleum ether as the solvent. The extracted essential oils were dehydrated using sodium sulphate. The pure extracted essential oil was added in air tight amber glass bottle and stored in clean and dry place. The schematic diagram of the steam distillation apparatus is shown in Figure (1).

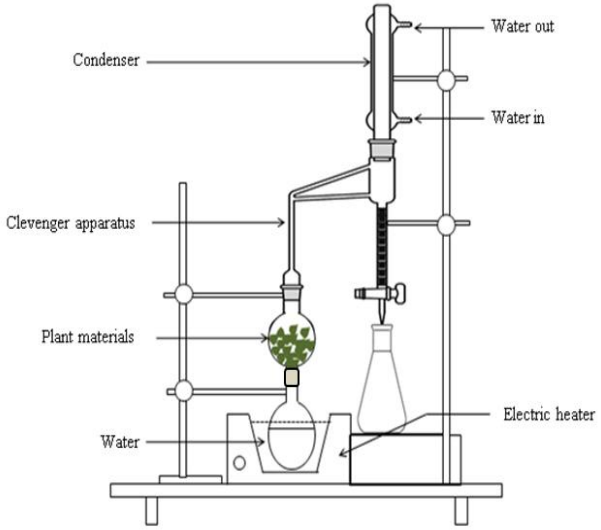


Figure 1 Schematic Diagram of Steam Distillation Apparatus

Determination of Physico-chemical Properties of Extracted Essential Oil

The physico-chemical characteristics such as specific gravity using density bottle (AOAC, 2000), refractive index using Abbe's refractometer and solubility of the extracted essential oil from betel leaves were analysed at room temperature.

Identification of Extracted Essential Oil by Gas Chromatography-Mass Spectroscopy (GC-MS) Analysis

The compounds in extracted essential oil from betel leaves were identified by GC-MS (PerkinElmer, Clarus 680 GC coupled to PerkinElmer, Clarus 600 MS Detector equipped with an Elite 5MS capillary non polar column -30.0 m length x 0.25 mm ID x 0.25 μm film thickness). The components of essential oil were identified on their retention time and mass spectra, matching with National Institute of Standards and Technology (NIST05) libraries provided with computer controlling the GC-MS system. The GC-MS analysis of the essential oil extracted was carried out at the National Analytical Laboratory, Department of Research and Innovation, Ministry of Education.

Screening of Antimicrobial Activity of Extracted Essential Oil

Antimicrobial activity of extracted essential oil from betel leaves was determined by agar-well diffusion method (Cruichshank, 1975). Tested microorganisms were *Bacillus subtilis* (N.C.T.C–8236), *Staphylococcus aureus* (N.C.P.C–6371), *Pseudomonas aeruginosa* (6749), *Bacillus pumilus* (N.C.I.B – 8982), *Candida albicans,* and *E coli* (N.C.I.B – 8134). Screening test was carried out at the Microbiology Laboratory of Pharmaceutical Research Department, Ministry of Industry.

Formulation of Peel-off Facial Mask

The basic formulation of peel-off facial mask was carried out according to the previously literature study (Reveny *et al.,* 2016 and Beringhs *et al.,* 2013) and is described in Table (1). The composition of the main ingredients such as cucumber juice, PVA, ethanol and carbomer were varied according to the D-optimal mixture design. The experimental design of four main components systems was conducted by using Design Expert (version 7.0.0, State Ease Inc., Minneapolis, USA). In the preparation of peel-off facial mask, smooth and firm texture cucumber with no cut and no bruises or discoloration of cucumbers were selected. The selected cucumbers were peeled and chopped into 1 cm cubes. Then, cucumber juice was extracted by using a domestic juice extractor. Approximately, 100 g cucumber gave 90 g of cucumber juice. After that, polyvinyl alcohol (PVA) was dissolved in cucumber extract, heated over water bath at 80±2 °C and constantly stirred until PVA was dispersed in cucumber extract. And then, ethanol, carbomer, citric acid and disodium EDTA were added to the prepared mixture as the water phase. As an oil phase, olive oil and propylene glycol were mixed to the above prepared water phase. The mixture was agitated using ultraturrex at 2400 rpm for 5 minutes. Finally, the extracted essential oil was added as natural preservative into the mixture. The amount of essential oils for use in peel-off facial mask was determined by Hedonic Scale Test and Skin Irritation Test. The finished products were stored in a clean and dry place at room temperature (27-30°C) where direct sunlight could not reach.

Table 1 Basis Formulation of Peel-off Facial Mask

Ingredients	Composition
Cucumber juice (g)	94.07
Polyvinyl alcohol (g)	10.43
Ethanol (g)	15
Carbomer (g)	0.55
Olive oil (g)	2
Propylene glycol (g)	2
Disodium EDTA (g)	0.5
Citric acid (g)	0.2
Essential Oil (mL)	0.1-0.3

Evaluation of Consumers' Acceptance on the Added Amount of Extracted Essential Oil in Formulated Peel-off Mask

Nine point Hedonic Scale Test was used to evaluate the consumers' acceptance on the added amount of essential oil for the odour of the formulated peel-off facial mask. Ten panelists involved in the assessment of the overall acceptance of the samples.

Skin Irritation Test (Patch test)

Scores were given for skin irritation based on Patch test. Patch tests were performed on the forearms of each volunteer. The scores were recorded for the presence of erythema (skin redness) using a scale with 4 points from 0 to 3, where 0 stands for absence of skin irritation, 1 for mild skin irritation, 2 for moderate skin irritation while 3 stands for severe skin irritation. Each volunteer was asked to note their irritation/itching towards the patches and then assigned a score from the same scale (Frosch et al., 2006 and Akhtar *et al.,* 2011).

Observation on Shelf-life of Formulated Peel-off Facial Mask

The shelf-life of peel-off facial mask stored at room temperature (27-30°C) was observed monthly for 6 months by studying the parameters such as pH, viscosity and microbiological contamination (Yeasts and molds and Total Plate Counts). The pH of the sample was measured using a pH meter (Mi 150, pH/Temperature Bench Meter). The viscosity of the sample was measured using NDJ 8S Digital Rotational Viscometer. The microbiological examination of yeasts and molds, and total plate count (TPC) were conducted at Small Scale Industries Department, Ministry of Agriculture, Livestock and Irrigation. The growth of yeasts and molds, and total plate count (TPC) of the sample was determined by dry rehydratable film method (Petrifilm^{TM} Method).

Results and Discussion

Steam distillation was used to extract the essential oil from air-dried betel leaves. The yield percent was 1.41 ± 0.02 %v/w. Table (2) shows the physical properties of extracted essential oil. It was an assessment of the purity and quality of the essential oil as well as for identification. The specific gravity of the essential oil from betel leaves were found to be 0.91 - 0.99 that may be few containing oxygenated aromatic compounds in extracted essential oil (Barkatullah *et al.,* 2012). It was noted that the refractive index for betel leaves essential oil was

1.45±0.01. Caburian and Osi, (2010) and Sugumanran *et al*., (2011) stated that betel leaves essential oil was specific gravity for 0.9313 - 1.001 with 1.4526 - 1.529 as refractive index. The solubility of the essential oil was shown in Table (3). It was found that the essential oil with water (1:5) ratio was not separated as the layer and the oil dispersed in the water. However, the essential oil and water was not miscible in ratio of 1:1 but soluble in (1:1) ratio of ethanol and petroleum ether since it was the complex mixture of polar and non-polar compounds (Morsy, 2017). And also, it may be due to the specific gravity of betel leaves essential oil (0.91 -0.99).

The constituents of extracted essential oils was identified using Gas Chromatography-Mass Spectrometry (GC-MS). GC-MS chromatogram of essential oil is shown in Figure (2) and Table (4) indicates the expected compounds of the essential oil with their retention time. Eugenol (69.63%) was the major component of essential oils from betel leaves and followed by eugenyl acetate (11.93%), caryophyllene (2.68%) and 4-allyl-1,2-diacetoxy benzene (1.38%). Six oxygenated compounds such as 2-allyl phenol, eugenol, methyl eugenol, eugenyl acetate, 4-allyl 1,2-diacetoxybenzene and phytol were found in the essential oil extracted by steam distillation. According to Joshi, (2013), Lee *et al*., (2015), PubChem CID:15624 and Vanin *et al*., (2014), the oxygenated compounds mainly provide the antimicrobial activity of the essential oils.

Screening of antimicrobial activities of essential oil is presented in Table (5) and Figure (3). From the results, the maximum inhibition zone of 21 mm against *Bacillus pumilus* followed by a zone of 20 mm against *Staphyloccocus aureus* and *Pseudomonas aeruginosa* were studied. It was observed that the essential oil showed against all six test microorganisms. It was due to the presence of the bioactive compounds in the extracted essential oil such as eugenol, caryophyllene and eugenyl acetate that provide the antimicrobial activities.

Table (6) shows the overall acceptance for the added amount of essential oil in peel-off facial mask. As shown in Table (6), 0.25 %v/w the essential oil from betel leaves cooperated in peel-off facial mask was the most preferable added amount in the facial mask. It was found that 0.1- 0.2 % v/w was not sufficient for the odour of the peel-off facial mask and 0.3 %v/w gave the slightly pungent odour and caused skin redness in some panelists. Seow *et al.,* (2013) stated that when the essential oils are added over threshold, undesirable organoleptic effects may prevail. Therefore, it is mandatory to equalize concentrations of essential oil for the usage as preservative in order to adjust the antimicrobial activities and organoleptic qualities of finished products. Dreger and Wielgus, (2013) also revealed that high concentration of essential oils leads to many problems like: phase separation, unfavorable viscosity of formulation and also strong undesirable odor. Skin irritation test was also conducted for sensitivity on the added amount of essential oil and is shown in Table (7). Skin irritation was found by the addition of above 0.25 %v/w. Sarkic and Stappen, (2018) stated that although essential oils are considered as safe and nontoxic when used at low concentration, skin sensitivity and irritations as well as other symptoms may arise after their application.

Changes in physico-chemical properties of peel-off facial mask formulated with 0.25 % v/w of essential oil for 6 months storage is shown Table (8)**.** It was observed that negligible change in pH of facial mask was found to be from 5.34-5.27 during 6-month storage. The pH of human skin typically ranges from 4.5 to 6.0 and that was considered to be average pH of the skin (Akhtar *et al.,* 2011). This negligible change in pH (±0.07) may be due to the oxidation of olive oil formulated in peel-off facial mask to aldehyde and organic acid (Raymond, Paul and Marian, 2009). In addition, very slightly change in viscosity was found to be increase in the storage time.

Raymond, Paul and Marian, (2009) also stated that the microorganisms grow well in unpreserved aqueous carbomer dispersion. Therefore, carbomer was only slightly reduced the viscosity at elevated storage temperatures if even antimicrobial agents is included in the formulation. It was noted that pH and viscosity of the samples with essential oil were not significantly changed until six-month storage time.

Table (9) shows the microbial count in peel-off facial mask formulated with 0.25 % v/w of essential oil . The count of yeasts and molds and total plate count were observed less than 10^3 cfu/g until five-month storage time but the growth of yeasts and molds and total plate count was found greater than 10^3 cfu/g at the sixth-month. Up to five months of storage time, CFU can be accepted under the guidelines of Cosmetic, Toiletry, and Fragrance Association (CTFA) and guidance of EU's Scientific Committee of Consumers Products (SCCP) (not more than 10^3 cfu/g for – bacteria, yeasts and molds) (Geis, 2006 and Detmer, Jørgensen and Nylén, 2007). It may be due to loss of activity in dilution and volatility of essential oil which can be highly undesirable for the products (Halla *et al.*, 2018). Moreover, the container may allow for the diffusion of oxygen and carbon dioxide and the condensation of water can occur in the container and it can facilitate the microbial growth in the package. Therefore, the containers and bottles should be designed to the difficult entry of the microorganisms into the products for protection from all environmental insults. (Smart and Spooner, 1972 and Varvaresou *et al*., 2009).

Table 2 Physical Characteristics of Extracted Essential Oil

Sr No.	Characteristics	Extracted Essential Oil
1.	Specific Gravity	0.95±0.04
2.	Refractive Index	1.45±0.01

Table 3 Solubility of the Extracted Essential Oil

Sr No.	Solvent	Solvent Ratio (oil : solvent)	Solubility
1.	Water	1 : 1	Immiscible
		1 :5	Not separating as a layer and oil disperses in water
2.	Ethanol	1 :1	Miscible
3.	Petroleum ether	1 :1	Miscible

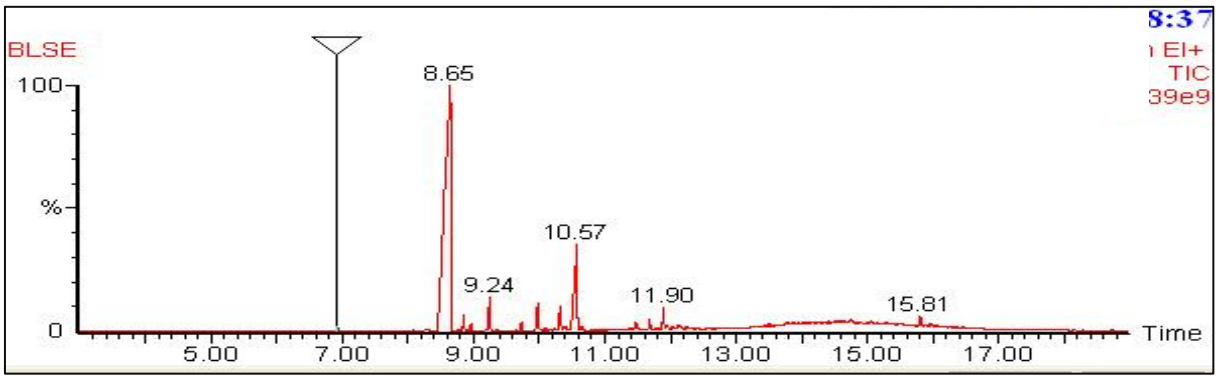


Figure 2 GC-MS Chromatogram of Extracted Essential Oil from Air-dried Betel Leaves by Steam Distillation

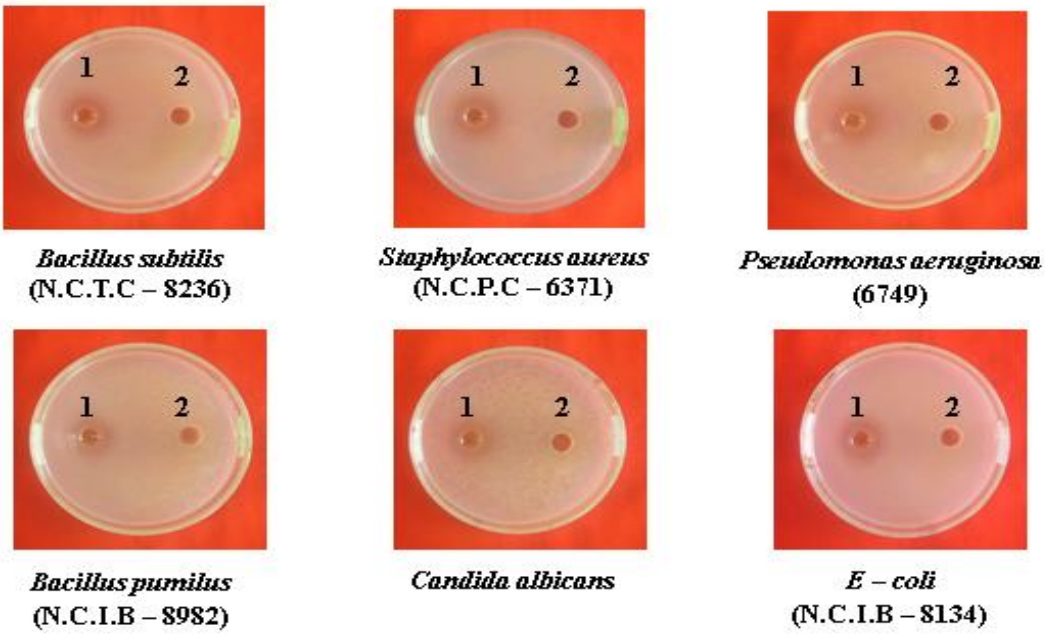
Table 4 Expected Compounds Present in Extracted Essential Oil from Air-dried Betel Leaves by Steam Distillation

Sr No.	Compounds	Retention Time (min)	Peak Area (%)	Formula	Compound Nature	Function	References
1	2-allyl phenol	6.92	0.38	$C_9H_{10}O$	Phenolic compound	Antifungal agent	PubChem CID:15624
2	Eugenol	8.65	69.63	$C_{10}H_{12}O_2$	Phenolic compound	Antimicrobial agent	Lee *et al.*,2015
3	Methyl eugenol	8.85	1.06	$C_{11}H_{14}O_2$	Phenolic compound	Antibacterial agent	Joshi, 2013
4	Caryophyllene	9.24	2.68	$C_{15}H_{24}$	Bicycilc sesquiterpene	Antimicrobial agent	Mohammed *et al.*, 2016
5	γ-muurolene	9.98	2.47	$C_{15}H_{24}$	Bicycilc sesquiterpene	Antibacterial agent	Mohan *et al.*, 2011
6	Eugenyl acetate	10.57	11.93	$C_{12}H_{14}O_3$	Aliphatic dicarboxylic ester	Antiseptic	Vanin *et al*,2014
7	4-allyl 1,2-diacetoxy benzene	11.9	1.38	$C_{13}H_{14}O_4$	Phenolic compound	Anti-platelet aggregation	Chen *et al.*, 2004
8	Phytol	15.81	0.73	$C_{20}H_{40}O$	Acyclic diterpene alcohol	Antimicrobial agent	Lee *et al.*,2015

Table 5 Antimicrobial Activities of Extracted Essential Oils from Air-dried Betel Leaves by Steam Distillation

Organisms	Essential Oil from Air-dried Betel leaves
Bacillus subtilis	18mm (++)
Staphylococcus aureus	20mm (+++)
Pseudomonas aeruginosa	20mm (+++)
Bacillus pumilus	21mm (+++)
Candida albicans	19mm (++)
E –coli	18mm (++)

Note : Agar well – 10mm, 10mm ~ 14 mm (+), 15mm ~ 19mm (++), 20 mm above(+++)



1 = Essential oil, 2 = Control

Figure 3 Antimicrobial Activities of Extracted Essential Oil of Air-dried Betel Leaves by Steam Distillation

Table 6 Overall Acceptance by Hedonic Scale Test for Added Amount of Extracted Essential Oil in Formulated Peel-off Facial Mask

Panelists	**Amounts of Betel Leaves Essential Oil (% v/w)**				
	0.1	**0.15**	**0.2**	**0.25**	**0.3**
1	8	8	9	9	3
2	7	7	8	9	4
3	7	8	8	8	4
4	7	8	8	8	4
5	7	7	7	7	4
6	7	7	7	9	4
7	6	7	7	8	4
8	8	8	8	9	3
9	8	8	8	8	3
10	7	7	7	7	4
Total Score	72	75	77	82	37
Average	7.2	7.5	7.7	8.2	3.7

Table 7 Score Given for the Added Amount of Essential Oil in the Formulated Peel-off Facial Mask Based on the Skin Irritation Test

Score	Numbers of Panelists for Various Amounts of Betel Leaves Essential Oil (%v/w)				
	0.1	**0.15**	**0.2**	**0.25**	**0.3**
0	10	10	10	10	5
1	-	-	-	-	3
2	-	-	-	-	2
3	-	-	-	-	-

Note : Number of Panelist = 10
0 - absence of skin irritation, 1 - mild skin irritation ,
2 – moderate skin irritation , 3 - severe irritation

Table 8 Changes in Physico-chemical Properties of Peel-off Facial Mask with 0.25 %v/w of Essential Oil during Storage

Sr No.	Parameters	Storage time/Shelf-life (month)						
		Fresh	**1**	**2**	**3**	**4**	**5**	**6**
1	pH	5.34 ±0.01	5.33 ±0.02	5.31 ±0.02	5.31 ±0.03	5.31 ±0.02	5.28 ±0.01	5.27 ±0.02
2	Viscosity (cP)	10853	10853	10850	10822	10791	10764	10725

Table 9 Microbial Count in Peel-off Facial Mask Formulated with 0.25 %v/w of Essential Oil from Betel Leaves

Sr No.	Parameters	Type of Sample	Storage time/Shelf-life (month)						
			Fresh	**1**	**2**	**3**	**4**	**5**	**6**
1	Yeasts & Molds (cfu/g)	BLEO	$<10^3$	$<10^3$	$<10^3$	$<10^3$	$<10^3$	$<10^3$	3×10^3
		Control	3×10^3	5×10^3	-	-	-	-	-
2	TPC (cfu/g)	BLEO	$<10^3$	$<10^3$	$<10^3$	$<10^3$	$<10^3$	$<10^3$	1×10^3
		Control	40×10^3	59×10^3	-	-	-	-	-

Note : TPC – total plate count,
Control sample – no chemical preservatives and no essential oil sample

Conclusion

The added amount (0.1-0.3% v/w) of essential oil from betel leaves were used in formulated peel-off facial mask as natural preservative and the satisfactory amount of essential oil was determined based on the overall acceptance by Hedonic Scale Test. Above 0.25 %v/w essential oil from betel leaves were found to cause the irritation to skin. 0.25 %v/w of essential oil from betel leaves was found to be prolong the shelf-life of peel-off facial mask until 5-month storage under limitation. Therefore, it is recommended for conducting the synergistic action of the essential oil and other chemical components to prolong the shelf-life of cosmetic products. In addition to utilizing the essential oil as natural preservatives, adjusting pH of the formulation,

lowering the water activity, proper packaging of the final products and good manufacturing practices (GMPs) are the alternative approaches to preserve the products for longer shelf-life.

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EVALUATION AND CHARACTERIZATION OF FREEZE-DRIED FRUIT JUICE POWDER

Nwe Nwe Aung[1], Swe Swe Hlaing[2], Wai Phyo Mon[3]

Abstract

In addition to liquid foods and semi-solid foods, agricultural products such as vegetables and fruits are currently being dried and distributed to food processing companies. The purpose of such food drying processes is not only to reduce the volume and weight of the products but also to improve the product stability (shelf- life) whereas the product quality must be maintained during and after drying. For drying of vegetables and fruits, freeze-drying (FD) is known to be a good method, by which product shrinkage is eliminated or minimized, and a near-perfect preservation results are expected. The aim of this study was to investigate the production of freeze-dried papaya and watermelon in the form of powder that can be used as a natural alternative to synthetic additives used in food products such as pudding, beverages, jam, jelly and sauces for improving their flavor and aroma. To evaluate the characteristics of freeze-dried fruit powder, physico-chemical characteristics such as moisture, total fiber, ash, protein, vitamin C, total soluble solids, pH and rehydration ratio were determined. The phytochemical investigation and chemical analysis of fresh fruits were also carried out. The analysis of major and traces elements in freeze-dried papaya and watermelon were determined by using X-ray Fluorescence Spectrometry (XRF).

Keywords: freeze-drying (FD), physico-chemical characteristics, rehydration ratio, XRF

Introduction

Nowadays, the fast economic development has changed the trend of food consumption from calories assurance to diet nutrient enrichment. The consumers today are well aware of the importance of vitamins and minerals. This scenario has increased the global market demand towards the fresh fruits. In order to handle the market demand throughout the year, the fresh fruits are preserved using different drying techniques. High moisture content in the fruit leads to having high water activity which leads the quality loss in fruits by increasing the enzyme activity and microbial growth. Therefore, the reducing moisture content and water activity in fruits is always desirable to maintain the quality.

The aim of drying process is to remove moisture from raw material which generally refers to heating. The mechanism of drying consists of heat and mass transfer which affects on inferior product quality such as degradation of colour, nutritional values (vitamins and minerals), and antioxidant activities (Que et al., 2008). Freeze-drying has been used to eliminate water from a frozen material at low temperature under vacuum condition. It is a dehydration process of frozen solvent, which normally refers to water in foodstuff, at very low temperature which sublimed directly from a solid phase into a vapour phase.

Fruit powders are convenient, easy to handle and can be used to prepare several products such as snacks, beverages, jam, jellies, bakery goods and pastes (Khalil et al., 2002).The advantages of freeze-drying are to produce a highly dried product where the main constituents are preserved compared to other methods of food drying due to a low temperature processing

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(Khalloufi and Ratti,2003; Marques et al., 2007). However, freeze-drying is an expensive method because of its equipment and operation cost compared to the other drying methods.

In this study, it was intended to investigate the production of freeze-dried papaya and watermelon in the form of powders. In order to obtain the powder products, each fruit with maltodextrin (Dextrose Equivalence of 10-12, as 10 % by weight) addition were freeze dried. The maltodextrin contributed to the reduction in moisture content and hygroscopicity of the material.

Papaya

English name - Papaya
Botanical name - *Carica papaya.* L
Kingdom - Plantae
(Unranked) - Angipsperms
(Unranked) - Eudicots
(Unranked) - Rosids
Order - Brassicales
Family - Caricaceae
Genus - *Carica*
Species - *C.papaya*
Part of uses - Leaves, Fruits, Bark
(http://www.en.wikipedia.org/wiki/papaya)

Figure 1 Fresh and Mature Papaya

Watermelon

English name - Watermelon
Botanical name - *Citrulluslanatus* var. *lanatus,*
(Unranked) - Angiosperms
(Unranked) - Eudicots
(Unranked) - Rosids
Order - *Cucurbit ales*
Family - *Cucurbitaceae*
Genus - *Citrullus*
Species - *C.lanatus*
Part of uses - Leaves, Fruits, (http://www.en.wikipedia.org/wiki/watermelon)

Figure 2 Fresh and Mature Watermelon

Materials and Methods

Fresh, mature and ripe papaya fruits (*Carica papaya* L.) were collected from Pyin Oo Lwin Township, Mandalay Region. Fresh papaya was washed thoroughly with water. Then, it was peeled and chopped into pieces, about 1 cm length and 0.7 mm thickness, air-dried and then ground to obtain a fine powder form. The fine powder was stored in air-tight bags until analysis.

Fresh mature watermelons from Tada- U Township, Mandalay Region were collected and thoroughly washed with distilled water to remove dirt, dust, pesticide residues and then rinds were washed with pure ethanol to remove micro flora on the surface of the fruit. Then, it was peeled and chopped into pieces, about 1 cm length and 0.7 mm thickness, air-dried and then ground to obtain the fine powder form. Fine powder was stored in air-tight bags until analysis.

Phytochemical Investigation of Fresh Papaya and Watermelon

Phytochemical tests for fresh papaya and watermelon were carried out according to the following standard procedures; test for Alkaloids, Tannins, Protein, Phenol, Glycosides, Flavonoids, Saponins, Carbohydrates, and Phenolic compounds. The results of phytochemical investigation of papaya and watermelon are shown in Table (1).

Preparation of Tropical Fruit Powders Using Freeze-Drying Method

Fresh mature papaya fruit was washed thoroughly with water and cut into about 15mm length and homogenized by a juice extractor to obtain a homogeneous juice. The juice was mixed with maltodextrin (Dextrose Equivalence of 10-12, as 10 % by weight) and poured into each 300ml autoclaved screw-top glass bottles and freeze-dried by using a freeze dryer (**Operon** Ultra-Low Temperature Freezer -86°C, Korea) with total pressure and temperature inside a vacuum chamber(between 0.1 to 2.0 torr) and -50°C, respectively. Average freeze-drying time was approximately 72 h and after the stage of drying, the final product reached a final temperature of about 25°C. The freeze-dried papaya was ground by grinder and sieved through 60-mesh sieve to obtain a homogeneous powder. The freeze-dried powder was stored in a desiccator until further analysis. To compare the characteristics of freeze-dried papaya powder with hot- air oven dried papaya powder, fresh mature papaya juice mixed with maltodextrin was dried at 60°C by using hot -air oven dryer.

Fresh mature watermelons with uniform in color and size were collected from Tada-U Township, Mandalay Region and stored at 25˚ C. The fruits were thoroughly washed with distilled water to remove dirt, dust, pesticide residues and then cut into quarters and the flesh was scooped out and cut into small cubes. The cubes were placed in a juice processor. The extracted juice was then centrifuged and filtered. The filtered juice was placed in autoclaved screw-top glass bottles. The filtered watermelon juice, in screw-top glass bottles, was freeze-dried as previously mentioned for encapsulated papaya powder and stored for further analysis.

Evaluation of the Characteristics of Fresh Fruit and Freeze-dried Fruit Powders

Physico-chemical Analysis of Fresh Papaya and Watermelon

The physico-chemical characteristics such as moisture content ,crude fiber content, ash content, protein content, vitamin-C, total soluble solids content and pH of fresh fruits and freeze-dried fruit powder were determined by AOAC(2000) method.

The solubility test and color reaction test of freeze-dried papaya powder were carried out with various solvents and reagents. Water absorption index, water solubility index, loose bulk density, packed bulk density and rehydration ratio of freeze-dried papaya powder were compared with those of hot- air oven dried papaya powder.

Determination of Elements Content by X-ray Fluorescence Spectrometry (XRF)

The analysis of major and trace elements in fresh fruit (papaya and watermelon) and freeze-dried fruit powder were determined by using Energy Dispersive X-ray Fluorescence Spectrometry and data are shown in Table (4) and (8).

Results and Discussion

Fruits contain various phytochemical compounds that are similar in composition to vegetables. All contain a high percentage of water averaging 85%. Fat, protein and carbohydrate (cellulose and starch) are present in small amounts (Ihekoronye and Ngoddy, 1985). Most fruits are eaten as desserts and they can be processed into liquid product which includes fruit juices, wines and other preserves like; marmalade, jams, jellies etc. Fruit products are marketed canned, bottled or packaged in tetra-packets. Spoilage of fruits usually occurs during storage, transportation and while waiting to be processed. It has been recognized for many years that fruits continue undergoing biochemical changes even after harvest until spoilage occurs by microorganisms. This contributes to high post- harvest losses (Akande, 1995). An effective method of fruit preservation should retain the original characteristics of fruit as convenient as possible.

Papaya (*Carica papaya* L.) is well known for its nutritional and medicinal properties throughout the world. The whole papaya plant including its leaves, seeds, ripe and unripe fruits and their juice is used as a traditional medicine.

Watermelons are an excellent source of lycopene and are a great option for consumers who want to consume lycopene but do not like the taste or texture of tomatoes and tomato products.

The aim of the present research was to prepare the freeze-dried tropical fruit powders such as papaya and watermelon. The phytochemical investigation, chemical analysis of fresh fruits and the solubility of product with different reagents were also determined.

The phytochemical analysis revealed that the presence of tannins, proteins, phenols, glycosides, flavonoids, saponins, reducing sugar, carbohydrates and phenolic compounds in both fruits except alkaloids which is absent in watermelon. The presence of tannin contributes towards antioxidant activity of food. According to the literature, main papayas nutrition's are phenolic compounds, carotenoids, soluble dry matter, β carotene, sugars and others.

Compounds found in the papaya influence the flavor of the papaya include the sugar (glucose, fructose, sucrose) which contribute to sweetness and alkaloids compounds which impart bitterness (Table 1).

Table 1 Phytochemical Investigation of Fresh Fruit

No.	Text	Solvent	Reagent	Observation	Result	
					Papaya	Water-melon
1.	Alkaloids	1% HCl	Wagner's reagent, Dragendroff's reagents	Blue and bluish black	+	-
2.	Tannins	H_2O	Gelatin and 1% $FeCl_3$	Pale Yellow	+	+
3.	Protein	H_2O	10% NaOH solution and 3% $CuSO_4$ solution	Reddish brown	+	+
4.	Phenols	H_2O	10% aqueous $FeCl_3$	Greenish yellow color	+	+
5.	Glycosides	H_2O	10% NaOH solution	Pale yellow ppt.	+	-
6.	Flavonoids	EtOH	Conc: HCl & Mg ribbon	Pale green color	+	+
7.	Saponins	H_2O	Distilled Water	Frothing	+	+
8.	Reducing Sugar	H_2SO_4	5 N NaOH solution & Benedict's solution	Pale brown color	+	+
9.	Carbohydrates	H_2O	10% α-naphthol and H_2SO_4	Purple ring	+	+
10.	Phenolic Compounds	H_2O	Benedict's solution	Brown ppt.	+	+

(+) Present (-) Absent ppt. = precipitate
These experiments were carried out at Laboratory of Industrial Chemistry Department, Yadanabon University

For the preparation of fruit juice powder, Maltodextrin is an adjuvant widely used in drying processes, including freeze-drying process. Maltodextrin is especially used in hard drying materials, such as fruit juices in order to reduce the problems of agglomeration during storage and to improve product stability. Currently, maltodextrin is the most widely used additive to obtain fruit juice powders since it satisfies the demand and is also reasonably cheap. The addition of drying aids is necessary during drying of fruit juices, adding amount not exceeding the operational limits of the equipment or altering the flavour properties.

From Table (2), although the moisture content and pH of fresh papaya were in close agreement with the literature value, other constituents such as total soluble solids, dietary fiber, ash and protein content were quite different with literature value because of the cultivated conditions, the climate and the nature of soil. The decrease in total soluble solids was due to the respiration process of the fruit. Respiration is the oxidative breakdown of the more complex materials such as starch, sugars and organic acids into simpler molecules such as carbon dioxide and water.

Table 2 Physico-chemical Characteristics of Fresh Papaya Fruit (*Carica papaya* .L)

No.	Characteristics	Experimental Values (per 100 g)	Literature Values *
1.	***Moisture (%)	84	85.7
2.	**Crude Fiber (%)	0.065	1.7
3.	**Ash (%)	4.42	5.8
4.	**Protein (%)	0.375	0.47
5.	Vitamin-C	0.215	0.3549
6.	***Total Soluble Solids (^{o}Brix)	10	12.94
7.	***pH	5.3	5.36

* Source: Nutritive Value of Indian Foods., National Institute of Nutrition, Indian council of Medical Research, Hyderabad. India 1984.

** These values were determined at Myanmar Pharmaceutical Factory (Sagaing), Ministry of Industry.

***These values were determined at Laboratory of Industrial Chemistry Department, Yadanabon University.

To evaluate the characteristics of freeze-dried papaya powder, the physico-chemical characteristics of freeze-dried papaya powder were compared with hot-air oven dried papaya powder and the results are shown in Table (3). According to Table (3), some characteristics of freeze-dried papaya powder was differed slightly from that of hot-air oven dried papaya powder. It was found that the prepared sample by using freeze-drying had low moisture content and high Vitamin C content. However, protein content, the water absorption index, water solubility index and rehydration ratio of both of papaya powders were not quite different.

Table 3 Comparison of Physico-chemical Characteristics of Freeze-dried Papaya Powder with Hot- Air Oven dried Papaya Powder

No.	Characteristics	Experimental Values (per 100 g)	
		Freeze-dried Papaya Powder	Hot Air Oven –dried Papaya Powder
1.	*** Moisture (%)	1.65	7.10
2.	** Crude Fiber (%)	0.021	0.065
3.	** Ash (%)	4.83	4.42
4.	** Protein (%)	5.37	5.31
5.	** Vitamin-C (%)	0.473	0.215
6.	*** Total Soluble Solids (^{o}Brix)	89.00	78.0
7.	*** pH	4.61	4.5
8.	WAI**	1.9	1.83
9.	WSI*	50.78	50.8
10.	Loose Bulk Density (g/ml)	0.38	0.55
11.	Packed Bulk Density (g/ml)	0.53	0.35
12.	Rehydration Ratio (%w/v)	5.3	5.455

** These values were determined at Myanmar Pharmaceutical Factory (Sagaing), Ministry of Industry

*** These values were determined at Laboratory of Industrial Chemistry Department, Yadanabon University

WSI*= Water Solubility Index WAI** = Water Absorption Index

It was also noted that the potassium content, calcium content and iron content of freeze-dried papaya powder were lower than that of fresh papaya (Table 4).

Table 4 Elements Content in Fresh Papaya and Freeze-dried Papaya Powder by X-ray Fluorescence Spectrometry (XRF)

No.	Elements	Experimental Values* (% by weight)		Literature Values * (% by weight)
		Fresh Papaya	Freeze-dried Papaya Powder	
1.	Potassium (K)	4.37	0.196	5.0
2.	Calcium (Ca)	0.45	0.060	1.52
3.	Iron (Fe)	0.32	0.005	1.0

*These values were investigated at Universities' Research Centre, Yangon.
*http://www.en.wikipedia.org/wiki/papayas

On comparing the solubility of papaya powders, the powder using freeze-drying method was more soluble in water, ethyl alcohol, acetic acid, acetone, and formaldehyde than the papaya powder using hot air oven method but both of them were insoluble in petroleum ether, shown in Table (5).

The different drying methods of papaya powder affected the physical and physico-chemical properties as well as aroma retention of powders. From the analysis of solubility and color reaction of prepared papaya powder using different drying methods, freeze-drying method was a suitable method for the preparation of fruit powders, shown in Table (5) and (6).

Table 5 Comparison of Solubility on Papaya Powder Using Different Drying Methods in Different Solvents

Amount of Papaya Powder = 0.01 g
Volume of Solvent = 15 drops

No.	Drying Methods	Solubility						
		Water	Vegeta-ble Oil	Ethyl Alcohol	Acetic Acid	Acetone	Formal-dehyde	Petroleum Ether
1.	Freeze-drying	Very Soluble	Soluble	Soluble	Very Soluble	Slightly Soluble	Very Soluble	Very Soluble
2.	Hot Air Oven	Slightly Soluble	Slightly Soluble	Slightly Soluble	Slightly Soluble	Slightly Soluble	Slightly Soluble	Insoluble

Table 6 Color Reactions of Papaya Powder using Different Drying Methods

Amount of Papaya Powder = 0.01 g
Volume of Reagent = 15 drops
Coating Material = maltodextrin

No.	Types of Method	Different Reagents		
		(% w/v) Sodium Hydroxide Solution	Hydrochloric Acid (Conc:)	Sulphuric Acid (Conc:)
1.	Freeze-dried Method	orange	orange	pale yellow
2.	Hot Air Oven Method	orange	orange	pale yellow

From Table (7), the findings of investigation for moisture content, pH and total soluble solids of fresh watermelon are in accordance with the literature value, other constituents such as crude fiber, ash and protein content were quite different with the literature value because of the cultivated conditions, the climate and the nature of soil and the physico-chemical characteristics of freeze-dried water melon powder was slightly differed from that of fresh watermelon.

Table7 Physico-chemical Characteristics of Fresh Watermelon and Freeze-dried Watermelon Powder

No.	Characteristics	Experimental Values of fresh watermelon (per 100 g)	*Literature Values of fresh watermelon(per 100 g)	Experimental Values of freeze-dried watermelon powder (per 100 g)	*Literature Value for Dried Watermelon (per 100 g)
1.	***Moisture (%)	90.1	92.02±1.65	4.78	11.92
2.	**Crude Fiber (%)	0.02	0.32±0.06	0.29	1.25
3.	**Ash (%)	1.5	0.27±0.03	3.42	3.56
4.	**Protein (%)	1.81	0.49±0.02	10.09	6.51
5.	** Vitamin-C (%)	0.037	0.6	0.11	0.06
6.	***Total Soluble Solids (^{o}Brix)	9.3	9.4	12.0	9.4
7.	***pH	5.43	5.3	5.43	5.72
8.	Rehydration Ratio (%w/v)	-	-	0.88	0.91

* Source: Nutritive Value of Indian Foods., National Institute of Nutrition, Indian council of Medical Research, Hyderabad. India 1984.
** These values were determined at Myanmar Pharmaceutical Factory (Sagaing), Ministry of Industry.
***These values were determined at Laboratory of Industrial Chemistry Department, Yadanabon University.

According to Table (8), it was also observed that the potassium content and calcium content of freeze-dried watermelon powder were lower than that of fresh watermelon although the iron content of freeze-dried watermelon powder was higher than that of fresh watermelon.

Table 8 Elements Content in Fresh Watermelon and Freeze-dried Watermelon Powder by X-ray Fluorescence Spectrometry (XRF)

No.	Elements	Experimental Values* (mg/100g) Fresh Watermelon	Experimental Values* (mg/100g) Freeze-dried Watermelon Powder	Literature Values * (mg/100g)
1.	Potassium (K)	112	30.49	126±2.36
2.	Calcium (Ca)	7	2.45	5.60±0.21
3.	Iron (Fe)	0.24	0.35	0.26±0.01

*These values were investigated at Universities' Research Centre, Yangon.
*http://www.en.wikipedia.org/wiki/papayas

According to Figures [4. (a),(b),(c),(d),(e),(f),(g) and (h)],the color and appearance of tropical fruits powder (papaya and watermelon) were the same as those of fresh fruits .In addition it was noted that the color of powders was darker than the that of fresh fruits after the shelf life was nine months because of storage condition. Nevertheless the freeze dried powder could be reconstituted instantly with water at room temperature.

Figure 3 Preparation of Tropical Fruit Powders Using Freeze-Dryer

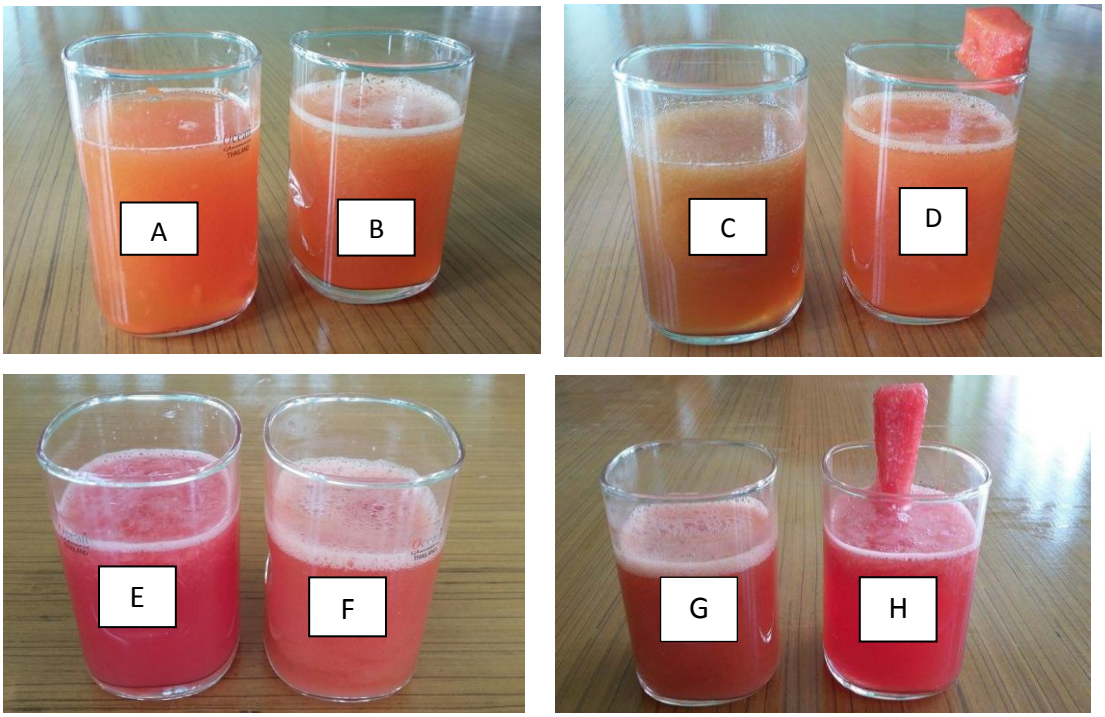

Figure 4 (A) Freeze-dried Papaya Juice (B) (D) Fresh Papaya Juice
(C) Freeze-dried Papaya Juice (shelf-life - 9 months)
(E) (H) Fresh Watermelon Juice (F) Freeze-dried Watermelon Juice
(G) Freeze-dried Watermelon Juice (shelf-life - 9 months)

Conclusion

For the food industry, the application of fruit juice powders has many advantages because of the reduced volume or weight, reduced packaging, easier handling and transportation, and much longer shelf life. Besides, their physical state provides a stable, natural, and easily measurable ingredient, which generally finds usage in many foods and pharmaceutical products such as flavoring and coloring agents.

In this research, preparation of fruit juice powder was carried out by using freeze drying with drying aid (maltodextrin).The addition of maltodextrin reduced the hygroscopicity of the powders. The instant properties of powders involve the ability of a powder to dissolve in water. The prepared fruit powder would be utilized in coloring some food products included jam, jelly, candy, and juice.

The different drying methods of papaya powder affected the physical and physicochemical properties as well as aroma retention of powders. From the analysis of solubility and color reaction test, freeze-drying method was the suitable method for the preparation of papaya powder.

Therefore, freeze-drying method was again chosen for the preparation of watermelon powder. Although some of the physico-chemical characteristics were quite different with the literature value, the aroma and flavor of fresh freeze-dried watermelon were also attractive as well as those of original fresh fruit. Moreover, packaging was important factor for fruit powders because the color was slightly changed after nine months. If stored in air-tight container, dried fruit powder will last for six to twelve months in the pantry and up to twenty -four months in the refrigerator. For maximum shelf-life, the sample should be stored in a vacuum sealed bag in the freezer.

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INVESTIGATION ON THE CHARACTERISTICS OF BIODIESEL PREPARED FROM MEZE SEED OIL

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Abstract

Production of biodiesel, a renewable fuel having environmental advantages, is a valuable process. This research is emphasized on the investigation of characteristics of biodiesel prepared from expelled Meze seed oil. Meze seed oil was firstly analyzed for its physicochemical properties and some of fuel properties. Biodiesel was prepared by two stage process; esterification followed by transesterification. Esterification step reduces the free fatty acid (FFA) value of the Meze seed oil to about 2% by using acid catalyst. Variation of different parameters such as volume of methanol, reaction time, reaction temperature, volume of acid catalyst (H_2SO_4) and amount of alkaline catalyst (KOH), were conducted to achieve the maximum yield of biodiesel. (400) mL (4:1%v/v) of methanol and (1.5) mL (1.5% v/v) of H_2SO_4 for 90 min reaction time at 60°C were chosen as the most suitable conditions for esterification stage. In the transesterification stage, (50) mL (1:2%v/v) of methanol, (1) g (1% w/v) of KOH, 90 min reaction time and reaction temperature of 60°C were also selected as the most favourable conditions for the preparation of biodiesel from esterified Meze seed oil. Functional groups present in the prepared biodiesel was also investigated by FT-IR analysis. Determination of the fuel properties of prepared biodiesel such as flash point, pour point, kinematic viscosity, specific gravity and cetane number were conducted and compared with ASTM standard. Prepared biodiesel was found to have a moderate kinematic viscosity but relatively high flash point when compared with the standards outlined in ASTM Standards for Biodiesel.

Keywords: Meze seed oil, Biodiesel, Transesterification.

Introduction

Biodiesel, a product of the transesterification of fats and oils by lower alcohols like methanol, is becoming increasingly popular as a fuel or as biodiesel blends through mixing with different levels of fossil diesel, in developing and developed countries. Vegetable oils, off-quality oils, used cooking oils and animal fats have been used as raw materials for biodiesel production using transesterification. Transesterification of these raw materials can be carried out in the presence of a catalyst, such as alkali, acid, or an enzyme (N. Saifuddin et al, 2009).

Mahua (*Madhuca longifolia),* belonging to the family Sapotaceae, is an important economic plant. It usually grows throughout the tropical and subtropical region. It is found abundantly in India, Sri Lanka and also Myanmar (tropical region). In Myanmar, it is called Meze or Myintzuthaka. It grows up to approximately 20 meters in height. Although having tremendous therapeutic and potential use, *Madhuca longifolia* is not fully utilized because of unawareness of people (Mishra & Padhan, 2013).

The flowers of *Madhuca longifolia* bloom with new leaves between February and April and the fruits ripen from May to August. Fruits are generally 2.5 to 5 cm long and ovoid or sub-globose shape. Their greenish colour changes to reddish-yellow or orange when they ripe and fleshy. (Mishra & Padhan, 2013).

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Seed oil can be produced by different methods such as, mechanical expression method, solvent extraction method, or both methods. The most common method for oil extraction is mechanical pressing using different kinds of press such as hydraulic press, screw press and rolling press. But, solvent extraction method is the one which is able to extract over 98% oil. However, specific process before conducting the next process is needed after extraction because of its disadvantages such as the necessary equipment for extraction is high in cost, the process is quite dangerous in correlation with fire and explosion and the solvent used (Deli, et al., 2011).

Meze (Mahua) seed oil can be used for skin care, for the manufacture of soaps, detergent, as vegetable butter and as fuel oil. Having emulsion property, it can also be used as an emulsifying agent in few pharmaceutical industries (Mishra & Padhan, 2013).

Thus, the objectives of this study are to extract the Meze (Mahua) seed oil and to apply for biodiesel preparation.

Materials and Methods

Materials

Mature meze seeds were purchased from Simehtun village, Amarapura Township, Mandalay Region. Best Oil Press machine was used to extract Meze seed oil. Analar grade of 98% methanol, sulphuric acid, potassium hydroxide and sodium hydroxide were also used in the preparation of biodesel.

Meze Tree and Fruits

Meze Seeds

Meze Seed kernels

Methods

Pretreatment of Meze Seeds

Dehulling of Meze seeds were done manually to obtain the inner kernels. Then, the kernels were sun-dried to reduce the moisture content (4-6%) for 2-3 days. The dried Meze seed kernels were stored in storage bin at room temperature before extraction.

Extraction of Meze Seed Oil

The dried Meze seed kernels were ground using expeller (Best Oil Press Machine with Heater ,Model No.02, Taiwan made) between 45-50^{o}C. Meze seed oil and oil cake residues were obtained separately. The oil obtained was settled and filtered to remove the residual solids.

Preparation of Biodiesel using Meze Seed Oil

In the preparation of biodiesel, a two-step transesterification process namely, acid esterification and alkali esterification was carried out. The esterification step firstly reduced the free fatty acid (FFA) content of Meze seed oil (MSO) to less than 2% using acid catalyst. Then, the alkaline catalyst transesterification process converted the low FFA of MSO to its ester.

About 100 mL of MSO was poured into 1L of flat-bottomed flask and heated to 60ºC. Then 400 mL of 98% methanol and 1.5 mL of sulphuric acid was added to the preheated MSO and heating was continued at 60ºC while stirring with magnetic stirrer at a speed of 500-600 rpm for 90 min. The product obtained was poured into a separating funnel and left for 2 hr. The excess methanol with sulphuric acid and impurities moved to the top surface and was removed. The lower layer was low free fatty acid esterified MSO. The oil sample was heated to 70 ºC to remove the excess methanol and washed with distilled water in a separating funnel. Finally, the acid value of the product separated at the bottom was determined. The effects of amount of methanol, amount of catalyst, reaction time and reaction temperature on reduction of acid value of esterified MSO were investigated.

Then, 100 mL of esterified MSO was put into a 500 mL of flat-bottomed flask and heated to 60ºC and stirred. The catalyst, 1g of potassium hydroxide which had already been mixed with 50 mL of 98% methanol, was gradually added into the preheated esterified MSO. Then, the mixture was stirred for 90 min at 60ºC. When the reaction was complete, the reaction mixture was transferred into a separating funnel and allowed to settle overnight to enhance the separation at room temperature. When two layers separated sharply, the bottom glycerine layer was drawn off. The biodiesel was purified by washing gently with warm water to remove residual catalysts or soap. The washed solution was checked with phenolphthalein indicator to ensure that the biodiesel was free from residual catalysts or soap. The washed biodiesel was dried in an oven at 110 °C to remove residual methanol. The effects of amount of methanol, amount of catalyst, reaction time and reaction temperature on yield of prepared biodiesel were determined respectively.

Methods of Identification and Analysis

Physico -chemical properties of Meze seed kernel were firstly investigated. To evaluate the quality of Meze seed oil, the characteristics such as specific gravity, refractive index, colour, moisture, acid value, saponification value, unsaponifiable matter, iodine value and peroxide value, kinematic viscosity, flash point and pour point were determined. Gas Chromatography analysis was also conducted to find out the fatty acid composition of Meze seed oil. The functional groups present in the prepared biodiesel were identified by FT-IR analysis. Fuel properties of biodiesel were also determined.

Results and Discussion

The physico-chemical properties such as moisture, ash, protein, crude fibre, crude fat and carbohydrate content of Meze seed kernels are shown in Table(1). It is seen that the percentage of crude fat is the highest in Meze seed kernel and it is also higher than literature values. Its protein content is significantly different from the literature values (Mishra. & Padhan, 2013). It can be clearly seen that the kinematic viscosity of Meze seed oil is much greater than that of literature value (Padhi, 2010). Fatty acid composition of extracted oil investigated by Gas Chromatography is indicated in Figure (1) and Table (2). The results shown in Table (2) represent that extracted Meze seed oil mainly contains palmitic acid (25.9%), oleic acid (43.7%), stearic acid (19.3%) and linoleic acid (9.8%) as predominant compounds.. The results are in agreement with Mishraet al. (2013).The results shown in Table (3) represent the physico-chemical properties of extracted Meze seed oil. Peroxide value, acid value and iodine value of extracted Meze seed oil indicate the freshness and lower rancidity of extracted oil.

In this study, Meze seed oil was applied in the preparation of biodiesel by two stage process; esterification followed by transesterification. The first step reduced the free fatty acid (FFA) value of the Meze seed oil to about 2% using acid catalyst. The crude Meze seed oil had an initial acid value of 8.27 mg KOH/g of oil corresponding to FFA level of 4.16%. According to Kathirvelu (2014), the process of transesterification is complicated, if oil contains large amount of FFA content that will form soap with alkaline catalyst. The soap can prevent the separation of the biodiesel from the glycerine fraction. Therefore, pretreatment process using an acid catalyst (sulphuric acid) was conducted to reduce the acid value of Meze seed oil. The reduction of acid value of Meze seed oil on different volumes of methanol used is shown in Table (4). It is seen that the acid value reduced quickly at the initial stage and reduced slowly later. According to Kathirvelu (2014), this might be due to the effect of water formation during the esterification of FFAs, which prevented further reaction. So, 400 mL (4:1% v/v) of methanol was selected as the optimum amount to reduce the acid value of Meze seed oil.

Table (5) shows the effect of amount of acid catalyst on the reduction of acid value of Meze seed oil. The catalyst amount was varied in the range of 0.5-2.5 mL for five different volumes (0.5, 1.0, 1.5, 2.0, 2.5 mL) of sulphuric acid. At lower catalyst concentration, acid value could not be reduced sharply. In accordance with Kathirvelu (2014), the ester formation rate increased with increasing catalyst concentration. However, it was observed that addition of sulphuric acid in excess darkened the colour of the product and adding lower volume than a certain volume affected the yield of the subsequent step. Therefore, 1.5 mL (1.5% v/v) of H_2SO_4 was selected as the suitable catalyst concentration that can provide the FFA content of less than 2%.

Effect of reaction time on reduction of acid value of esterified Meze seed oil is tabulated in Table (6). It is clearly seen that the acid value of the esterified oil dropped down to 2.37 (71.34% reduced) after 90 min. So, 90 min was chosen as the optimum reaction time for reducing the acid value of esterified Meze seed oil. Table (7) shows the effect of reaction temperature (40, 50, 60, 70, 80°C) on the reduction of acid value of Meze seed oil. The results show that the reaction temperature had an important role in the acid catalyzed esterification. The rate of reaction is increased by increasing the reaction temperature. This result was in accordance with Kathirvelu (2014). It was observed that the acid value of Meze seed oil could not be reduced to less than 2 mg KOH/g of oil at 40 ºC. At 60 °C, the acid value was reduced to 2.37 (FFA content of 1.19%). Hence, the reaction temperature 60°C was considered to be the optimum temperature for this reaction.

The effect of methanol on yield of biodiesel in transesterification is described in Table (8). It can be seen that higher amount of methanol used (ie, 60, 70, 80 mL) gave high yield percent of biodiesel. But it is not too different of the yield percent that can be given by the use of 50 mL of methanol. In addition, high amount of methanol added to vegetable oil interfered with the separation of glycerine because there was an increase in solubility. When glycerine remains in the solution, it helps drive the equilibrium back to the left side of the equilibrium, resulting in lower yield of esters (Kathirvelu, 2014). So, 50 mL (1:2 v/v) of methanol was selected as the suitable condition for alkali catalyst esterification. The variation of the amount of alkali catalyst (KOH, 0.4-1.2 g/100 mL of oil) was carried out to prepare biodiesel and it is shown in Table (9). From this Table, the yield percentage of biodiesel at different amount of catalyst used can be seen. The results show that the reaction was highly dependent on the catalyst amount. It was

observed that the maximum yield of biodiesel was obtained at 1g of KOH. With further increase in amount of catalyst, there was a decrease in the yield of biodiesel. Addition of excess amount of catalyst give rise to the formation of soap and glycerol phase. Esterification does not take place effectively for insufficient amount of catalyst (Kathirvelu, 2014). Therefore, 1g (1% w/v) of KOH was chosen as the suitable amount for alkali catalyst esterification.

The different reaction times selected for this study was 45, 60, 75, 90 and 105 min and the effect of reaction time on yield of biodiesel is shown in Table (10). The results clearly show that the yield of biodiesel increased with increasing reaction time. In this study, 90 min was selected as the suitable reaction time for alkali catalyst esterification. Table (11) describes that the effect of different reaction temperature on the yield of biodiesel. It was observed that the yield of biodiesel decreased far beyond the boiling point of methanol. According to (Kathirvelu ,2014), the reaction temperature above boiling point of methanol is avoided since at high temperature it tends to accelerate the saponification of glycerides by the alkaline catalyst before completion of the alcoholysis. Therefore, 60°C was chosen as the optimum reaction temperature for alkali catalyst esterification.

The prepared biodiesel was identified by FT-IR Analysis. The spectra are shown in Figure (2). The functional groups in the compounds contained in prepared biodiesel are interpreted in Table (12). A strong signal was observed between wave number 1500 cm^{-1} and 2000 cm^{-1}. It was confirmed that the prepared biodiesel contained fatty acid methyl esters group. A group of carboxylic acid compounds was investigated between 2858.87 cm^{-1} and 2926.11 cm^{-1}. Aliphatic chloro and fluoro compounds were also found between 723 cm^{-1} and 1170.83 cm^{-1}. The comparison of fuel properties was conducted between prepared biodiesel with ASTM standard and it is described in Table (13). The results show that the properties of prepared biodiesel mostly agree with ASTM standard.

Meze Seed Oil by expeller

Biodiesel prepared from Meze Seed Oil

Table 1 Physico-Chemical Properties of Meze Seed Kernels(Myanmar)

Sr. no.	Characteristics	Meze seed kernel	Literature values *
1.	Moisture content (w/w%)	4.18	7.8
2.	Ash content (w/w%)	1.64	3.4
3.	Protein (w/w%)	5.90	16.9
4.	Crude fiber (%)	8.86	3.2
5.	Crude fat (%)	53.71	46.7
6.	Carbohydrate (%)	25.71	22

* Mishra. & Padhan (2013)

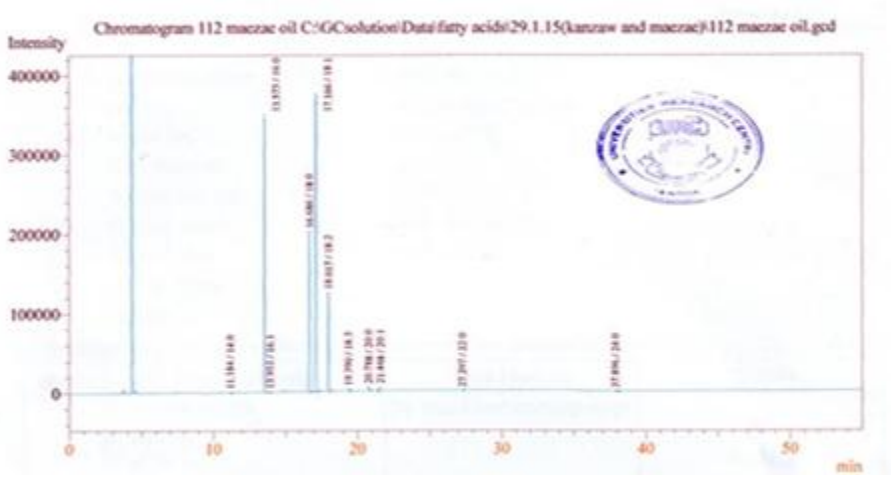

Figure 1 Fatty Acid Composition of Extracted Meze Seed Oil

Table 2 Fatty Acid Composition of Expelled Meze Seed Oil by GC Analysis

Fatty acids composition analyzed by GC (%w/w)	Experimental value	Literature value *
C 12:0 (Lauric)	Not detected	-
C 14:0 (Myristic)	0.07	-
C 14:1 (Myristoleic)	Not detected	-
C 16:0 (Palmitic)	25.91	24.5
C 16:1 (Oleo-Palmitic)	0.06	-
C 18:0 (Stearic)	19.33	22.7
C 18:1 (Oleic)	43.72	37
C 18:2 (Linoleic)	9.82	14.3
C 18:3 (Linolenic)	0.28	-
C 20:0 (Arachidic)	0.53	-
C 20:1 (paullinic)	0.13	-
C 22:0 (Behenic)	0.05	-
C 22:1 (Erucic)	Not detected	-
C 24:0 (Lignoceric)	0.1	-

(*) Mishra. & Padhan, 2013.

Table 3 Physico-chemical Properties of Expelled Meze Seed Oil

Sr.no.	Characteristics	Expelled Meze seed oil	Literature value *
1.	Refractive index	1.465	1.452-1.462
2.	Specific gravity	0.9089	0.862-0.875
3.	Colour	2.3R, 1.5B, 20Y	Pale Yellow
4	Moisture (loss on drying,%)	0.046	-
5.	Acid value (mg KOH per g)	8.27	0.5-20
6.	Iodine value (mgI_2/g)	53.170	58-70
7.	Saponification value (mg KOH per g)	195.499	187-196
8.	Unsaponifiable matter (%)	0.984	1-3
9.	Peroxide value (milliequivalents oxygen per kg)	9.369	-
10	Kinematic viscosity (Cst) * *	51.8	25
11	Flash point (°C) * *	204	226
12	Pour point (°C) * *	15	15

(*) Mishra and Padhan (2013), (* *) (Padhi, 2010)

Table 4: Effect of Volume of Methanol on Reduction of Acid Value (AV) of Esterified Meze Seed Oil Using Acid Catalyst

Sr. no.	Volume of methanol (mL)	AV of Meze seed oil after reaction (mg KOH/g of oil)	(%) Reduction of acid value of esterified Meze oil
1	100	3.38±0.03	59.17±0.35
2	200	3.01±0.03	63.64±0.25
3	300	2.53±0.02	69.45±0.25
4	400*	2.37±0.03	71.34±0.10
5	500	2.35±0.04	71.54±0.45

(*) Most suitable condition

Table 5 Effect of Volume of Acid Catalyst on Reduction of Acid Value (AV) of Esterified Meze Seed Oil

Sr. no.	Volume of catalyst (H_2SO_4) (mL)	AV of Meze seed oil after reaction (mg KOH/g of oil)	(%) Reduction of acid value of esterified Meze seed oil
1	0.5	3.25±0.09	60.7±1.12
2	1.0	2.76±0.1	66.71±1.19
3	1.5 *	2.37±0.06	71.34±0.71
4	2.0	2.25±0.02	72.83±0.21
5	2.5	2.05±0.03	75.17±0.37

(*) Most suitable condition

Table 6 Effect of Reaction Time on Reduction of Acid Value (AV) of Esterified Meze Seed Oil Using Acid Catalyst

Sr. no.	Reaction time (min)	A V of Meze seed oil after reaction (mg KOH/g of oil)	(%) Reduction of acid value of esterified Meze seed oil
1	45	3.9±0.06	52.84±0.78
2	60	3.3±0.07	60.10±0.84
3	75	2.97±0.07	64.09±0.89
4	90 *	2.37±0.08	71.34±0.99
5	105	2.36±0.04	71.46±0.49

(*) Most suitable condition

Table 7 Effect of Reaction Temperature on Reduction of Acid Value (A.V) of Esterified Meze Seed Oil Using Acid Catalyst

Sr.no.	Reaction temperature (◦C)	A V of Meze seed oil after reaction (mg KOH/g of oil)	(%) Reduction of acid value of esterified Meze seed oil
1	40	5.22±0.06	36.84±0.67
2	50	4.35±0.03	47.4±0.39
3	60 *	2.37±0.04	71.34±0.20
4	70	2.36±0.07	71.46±0.89
5	80	2.36±0.05	71.42±0.64

(*) Most suitable condition

Table 8 Effect of Volume of Methanol on Yield of Biodiesel Prepared from Esterified Meze Seed Oil in Transesterification

Sr.no.	Volume of methanol (mL)	Volume of biodiesel (mL)	Yield (% v/v)
1	40	50.2±0.08	42.47±0.21
2	50*	65.5±0.17	55.55±0.16
3	60	65.8±0.12	55.8±0.11
4	70	66.3±0.22	56.23±0.11
5	80	66.8±0.12	56.66±0.19

(*) Most suitable condition

Table 9 Effect of Weight of Alkali Catalyst on Yield of Biodiesel Prepared from Esterified Meze Seed Oil in Transesterification

Sr.no.	Weight of catalyst (KOH) (g)	Volume of biodiesel (mL)	Yield (% v/v)
1	0.4	62±0.45	52.58±0.09
2	0.6	62.3±0.12	52.84±0.08
3	0.8	63.5±0.22	53.86±0.07
4	1 *	65.5±0.25	55.55±0.05
5	1.2	64.4±0.02	54.62±0.07

(*) Most suitable condition

Table 10 Effect of Reaction Time on Yield of Biodiesel Prepared from Esterified Meze Seed Oil in Transesterification

Sr.no.	Reaction time (min)	Volume of biodiesel (mL)	Yield (% v/v)
1	45	59.8±0.8	50.72±0.02
2	60	62.2±0.7	52.75±0.07
3	75	63±0.26	53.43±0.02
4	90 *	65.5±0.39	55.55±0.22
5	105	64.5±0.16	54.70±0.02

(*) Most suitable condition

Table 11 Effect of Reaction Temperature on Yield of Biodiesel Prepared from Esterified Meze Seed Oil in Transesterification

Sr.no.	Reaction temperature (°C)	Volume of biodiesel (mL)	Yield (% v/v)
1	40	55.7±0.16	47.24±0.02
2	50	62.3±0.12	52.84±0.08
3	60 *	65.5±0.16	55.55±0.05
4	70	65.8±0.33	55.81±0.12
5	80	60.2±0.14	51.06±0.09

(*) Most suitable condition

Table 12 FT-IR Spectra Data of Prepared Biodiesel

Wave number, cm^{-1}		Functional group	
Observed	**Literature ***		
3468.13	3500-3300	υ – NH	Stretching vibration of amines groups
2926.11 2856.67	< 3000	υ – CH	Stretching vibration of alkane group
1743.71	~1700	υ – C=O	Stretching vibration of carboxylic acid
1452.45	> 1400	δas- CH	CH asymmetric bending vibration
1369.50	< 1400	δs- CH	CH symmetric bending vibration
1170.83 1012.66	1260-1000	υ – C-O	Stretching vibration of alcohol and phenol group
850.64 723.33	900-700	υ – CH	Stretching vibration of alkene group

* Mohan(2000)

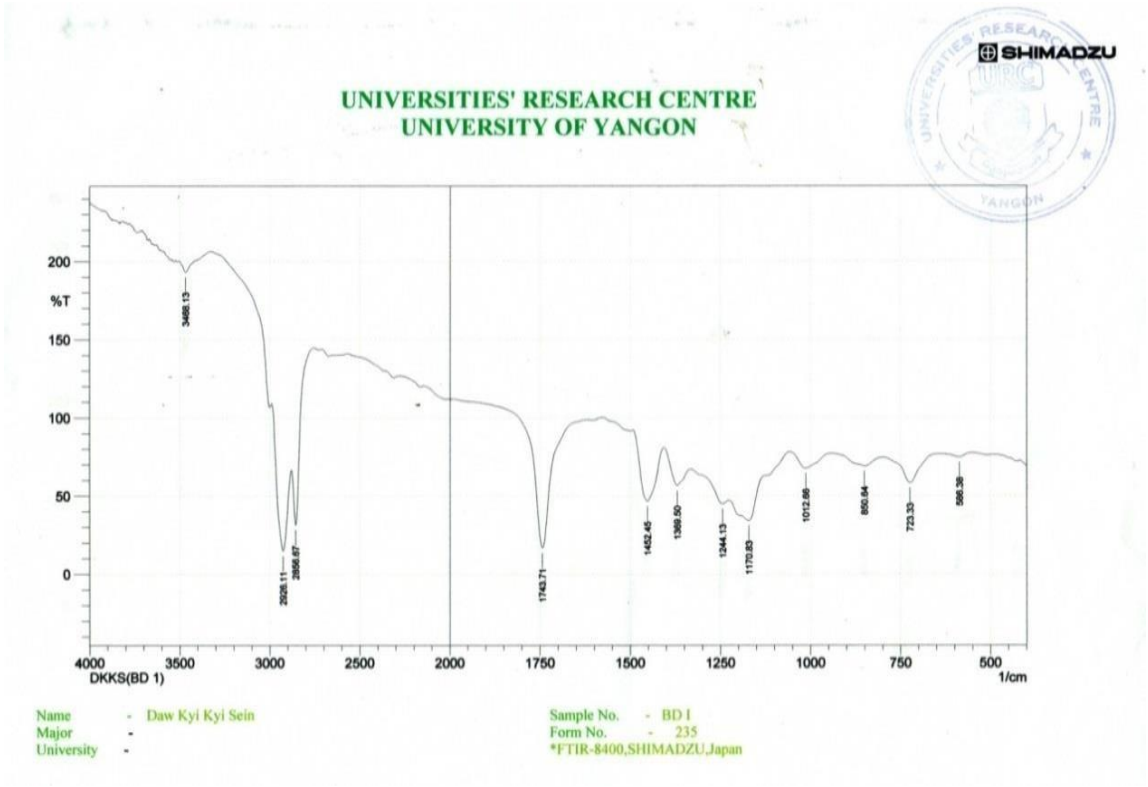

Figure 2 FT-IR Spectra of Prepared Biodiesel

Table 13 Fuel Properties of Meze Seed Oil Biodiesel (MSOB)

Sr.no.	Property	MSOB*	ASTM (D 6751)
1	Flash point (ºC)	226	130 minium
2	Pour point (ºC)	12	Not available
3	Colour	< 2	-
4	Kinematic viscosity at 40ºC (Cst)	5.4	1.9-6.0
5	Specific gravity at 30ºC	0.8773	0.88
6	Cetane number	72.91	47 minium
7	Estimated heat of combustion (cal/g)	9442.228	-
8	Copper strip corrosion test	1a	Maximum-3
9	Saponification value (mg KOH/g)	205.08	-
10	Iodine value (mg I_2/g)	61.032	-

1= slight tarnish , a= light orange, almost the same as fresh, 3 = dark tarnish

Conclusion

In the preparation of biodiesel, a two stage transesterification process was selected to convert high free fatty acid (FFA) oil to its methyl ester. In the first stage of pretreatment process, the FFA content of Meze seed oil could be reduced to less than 2% using acid catalyst (1.5 % v/w H_2SO_4) reacting with methanol to oil ratio of 4:1 at 60°C and 90 min reaction time. Excess addition of sulphuric acid darkens the product. The first stage product having acid value less than 2% of FFA content was used for the second stage alkali catalyzed (1% w/w KOH) transesterification reaction. Methanol to oil ratio of (1:2 %v/w) favoured the completion of the reaction in 90 min at 60°C. The process gave a yield of 55.5% of biodiesel, which had comparable fuel properties with that of ASTM standard.

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GC-MS ANALYSIS OF BIOMEDICAL CONSTITUENTS IN THE *EUPATORIUM ODORATUM L.* LEAVES (JAMANI) EXTRACTS AND THEIR ANTIBACTERIAL ACTIVITY AGAINST MULTIPLE BACTERIAL PATHOGENS

Ae Mar[1], Chaw Ei Hlaing[2], Thu Zar Lwin[3]

Abstract

The purpose of this study was to determine the biomedical constituents and antimicrobial properties of the crude extracts isolated from the leaves of *E. odoratum* collected in 2017 at Yadanabon University campus, Mandalay, Myanmar. The crude extracts, isolated by using maceration method with ethanol and hexane as solvents, were analyzed by gas chromatography coupled with mass spectrometry (GC-MS). The result showed the presence of *n*-Tetradecanol (52.98%, 37.42%) in both ethanol and hexane extracts but methyl hexadecanoate (40.47%) was recorded only in hexane extract as the main constituent. 2E-dodecanol (55.25%) was found to be the dominant constituents in ethanol extract of the leaves. The antimicrobial activity was evaluated by the paper disc diffusion method. Antimicrobial tests showed that ethanol extract of the leaves was active against all the clinically isolated gram-positive and gram-negative bacteria tested. The hexane extract was active against *E. coli* and *V. cholerae* but inactive against *K. pneumoniae* and all tested gram positive bacteria. The ethanol extracts which demonstrated good antibacteria activities and also showed better minimum inhibitory concentration (MIC) values on *V. cholera and S. aureus* (31.25 µg/ml). The results suggest once again that the antimicrobial activity of the extracts of the leaves is a resultant of the antibacterial properties of the major and minor components in their chemical composition. The skin irritant property of prepared ointments were examined by albino rabbit and there were not shown skin irritant property.

Keywords: *E. odoratum,* hexane, 2E-dodecanol, *E. coli,* antibacteria

Introduction

Globally, there is an ascending trend in life threatening diseases and emergence of drug resistant- bacterial pathogens which has become a serious health concern (WHO, 2001). Therefore, there is an urgent need to search for newer and effective novel compounds from various biota. For a long period of time, plants have been a valuable source of natural products for maintaining human health, especially in the last decade, with more intensive studies for natural therapies. The use of plant compounds for pharmaceutical purposes has gradually increased in the world. According to World Health Organization, medicinal plants would be the best source to obtain a variety of drugs (Santos, Oliveira & Tomassini, 1995). About 80% of individuals from developed countries use traditional medicine, which has compounds derived from medicinal plants. Therefore, such plants should be investigated to better understand their properties, safety and efficiency (Pierangeli & Windell, 2009).

Eupatorium odoratum (formerly *Chromolaena odorata*) belongs to the family Asteraceae and it consists of about 60 species spread over tropical, sub-tropical and warmer parts of temperate regions of the world. The fresh leaves of *E. odoratum* or the decoction has been used by practitioners of traditional medicine for the treatment of human burns, soft tissue wounds, ulcerated wounds, burn wounds, postnatal wounds and also for the treatment of leech bites,

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indigestion and skin infection (Panyaphu et al. 2011). *E. odoratum* is used as a traditional medicine in Myanmar, where its Burmese common name is "Jamani or Bi-Zat". While it has been widely considered a weed by agriculturalists (Vaisakh & Pandey, 2012), the aqueous extract and the decoction from the leaves of this plant have been used throughout Vietnam for the treatment of soft tissue wounds, burn wounds, and skin infections. A number of clinical studies done by Vietnamese as well as foreign medical workers has demonstrated the efficacy of this extract on the wound-healing process. In Thailand, the leaves are also used as cataplasm to stem external hemorrhage (Tonzibo *et al*, 2007).

In the last few years gas-chromatography mass-spectrometry has become firmly established as a key technological platform for metabolite profiling in plant. Gas chromatography mass -spectrometry (GC-MS) based metabolome analysis has profound applications in discovering the mode of action of drugs or herbicides and helps unravel the effect of altered gene expression on metabolism and organism performance in biotechnological applications (Kanthal, 2014). Thus, the aim of this work was to characterize and chemically quantify the crude extracts of the *E. odoratum* leaves as well as to evaluate antimicrobial activities. Moreover, this work illustrates that traditional remedies that are used by folk practitioners to improve healing can be examined in a scientific manner using in vitro wound-healing models. It could be that the synergistic properties of components of the natural extract contribute to the positive effects demonstrated on various wound-healing mechanisms.

Materials and Methods

Materials

Eupatorium odoratum (Taw-Bizat) leaves were collected from around of the Yadanabon University, Amarapura Township. The plant was identified by the botanist, Dr. Soe Myint Aye, Professor of the Department of Botany, University of Mandalay, Myanmar. Ethanol, and hexane were used to extract the absolutes (crude extract) from the odorata leaves. These chemicals were purchased from Able Chemical Store, Mandalay.

Figure 1 *Eupatorium odoratum L.*

Methods

Maceration

The *E. odoratum* leaves were cleaned with water, dried under shade for about 7 days and powdered by an electrical grinder (MX-GM 1011, 1000 ml). The extract of the leaves was also obtained by using the maceration of 150 g of this leaves with hexane, and ethanol (500 ml each). The extraction process was done over the period of seven days at room temperature. After filtration and evaporation of all solvent under reduced pressure yielded *E. odoratum* crude extracts of hexane (yellow extract; 0.54 g), and ethanol (dark-brown extract; 2.62 g), respectively. The extracts were then stored at 4°C in the dark until further analysis.

Figure 2 (a) Dry *E. odoratum* leaves (b) Maceration

Analysis of Antibacterial Activity

The various extracts were tested for their antibacterial activities using the disc diffusion technique. *S. epidermidis* DMST 15505, *S. aureus* DMST 8804, *B. subtilis* TISTR 008, *K. pneumoniae* DMST 4739, *V. cholerae* DMST 2873 and *E.coli* DMST 4212 were testing of the antibacterial activity of *E. odoratum* crude extracts. The standard discs containing bacitracin and chloramphenicol were used as positive control while all used solvents including ethanol, and *n*-hexane were used as negative control.

Paper disc diffusion method: All extracts of *E. odoratum* leaves were tested for their antibacterial activity by disc diffusion method. To prepare the testing bacteria, a single colony of each bacterial culture was transferred to 3 mL nutrient broth (NB) pH 6.9 (HiMedia Laboratories Pvt. Ltd, Mumbai, India) and incubated for overnight at 37 °C and each bacterial culture was then spread on the surface of the nutrient agar medium (NA) obtaining from 8.0 g/L of NB and 15.0 g/L of agar (Union Science Co. Ltd, Chiang Mai, Thailand) using sterile cotton swab. Subsequently, filter paper discs (6 mm in diameter (Whatman No.1, Maidstone, UK)) were placed on surface of each inoculated plate. The crude extracts were prepared at two fold concentrations (0.135, 0.625, 1.25, 2.5, 5, and 10, 20 µg/mL). A small amount, 20 µL, of each was then added into a disc plate using a sterile micropipette. These plates were then incubated overnight at 37 °C, and then the diameter of the clear zone around each disc plate was measured in mm after incubation and was expressed as the mean value +/- the standard deviation (± SD). This experiment was performed 3 times on each extract.

Preparation of Ointment

Materials

The three ingredients (bee wax, coconut oil and crude extract of *E. odoratum*) were used in the preparation of ointment. Bee wax was purchased from Min Thar shop, Zay Cho market, Mandalay. Coconut oil was purchased from Tadau market, Mandalay Region.

Preparation Procedure

Coconut oil (4 ml) and bee wax (1.2 g) were placed into a cleaned, dried and weighed test tube and heated at double boiler with stirring at 70 °C for 15 mins. When the wax has completely melted, 0.1 ml of crude extract (ethanol & hexane) of *E. odoratum* were added into the mixture and allowed the entire mixture to remain on the hot plate until liquefied. The resulted liquefied mixture was poured into the sterilized bottle and cooled at room temperature for 20 min.

Figure 3 Prepared Ointment

Evaluation of Properties of Ointment

Skin Irritation Test by Albino Rabbit (Draize Test)

Three sexually mature female albino rabbits, weighing 2.5 kg, originated from the breeding colony of laboratory animals were used. Test animals were kept within a limited-access rodent facility with environmental conditions set to a temperature of 25±2 °C, a humidity of 60-90% relative humidity (RH). Animals were provided with commercial rabbit-diet and drinking water. The 3 cm^2 area of hair from back quarter of rabbit was shaved by using blade and cleaned by the clean water. This shaved area of the skin of each rabbit was divided into three marked area. The first marked area was control position (no treatment) and another two marked areas were swabbed with 0.2 ml of sample prepared ointment (hexane, & ethanol) on shaved area (Figure 4). The animals were return to the cages. The skin area of rabbit was checked after 2 h, 24 h, 48 h and 72 h. If the area was showed redness (erythema), the animal was suffering the skin irritation. It was checked those skin areas daily for 3 days. This experimental test was conducted at Laboratory Animal Services Division, Department of Medical Research, Yangon.

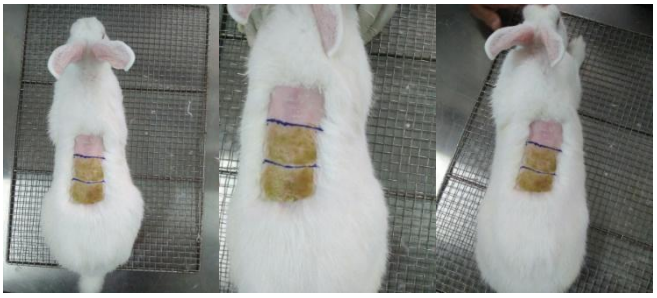


Figure 4 Skin Irritation Test

Results and Discussion

The extracts obtained from maceration were produced greenish colored with a strong pleasant odor and different yields. Higher yields were detected with ethanol maceration versus *n*-hexane maceration. A total of 64 volatile components were identified from two extracts by GC-MS analysis. The identified constituents, their percentages, and retention indices are listed in Table 1. Figure 5 and 6 have been shown for the chromatogram of extracts of *E. odoratum.* Significant qualitative and quantitative variations of most identified constituents were detected among these extracts. Thirty-seven components were detected in the *n*-hexane extract, representing 94.24% of the total. The major components were n-tetradecanol (52.98%) and methyl hexadecanoate (40.46 %), followed by canellal (0.15 %), and caryophyllene oxide (0.25 %).Thirty-five compounds were detected in the ethanol extract, representing 93.59% of the identified compounds, including 2E-dodecanol (55.24%), n-tetradecanol (37.42%), iso-longifolol acetate (8.09%), α-ylagene (4.73%), pregeijerene B (2.14%), and cis-carveol (1.70%) as the major constituents.

Most volatile components identified were similar to previous reports although the different quantities of compounds were detected. In the present investigation, monoterpenes and sesquiterpenes and their derivatives predominated in the hexane and ethanol extracts of *E. odoratum* leaves. Among identified compound, caryophyllene oxide, *n*-tetradecanol, methyl hexadecanoate, andn-heneicosane were found in this two extracts. In general, the application of extracts such as the biocide action are depend on the composition of these oils and extracts (Chamorro, Zambón, Morales, Sequeira, & Velasco, 2012).

Table 1 Chemical Composition of Various Extracts Obtained from *E. odoratum* Leaves

No.	Compound	RI	Extracts	
			Hexane	Ethanol
1	Isovaleric	835	-	0.057
2	2-Ethoxy ethyl acetate	904	-	0.012
3	Sabinene	975	0.02	-
4	<1,2,4>-Trimethyl benzene	1025	0.04	-
5	Methyl-(3E)-hexenoate	934	0.01	-
6	Mesitylene	995	0.02	-
7	Ethyl acetal-(Leaf Alcohol)	1088	-	0.002
8	Geijerene	1143	0.03	0.046
9	cis-Carveol	1229	-	0.185
10	3Z-Hexenyl 2-methyl butanoate	1232	t	-
11	Pregeijerene B	1276	t	0.127
12	Fenchol-<2-ethyl-endo>	1288	-	t
13	n-Tridecane	1300	t	-
14	2-Adamantanone	1311	t	-
15	a-Ylagene	1375	-	0.133
16	4aa,7a,7ab-Nepetalactone	1387	-	t
17	Damascone	1387	-	t
18	β-Cubebene	1388	0.042	-
19	β-Bourbonene	1388	t	-
20	3-Dodecanone	1390	-	t
21	n-Tetradecane	1400	0.006	-

No.	Compound	RI	Extracts	
			Hexane	Ethanol
22	α-Funebrene	1402	-	t
23	Z-Caryophyllene	1408	t	-
24	β-Ylangene	1420	0.014	-
25	Linalool butanoate	1421	t	-
26	1-Phenyl hexan-3-one	1425	-	0.016
27	Dictamnol	1429	-	t
28	γ-Elemene	1436	-	t
29	2E-Dodecanol	1466	-	55.24
30	cis-Muurola-4(14),5-diene	1466	-	t
31	neo-methyl lactate	1469	t	-
32	Dauca-5,8-diene	1472	t	-
33	β-Thujaplicin	1477	0.05	-
34	Butylated hydroxytoluene	1515	0.01	0.01
35	δ-Amorphene	1518	t	-
36	δ-Cadinene	1523	-	0.042
37	Laciniata furanone G	1529	t	-
38	Germacrene B	1561	-	0.023
39	Spathulenol	1578	-	0.003
40	Caryophyllene oxide	1583	0.256	t
41	1-Hexadecene	1589	-	0.003
42	β-Biotol	1613	-	t
43	Massoia dodecalactone	1686	t	0.0471
44	*n*-Tetradecanol	1672	52.98	37.417
45	Geranyl tiglate	1696	t	-
46	n-Heptadecane	1700	-	-
47	iso-Longifolol		-	0.094
48	iso-Longifolol acetate	1820	t	0.116
9	Acorone	1820	0.065	t
50	Avocadynofuran	1826	-	0.007
51	cis-Thujopsenic acid	1864	-	0.001
52	Methyl hexadecanoate	1921	40.469	-
53	Isohibaene	1937	0.03	-
54	Ethyl hexadecanoate	1993	-	t
55	Canellal	2046	0.157	-
56	Methyl linoleate	2085	t	-
57	Methyl linoleate	2095	-	t
58	Linoleic acid	2133	t	-
59	n-Heneicosane	2100	0.01	0.01
60	Methyl octadecanoate	2125	t	-
61	Linoleic acid	2133	-	t
62	Nezukol	2133	0.03	-
63	Incensole oxide	2237	t	-
64	<7-α-hydroxy->Manool	2237	t	-
Number of Compounds			38	35
% of Compounds			94.239	93.5911

RI: Retention index relative to C_8–C_{25}-alkanes on TG-5 column, MS: NIST and Wiley library, and the literature, t: trace (<0.1%).

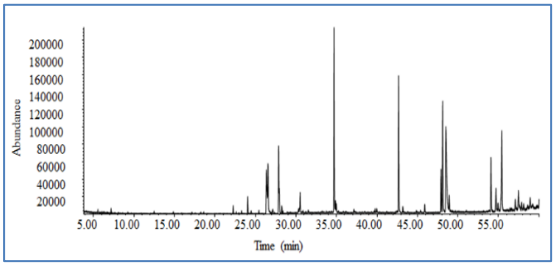


Figure 5 GC-MS Chromatogram of Hexane Extract of *E. odoratum* Leaves

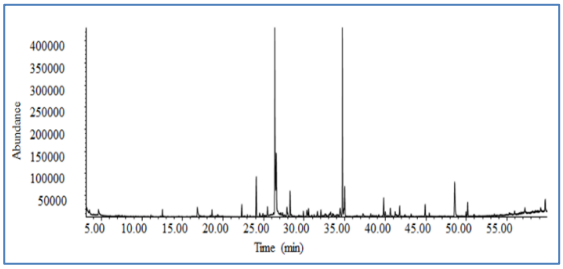


Figure 6 GC-MS Chromatogram of Ethanol Extract of *E. odoratum* Leaves

The observation of the antimicrobial activities has been tabulated in Table 2 and it was found to be varying between 31.25-1000 µg/mL, with respect to most of the test bacteria. The various extracts provided various efficiencies of antibacterial activity depending on the tested bacterial strains. The ethanolic extract had the greatest antibacterial activity against all tested Gram-positive and negative bacteria as compared with the hexane extract. An increased inhibition zone of the ethanol extract was observed with *B. subtilis*, followed by *S. epidermidis*, *K. pneumoniae*, and *E. coli* measured at 10.3, 10.1, 10 and 9.3 mm, respectively. Similar inhibition zone on *V. cholera* strains was detected in all extracts of the leaves. The hexane extract inhibited only two Gram-negative bacteria, *E. coli* (10.3 mm) and *V. cholera* (7.6 mm). As a result, *E. coli and V. cholera* were more sensitive to various extracts (see Table 2 & Figure7).

Table 2 Antibacterial Activity of Different Extracts of *E. odoratum* Leaves (diameter, mm ± SD)

Bacteria	Chloramphenicol	Bacitracin	Extract	
			Hexane	Ethanol
Gram-positive bacteria				
S. epidermidis	19.85 ± 0.75	27.8 ± 0.3	-	10.1±0.1
S. aureus	18.8 ± 0.1	19.45 ± 0.75	-	7.5±0.3
B. subtilis	9.75 ± 0.25	10.65 ± 0.35	-	10.3±0.35
Gram-negative bacteria				
E. coli	9.4 ± 0.4	16.4 ± 0.6	10.3±0.2	9.3±0.4
K. pneumoniae	12.7 ± 0.3	18 ± 0.5	-	10±0.5
V. cholera	11.05 ± 0.45	18.65 ± 0.65	7.6±0.13	8±0.2

–: not detected

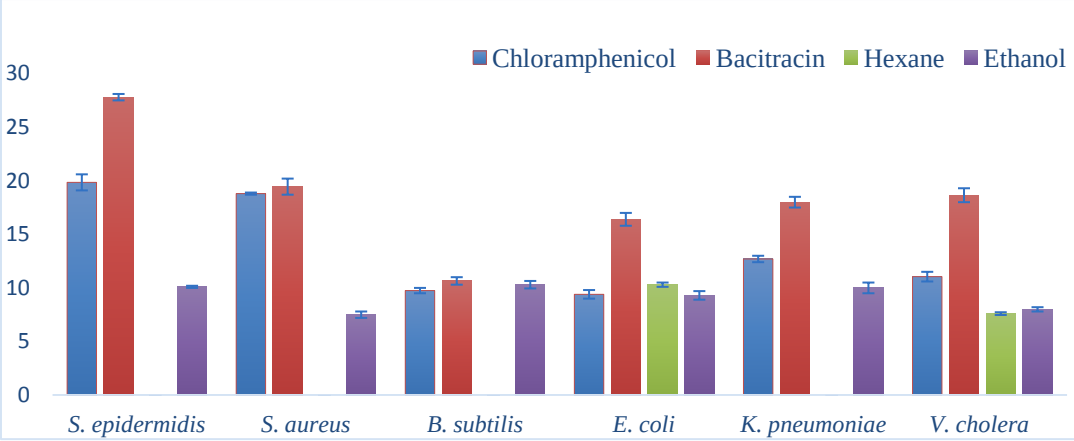


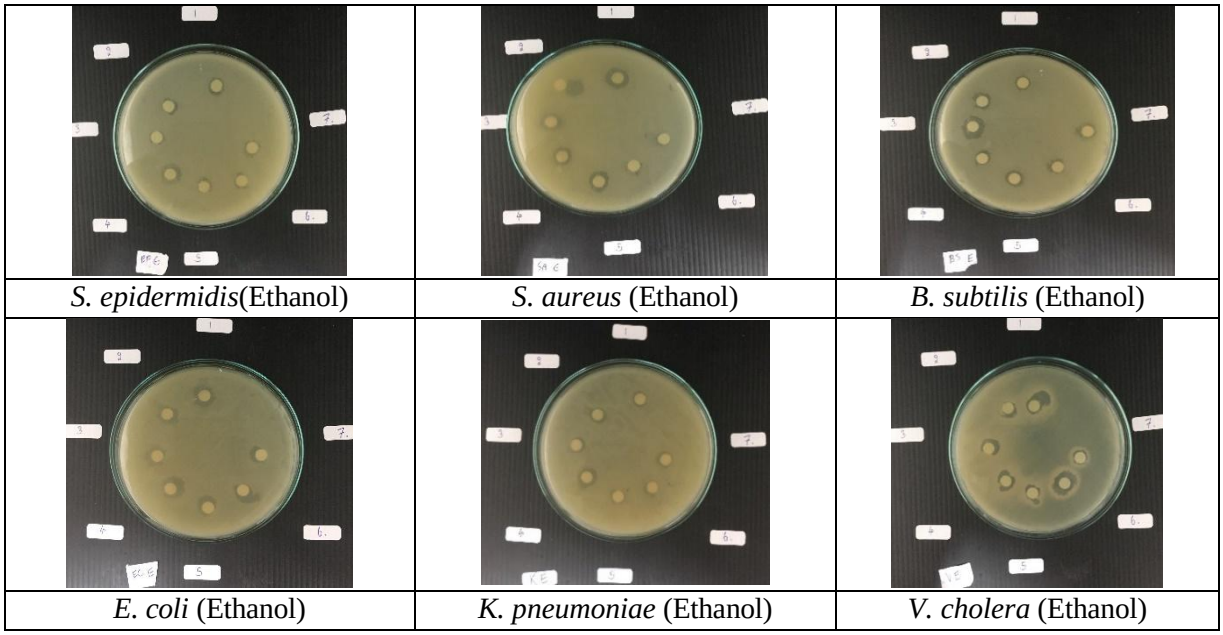
Figure 7 Zone of Inhibition produced by Crude Extracts of *E. odoratum* Leaves and Antibiotics

The antibacterial activities of all extracts were evaluated by the diameter of inhibition and MIC values compared with those obtained from positive control, bacitracin and chloramphenicol. MIC values of all samples are shown in Table 3, while the antibacterial activities of all samples on Gram-negative and Gram-positive bacteria are summarized in Figure 8 & 9. The ethanol extracts of *E. odoratum* leaves which demonstrated good antibacteria activities and also showed better minimum inhibitory concentration (MIC) values on *V. cholera and S. aureus* (31.25 µg/mL). The antimicrobial activities of crude extracts of *E. odoratum* for bacterial strains like *E. coli* DMST 4212 and *B. subtilis* TISTR 008 were inhibited at MIC value of 62.5 µg/mL. In addition, *S. epidermidis* DMST 15505, and *K. pneumoniae* DMST 4739 for the ethanolic extract were inhibited at MIC value of 125 µg/mL, while the hexane extract presented higher MIC values ranging from 62.5 to 500 µg/mL (see Table 3).). According to this information, it was clearly found that *E. odoratum* has antimicrobial activity against the pyogenic pathogens. Among the extracts selected for the study, the ethanolic extract of *E. odoratum* shows highest antimicrobial activity against the target microbial flora, when compared with hexane extract.

It was reported that the plant extract have significant antimicrobial activity against pyogenic microorganism due to the presence of various phytochemical constituents such as alkaloids, glycosides, flavonoids, terpenoids, saponins, tannins. Moreover, the presence of bioactive compounds and the supporting studies accompanied with them depicts that the *E. odoratum* is highly active against pyogenic pathogens. This shows that the phytochemical constituents can be used to treat pyogenic infection. The potency of the extract is also based on the method of extraction and concentration of plant extract. In addition, diversity of major and minor constituents in the extracts due to the synergistic effects could be affected on the consideration to account for their biological activity (Giweli *et al*., 2013).

Table 3 Minimum Inhibitory Concentration of Crude Extracts of *E. odoratum* Leaves

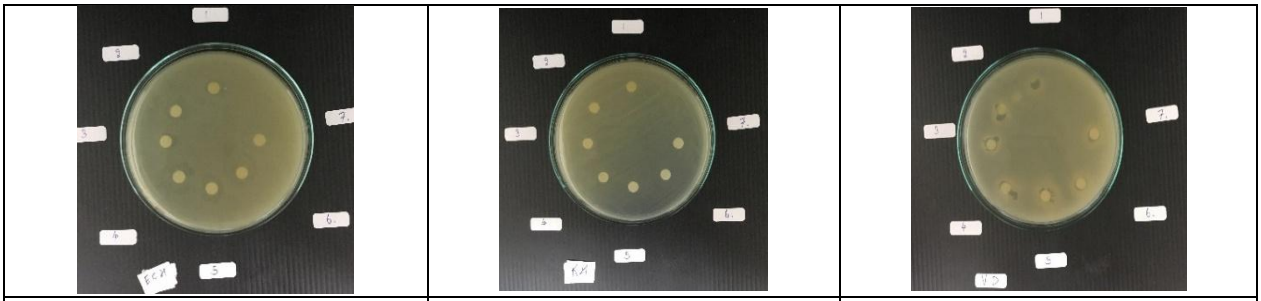
Sr. No.	Bacteria	MIC of Hexane Extracts	MIC of Ethanol Extracts
Gram-positive bacteria			
1.	*S. epidermidis* DMST 15505	-	125 µg/mL
2.	*S. aureus* DMST 8840	-	31.25 µg/mL
3.	*B. subtilis* TISTR 008	-	62.5 µg/mL
Gram-negative bacteria			
4.	*E. coli* DMST 4212	62.5 mg/ml	62.5 µg/mL
5.	*V. cholerae* DMST 2873	500 mg/ml	31.25 µg/mL
6.	*Ps. aeruginosa* DMST 4739	125mg/ml	125 µg/mL



S. epidermidis(Ethanol) | *S. aureus* (Ethanol) | *B. subtilis* (Ethanol)

E. coli (Ethanol) | *K. pneumoniae* (Ethanol) | *V. cholera* (Ethanol)

Figure 8 Inhibition Zones of the Tested Bacteria by Ethanol Extract of *E. odoratum* Leaves



E. coli (Hexane) | *K. pneumoniae* (Hexane) | *V. cholera* (Hexane)

Figure 9 Inhibition Zones of the Tested Bacteria by Hexane Extracts of *E. odoratum* Leaves

Skin irritant property of ointment was shown in Table (4). Rabbit with ointment was not shown the sign of skin redness (erythema) after 2 h, 24 h, 48 h and 78 h. From the above results, ointments formulated with the two crude extracts were not shown skin irritation sign. The results are shown in Table (4) and Figure (10). Plant products have been shown to possess good therapeutic potential as anti-inflammatory agents and promoter of wound healing due to the presence of active terpenes, alkaloids and flavonoids (Ibrahim, 2018). The study reveals that both ethanolic and hexane extracts of

E. odoratum possesses good skin irritation properties which may be attributed to the individual or combined action of phytoconstituents like alkaloids, and terpenoids present in the extracts.

Table 4 Skin Irritant Property of Prepared Ointments

Animal	Sample	Interval of skin exposure			
		2h	24h	48h	72h
Albino rabbit	Ointment (Ethanol)	0.0	0.0	0.0	0.0
	Ointment (Hexane)	0.0	0.0	0.0	0.0
	Control	0.0	0.0	0.0	0.0

Note: no erythema = 0.0, slightly erythema = 0.2-0.4, erythema = 0.6

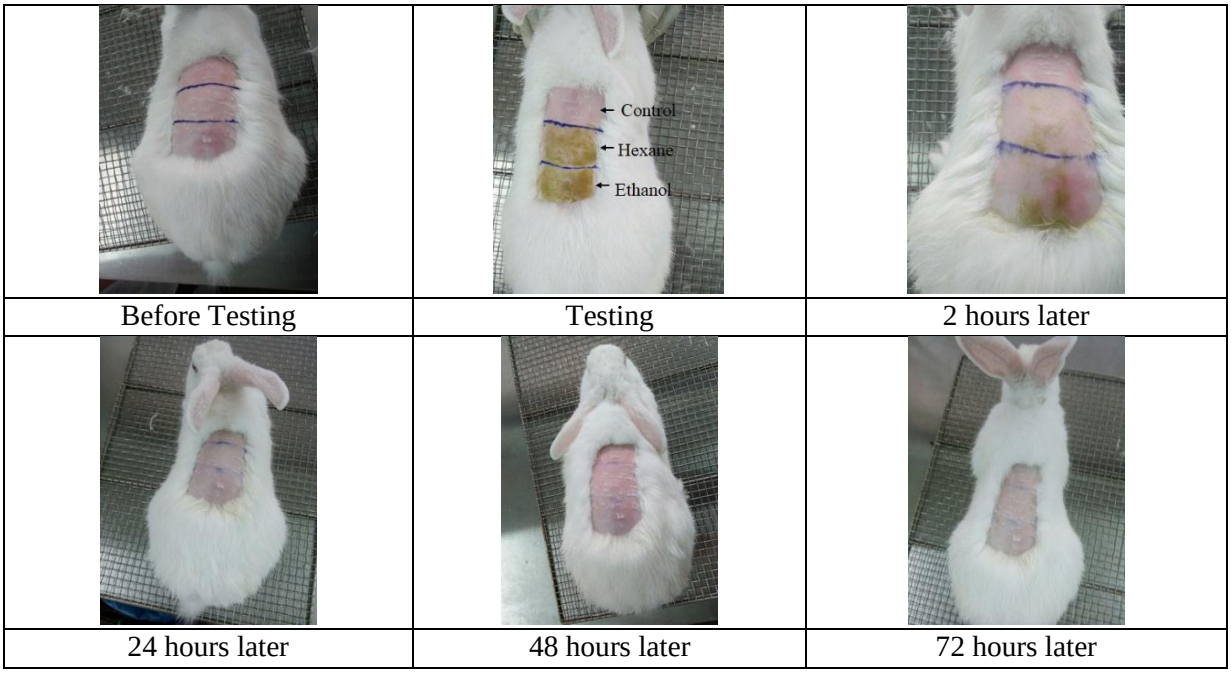


Figure 10 Skin Irritation score observation

Conclusion

The present work has been designed to evaluate the chemical constituents and antimicrobial potential of *E. odoratum* with a view to contributing to the search for beneficial uses of this invasive plant which is a menace to farmers. It is quite evident from this work that *E. odoratum* is an important medicinal plant. It contains a number of phytoconstituents, which are the key factors in the medicinal value of this plant. According to all of positive findings, it is concluded that the traditional plants may represent new sources of antimicrobial with stable, biologically active compounds that can establish a scientific base for the use of plants in modern medicine. Further investigations are necessary to determine the bioactive constituents present in the extracts to prove its potential in clinical studies.

Acknowledgement

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PREPARATION AND CHARACTERIZATION OF DRY CAT FOOD FROM FACTORY WASTES FISH MIXING WITH DIFFERENT CEREALS

Kyaing Thuzar Mon[1], Nwe Nwe Aung[2], Pansy Kyaw Hla[3]

Abstract

The aim of this research is to prepare the dry cat food using factory wastes fish and cereals. Pyi-taw-thar and Nga-khaung-pwa were collected as the rejected low grade fish from Ngwe Pin Lae fish factory, Hlaingthayar Township, Yangon Region. The ingredients were dried by using hot air oven. Dry cat food was prepared from fish by mixing with each cereal namely wheat, corn and soybean. The respective conditions were investigated for varying ingredient compositions and steaming time. The nutritive values, pH, water activity, size stability, metabolizable energy and cat feeding of dry cat food with different cereals were also determined. It was observed that the optimum parameters were 3:7 ratio of fish powder and wheat flour with steaming time of 25 min., 1:1 ratio of fish powder and corn flour with steaming time of 25 min. and 2:8 ratio of fish powder and soybean flour with steaming time of 20 min.. The optimum dry cat food (fish and soybean) was also selected based on the comparison of nutritive values of dry cat food (fish and wheat, fish and corn, and fish and soybean). It was found that the dry cat food prepared from fish and soybean had the highest protein content of 41.5 % and metabolizable energy of 3277.5 kcal/kg.

Keywords: Dry cat food, fish, cereals, nutritive value, protein content

Introduction

Dry cat food is usually the extruded product. Dry cat food (8-10 % moisture) is manufactured using extrusion cooking under the condition of high heat and pressure. Dry cat food has a long shelf-life on account of its low moisture content. Several types of cat food are graded depend on their ingredients and nutritive value especially the protein content. There are three types of commercial cat foods. They are dry cat food, semimoist cat food, and canned cat food. These products vary in moisture content, protein content and energy value. Proteins, fats and carbohydrates are the three major groups of nutrients in any cat food. Protein is necessary for the cat to grow (Rivera, 1998). Meat, meat byproducts, fish, poultry, cereals, fruits, and bones are used as the ingredients for the production of cat food**.** Cereals are used to supply energy, a proportion of protein and other nutrients such as thiamine and niacin. Cereals including corn, rice, wheat, barley or sorghum are good sources of carbohydrates in the cat food (Amir and Mona, 2013). And soybean which is a source of protein and energy, omega 6, B vitamins, fibre and minerals is also used as the ingredient (Potter, 1996). A favourable source of high quality protein in the cat food is fish. The soft tissue of fatty fish comprises vitamin A, D and omega 3 (Pet Food Manufacturers Association, 2010). The protein content in cat food recommended by AAFCO is 30-45 % (AAFCO, 2003).

The objectives of the study are:

(1) to develop dry cat food from fish and cereals namely wheat, corn and soybean using an extruder, and

(2) to evaluate the physico-chemical properties and the cat feeding of dry cat food by varying the ingredients composition.

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[2] Dr, Professor, Department of Industrial Chemistry, Yadanabon University
[3] Dr, Professor (Retired), Department of Industrial Chemistry, University of Yangon

Materials and Methods

Raw Materials

Torpedo scad **(**Pyi-taw-thar) and White mouth croaker (Nga-khaung-pwa) were collected as rejected fish from Ngwe Pin Lae Fish Factory, Ngwe Pin Lae Marine Industrial Zone, Hlaingthayar Township, Yangon Region. Wheat powder and table salt were also purchased from Ocean Supermarket, Mayangone Township, Yangon Region. Corn flour was purchased from Orange Supermarket, Kyeemyindaing Township, Yangon Region. Soybean was purchased from Nyaungpinlay Market, Lanmadaw Township, Yangon Region. Citric acid (Analar grade, BDH) was purchased from Academy Chemical Shop, 28th Street, Pabedan Township, Yangon Region.

Methods

Preparation of Fish Powder Using Hot Air Oven Drying

The (1:1) ratio of Pyi-taw-thar and Nga-khaung-pwa fish was cut into 1cm to 2 cm length. The cut fish was dried in a hot air oven at 60˚C for 4 hr and the dried fish was ground into fish meal using meat grinder. Then the fish meal was dried again in a hot air oven at 60˚C for 2.5 hr. After that the dried fish meal was ground into powder using grinder. Finally, the powder was screened with sieve (-14+20).

Nutritive Value of Fish Powder

The nutritive value of fish powder such as moisture content, ash content, protein content, crude fibre content, crude fat content, carbohydrate and energy value were determined.

Preparation of Dry Cat Food

Effect of Steaming Time on the Characteristics of Dry Cat Food (Fish and Wheat)

Dry ingredients such as 50 g of fish powder and 50 g of wheat powder were mixed together in a steel tray. Then 1 g of salt and 0.5 g of citric acid were added into the mixture and mixed thoroughly. And the mixture was pre-steamed at 90-95˚C for 20 min. to gelatinize the starch. The mixture was thoroughly mixed with 50 ml. of distilled water to form a moist mixture for cooking. The moist mixture was cooked with steam at 90-95˚C by varying the steaming time 10 min., 15 min., 20 min., 25 min., 30 min. and 35 min., respectively. After that the cooked mixture was dried in a hot air oven at 60˚C for 3 hr. The dried mixture was ground into powder and screened with sieve (-30+40). The moisture content, pH and yield percent of sample were determined.

Effect of Ingredients Composition Ratio on the Characteristics of Dry Cat Food (Fish and Wheat)

In order to get the proper ratio for the preparation of wheat mixed dry cat food, fish powder and wheat powder were firstly mixed together in a steel tray by varying the weight ratio of 10:90, 20:80, 30:70, 40:60, 50:50, 60:40 and 70:30. Then 1 g of salt and 0.5 g of citric acid were added into the mixture and mixed thoroughly. And then the mixture was pre-steamed at 90-95˚C for 20 min. to gelatinize the starch and it was thoroughly mixed with 50 ml. of distilled water to form a moist mixture for cooking. The moist mixture was then cooked with steam at 90-95˚C at the suitable steaming time of 25 min.. After that the cooked mixture was extruded by

using a meat grinder/extruder. Finally, the extrudates were dried at 60˚C for 3.5 hr. The protein content, moisture content, ash content, pH, yield percent and the cat feeding of wheat mixed dry cat food were determined. From these results, the most suitable sample was selected and the nutritive value of it was investigated.

Similarly, the effect of steaming time on the characteristics of dry cat food (fish and corn), (fish and soybean) was analyzed. The effect of ingredients composition ratio on the characteristics of these samples was also investigated.

Determination of Characteristics of Dry Cat Food

The nutritive value of dry cat food such as moisture content, ash content, protein content, crude fibre content, crude fat content, carbohydrate and energy value, and pH, metabolizable energy, water activity, size stability and cat feeding were determined.

Determination of Metabolizable Energy

ME (kcal/kg) = 10 [(3.5xCP) + (8.5 x CF) + (3.5 x NFE)]

Where ME= Metabolizable Energy

CP= % crude protein

CF= % crude fat

NFE = % nitrogen-free extract (carbohydrate)

(Asaro, Guevara and Berendt, 2017)

Determination of Feline Body Mass Index of Experimental Cats

Feline Body Mass Index is a simple measure of body fat content in cats.

$$\text{FBMI} = \frac{\left(\frac{rib \quad cage}{0.7062}\right) - \text{LIM}}{0.9156} - \text{LIM}$$

where, LIM = Lower Hind limb Measurement (cm)

rib cage = rib cage Measurement (cm)

(WALTHAM, 2003*)*

Figure 1 Pyi-taw-thar

Figure 2 Nga-khaung-pwa

Figure 3 Meat grinder/Extruder

Results and Discussion

The ingredients used in the production of dry cat food are usually powder form and therefore 1:1 ratio of both fish were firstly converted into fish powder by drying in hot air oven. The investigated nutritive value of prepared fish powder was compared with the prepared fish powder from tuna trimmings and the results are shown in Table (1). It was found that the moisture content and ash content were markedly higher than that of Tuna trimmings, although, the protein content was found slightly lower. The effect of steaming time on physico-chemical characteristics of dry cat food (fish and wheat) was studied and shown in Table (2). Although the yield percent of WST_1 to WST_5 were the same, the attractable fishy smell was observed in WST_4 to WST_6. Therefore WST_4 was selected as the suitable condition based on the odour, moisture content and short steaming time. Table (3) shows the effect of ingredient composition ratio on physico-chemical characteristics and cat feeding of extruded dry cat food (fish and wheat). Among the different composition ratio, HFW_3 was selected as the suitable sample because of the moisture content and yield percent. Although HFW_5 with the ratio of 50 g : 50 g had the highest consumption of cat, the ratio used in HFW_3 was chosen as the optimum ratio based on the protein content.

In this research work, the preparation of dry cat food was also prepared by mixing fish with other cereals such as corn and soybean. Table (4) and (6) pointed out the comparison of steaming time for corn and soybean. The optimum steaming time used for corn and soybean was found 25 min. and 20 min. because of the odour, moisture content and short steaming time. The physico-chemical characteristics obtained in Table (5) and (7) represented the value obtained for corn and soybean. The effect of ingredients composition ratio on cat feeding of dry cat food (fish and corn) and (fish and soybean) were also tasted with the domestic cat. It was found that all the samples were consumed by the domestic cat and HFC_5 and HFS_2 were found the highest consumption of dry cat food. According to the results of Table (5), HFC_5 was chosen as the suitable sample because of the moisture content and protein content. According to the results of Table (7), HFS_2 was chosen as the suitable sample based on the moisture content, protein content and nutrition of fish.

The comparison of the nutritive values of the suitable dry cat food containing fish mixed with wheat (HFW_3), corn (HFC_5) and soybean (HFS_2) is shown in Table (8). According to this Table, HFS_2 (fish and soybean) was chosen as the most suitable sample for the preparation of dry

cat food because of the highest protein content and metabolizable energy. The effect of different ingredients and recipes on water activity and size stability of the suitable dry cat food containing fish mixed with wheat (HFW_3), corn (HFC_5) and soybean (HFS_2) was studied and shown in Table (9). According to this Table, the water activity of all of dry cat food was around the value of 0.3. Bacteria, molds and yeast require water for growth and every microorganism has a minimum water activity below a_w =0.61. Size stability of dry cat food was determined by using drop test and it was found that they were not easily breakable. The feline body mass index (FBMI) of experimental cats were also determined and shown in Table (10). The kibbles with different ingredients and recipes were simultaneously fed to the neighbour's cats in the evening and the results are shown in Table (11) and it was observed that all of dry cat food were consumed by the cats.

Table 1 The Nutritive Value of Prepared Fish Powder

Sr. No.	Value of Sample	Fish Powder	Literature Value* (Prepared fish powder from Tuna Trimmings)
1	Moisture Content (% w/w)	11.37±0.2	4.8
2	Ash (% w/w)	9.51±0.1	3.4
3	Protein (% w/w)	72.33	80.71
4	Crude Fat (% w/w)	0.09	-
5	Crude Fibre (% w/w)	4.08	5.7
6	Carbohydrates	2.31	5.39
7	Energy Value (kcal/100 g)	299.37	344.4

(*) Abbey et al., (2016)

Table 2 Effect of Steaming Time on the Characteristics of Dry Cat Food (Fish and Wheat)

Sr. No.	Sample	Steaming Time (min.)	Moisture Content (% w/w)	pH	Yield (%)	Observations
1	WST_1	10	5.75±0.25	6	93.5	Relatively weak fishy smell, brown colour
2	WST_2	15	6.55±0.25	6	93.5	Moderately strong fishy smell, brown colour
3	WST_3	20	7.5±0.3	6	93.5	Moderately strong fishy smell, brown colour
4	WST_4	25*	8.25±0.25	6	93.5	Attractable fishy smell, brown colour
5	WST_5	30	8.88±0.2	6	93.5	Attractable fishy smell, brown colour
6	WST_6	35	11.5±0.1	6	88.1	Attractable fishy smell, brown colour

(*) Most suitable condition

Table 3 Effect of Ingredients Composition Ratio on the Characteristics and Cat Feeding of Extruded Dry Cat Food (Fish and Wheat)

Sr. No.	Sample	Weight of fish powder to wheat flour based 100 g	Moisture Content (% w/w)	Ash (% w/w)	Protein (% w/w)	pH	Yield (%)	Cat Feeding (%)
1	HFW_1	10:90	9.85±0.15	12.5±0.5	ND	6	89	28.1
2	HFW_2	20:80	9.6±0.3	12.5±0.5	24.65	6	89	69.5
3	HFW_3*	30:70	9.6±0.3	13.5±0.5	33.16	6	94	76.6
4	HFW_4	40:60	9.9±0.1	14.5±0.3	34.27	6	94	64.2
5	HFW_5	50:50	9.8±0.1	14.5±0.5	39.47	6	94	100
6	HFW_6	60:40	9.65±0.05	15.5±0.5	48.28	6	89	83.7
7	HFW_7	70:30	9.45±0.35	15.8±0.2	53.15	6	89	68.4

(*) Most suitable sample

Table 4 Effect of Steaming Time on the Characteristics of Dry Cat Food (Fish and Corn)

Sr. No.	Sample	Steaming Time (min.)	Moisture Content (% w/w)	pH	Yield (%)	Observations
1	CST_1	10	10.2±0.2	5.8	84	Relatively weak fishy smell, brown colour
2	CST_2	15	10.5±0.1	5.8	91	Moderately strong fishy smell, brown colour
3	CST_3	20	10.3±0.2	5.9	91	Moderately strong fishy smell, brown colour
4	CST_4	25*	10.4±0.1	5.9	93	Attractable fishy smell, brown colour
5	CST_5	30	11.8±0.4	6.1	94	Attractable fishy smell, brown colour
6	CST_6	35	12.3±0.9	6.1	89	Attractable fishy smell, brown colour

(*) Most suitable condition

Table 5 Effect of Ingredients Composition Ratio on the Characteristics and Cat Feeding of Extruded Dry Cat Food (Fish and Corn)

Sr. No.	Sample	Weight of fish powder to Corn flour based 100 g	Moisture Content (% w/w)	Ash (% w/w)	Protein (% w/w)	pH	Yield (%)	Cat Feeding (%)
1	HFC_1	10:90	9.1±0.1	9±0.1	ND	6	95	57
2	HFC_2	20:80	9.6±0.1	10±0.1	12.46	6	95	98
3	HFC_3	30:70	9.8±0.2	11.3±0.1	18.93	6	94	94
4	HFC_4	40:60	9.3±0.2	12.3±0.1	28.23	6	90	93
5	HFC_5*	50:50	9.4± 0.1	12.7±0.1	33.88	6	90	100

ND = Not determined

(*) Most suitable sample

Table 6 Effect of Steaming Time on the Characteristics of Dry Cat Food (Fish and Soybean)

Sr. No.	Sample	Steaming Time (min.)	Moisture Content (% w/w)	pH	Yield (%)	Observations
1	SST_1	10	7.5±0.15	6	91	Relatively weak fishy smell, brown colour
2	SST_2	15	7.8±0.1	6	91	Moderately strong fishy smell, brown colour
3	SST_3	20*	7.9±0.1	6	95	Attractable fishy smell, brown colour
4	SST_4	25	8.1±0.13	6	93	Attractable fishy smell, brown colour
5	SST_5	30	8.3±0.15	6	93	Attractable fishy smell, brown colour
6	SST_6	35	7.6±0.1	6	93	Attractable fishy smell, brown colour

(*) Most suitable condition

Table 7 Effect of Ingredients Composition Ratio on the Characteristics and Cat Feeding of Extruded Dry Cat Food (Fish and Soybean)

Sr. No.	Sample	Weight of fish powder to Soybean flour based 100 g	Moisture Content (% w/w)	Ash (% w/w)	Protein (% w/w)	pH	Yield (%)	Cat Feeding (%)
1	HFS_1	10:90	8.8±0.8	14±0.1	38.18	6	90	92
2	HFS_2*	20:80	10± 0.05	13± 0.1	41.50	6	92	94
3	HFS_3	30:70	10.2±0.02	13±0.1	45.51	6	92	54
4	HFS_4	40:60	10.9±0.05	12±0.07	47.33	6	93	54
5	HFS_5	50:50	9.8±0.2	11±0.05	51.01	6	93	89

(*) Most suitable sample

Table 8 Comparison of the Nutritive Value of Suitable Dry Cat Food (Fish and Cereals)

Sr. No.	Value of Sample	Fish + Wheat (HFW_3)	Fish + Corn (HFC_5)	Fish + Soybean* (HFS_2)
		30 g : 70 g	**50 g : 50 g**	**20 g : 80 g**
1	Moisture (% w/w)	9.6± 0.3	9.4±0.1	10± 0.05
2	Ash (% w/w)	13.5±0.5	12.7±0.1	13± 0.1
3	Protein (% w/w)	33.16	33.88	41.50
4	Crude Fat (% w/w)	1.3	3.15	14.89
5	Crude Fibre (% w/w)	0.35	0.17	3.13
6	Carbohydrates	37.69	34.4	15.98
7	Energy Value (kcal/100g)	295.1	301.47	363.93
8	pH	6.0	5.9	6.0
9	Metabolizable Energy (ME) (kcal/kg)	2590.3	2657.6	3277.5

(*) Most suitable sample

Table 9 Effect of Different Ingredients and Recipes on Water Activity, Size Stability of the Most Suitable Dry Cat Food Using Hot Air Oven Drying

Sr.No.	Sample	Water Activity	Size Stability Percent (Drop Test)
1	HFW_3	0.32	95
2	HFC_5	0.31	98
3	HFS_2	0.30	98

Table 10 The Feline Body Mass Index (FBMI) of Experimental Cats

Sr. No.	Cats	Gender	Age (yr)	Weight of Cat (lb)	Rib Cage (cm)	Lower Hind limb Measurement (cm)	Feline Body Mass Index (Percentage Body Fat)
1	C_1	Male	4	15	43	20	24.66
2	C_2	Male	7	15	44	22	22.02
3	C_3	Male	1	10	26	14	10.92

C_1= Ahlone Township
C_2= North Dagon Township
C_3= Kamaryut Township

Table 11 Effect of Different Ingredients and Recipes on Neighbour's Cats Feeding of the Most Suitable Dry Cat Food Using Hot Air Oven Drying

Sr. No.	Sample	Cat Feeding (%)	
		C_2	C_3
1	HFW_3	100	100
2	HFC_5	100	100
3	HFS_2	100	100

C_2= North Dagon Township
C_3= Kamaryut Township

Figure 4 Dry Cat Food (Fish and Wheat)

Figure 5 Dry Cat Food (Fish and Corn)

Figure 6 Dry Cat Food (Fish and Soybean)

Conclusion

The aim of this research was to investigate the process development on the production of dry cat food based on factory wastes fish by converting to acceptable products for cat consumption and import substitution. This research gave practically how various low grade fish resources could be processed to high grade protein fish powder and dry cat food. In Myanmar, the cat owners usually feed home-made diets. Hence, dry cat food was intended to produce from fish by incorporating cereals such as wheat, corn and soybean. The protein content of dry cat food was 33.16 % from fish and wheat, 33.88% from fish and corn, 41.5 % from fish and soybean. It can be concluded that soybean was the suitable cereal for the preparation of dry cat food because it gave the highest protein content.

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STUDIES ON THE QUALITY IMPROVEMENT OF FERMENTED FISH SAUCE BY TREATING WITH ADSORPTION TECHNIQUE

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Abstract

Fish sauce, salt-fermented product, is an economically important fishery product in most Southeast Asian countries. Its manufacturing processes have been conducted with various species of fish based on salt to fish ratios. In this research, the appearance and taste of fish sauce, was improved to reach the export quality product by treating with two effective methods, adsorption mechanism and high membrane technology. The characteristics of coconut coir pith activated carbon were determined and it was used as adsorbent. According to the SEM images, processed activated carbon had mesopore suitable for dye removal and micropores which were important for adsorption mechanism. The physico-chemical properties of processed fish sauce such as protein content, salt content, pH, turbidity, colour and total solids content before and after treatment by adsorption have been analyzed. Selected adsorbent, coconut coir pith activated carbon provided the colour bleaching efficiency 40.70 % and sodium chloride reducing efficiency 8.82 % in the treatment of fish sauce. After membrane filtration, turbidity and deep brown colour of fish sauce were distinctly reduced and clear fish sauce was obtained but dissolved solids that had less than (0.1 to 0.01) μm pore size cannot be removed by membrane filtration. So it was obviously seen that lignocellulosic adsorbent could effectively improve the quality of fish sauce than ultrafiltration.

Keywords: fish sauce, activated carbon, adsorption, membrane filtration

Introduction

To a large number of world's population, fish is one of the most important sources of dietary protein. Many fermented fish products are prepared in different parts of the world and the method of processing depends upon various factors, viz., availability of raw materials, consumer's preference and the climatic conditions of the region. Among other fermented fish products, fish sauce[1] can be made cheaply from various kinds of fish, which are not normally used for food. Fish sauce is a brown, liquid seasoning commonly used in Southeast Asia countries (Jones, N.R., 1962). In Myanmar, fish sauce is prepared by different ways. Fish sauce called Ngan Pya Ye is the main ingredient in Myanma dishes. Traditionally, fish sauce is produced by mixing 1:2 or 3 ratios of salt and fish and fermenting at ambient temperature (30 to 40) °C for 6 to 12 months or longer. Thus, this study analyzed the changes in the physico-chemical characteristics of processed fish sauce from snakehead mudfish with different fish to salt ratios during fermentation period. High salt content of fish sauce is leading to a health problem. And then, dark colour and strong smell of fish sauce is unattractive to the customer. So, the quality of Myanma traditional fish sauce was promoted by adsorption mechanism in this research.

Adsorption has gained importance as a purification, separation and recovery process on industrial scale. Activated carbon is perhaps one of the most widely used adsorbents in industry for environmental applications. Due to the high production cost of synthetic adsorbents, there is a

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need for an alternative material that cost less, renewable and environmentally friendly (http: //www.ijerst.com/currentissue. php).

Basically there are two different processes for the preparation of activated carbon, the so-called physical or thermal and chemical activation. Chemical activation has several advantages than physical activation (Gottipati, 2012). Fish sauce, Myanma traditional product was improved to reach the export quality product by treating with activated carbon. An attempt was made to get standardized quality fish sauce by applying high membrane technology. The focus of this research is to explore the feasibility of agricultural waste based activated carbon being utilized in improvement of processed fish sauce.

Materials and Methods

Materials

To improve the quality of fish sauce, fish sauce was produced by mixed with Nga-Pa-Naw (*Channa burmanica*) and 30 % salt. For adsorption mechanism, coconut coir pith activated carbon was applied as adsorbent and ultrafiltration process was used in membrane filtration.

Methods

Analysis of Fish Sauce

Characteristics of fish sauce (pH, total nitrogen content, protein content and sodium chloride content) were analyzed.

Analysis of Adsorbent

Physico-chemical properties (moisture, ash, volatile matter, fixed carbon content and bulk density) of coconut coir pith activated carbon were investigated. Moisture content of activated carbon was determined using ASTM D 2867-09. The volatile matter content was determined by ASTM D 5832-98.

Characteristics of activated carbon such as pH, surface area (Okibe, F.G., *et al*, 2013), iodine sorption capacity and methylene blue number (Gottipati, 2012) were studied**.** Prepared activated carbon was characterized by using SEM and XRD techniques.

Treatment of Fish Sauce by Adsorption Mechanism

Various ratios of fish sauce to activated carbon were used at room temperature (29 -31) °C for 1 hr. with stirring (200 rpm). The appropriate ratio of activated carbon to fish sauce was determined and the appropriate temperature and time for the reduction of salt content were further studied. The changes in physico-chemical properties of treated fish sauce were also analyzed. Effects of activated carbon to fish sauce ratio, agitation temperature and time on the reduction of sodium chloride content were determined.

Treatment of Fish Sauce by Membrane Technology

In this research, ultrafiltration membrane (0.01 to 0.1μm) was used in the treatment of fish sauce. Processed fish sauce by natural fermentation was treated with ultrafiltration. 30 min suction time and aeration time, 2 min were applied to complete one filtration cycle.

Figure 1 Treatment of Fish Sauce by Ultrafiltration Membrane Process Assembly Results

Results

Table 1 Physico-chemical Properties of Fish Sauce

Sr. No	Properties	Fish Sauce	Golden Boat Fish Sauce (Thailand Product)
1.	Protein content (% w/w)	20.32 ± 3.45	13.3 ± 4.66
2.	Total nitrogen content (% w/w)	2.45 ± 0.42	2.12 ± 0.03
3.	Fibre content (% w/w)	0.5	0.2
4.	Lipid content (% w/w)	0.393 ± 1.02	0.421 ± 0.1
5.	Salt content (% w/w)	28.77 ± 2.98	27.22 ± 4.55
6.	Ash content (% w/w)	12.38 ± 0.57	10.13 ± 1.01
7.	pH	6.5 ± 3.41	5.7 ± 2.61
8.	Colour (at 420 nm)	1.462 ± 1.224	0.678 ± 1.961

Table 2 Microbial Analysis of Fish Sauce

Sr. No	Examination	Fish Sauce	Golden Boat Fish Sauce (Thailand Product)
1.	Yeast and mould counts (cfu/L)	2×10^3	4×10^3
2.	Total plate count (cfu/L)	15×10^3	16×10^4
3.	*E. coli*	ND	ND
4.	Bacterial species	*Aspergillus niger, Aspergillus oryzae*	-

Table 3 Physico-chemical Properties of Activated Carbon

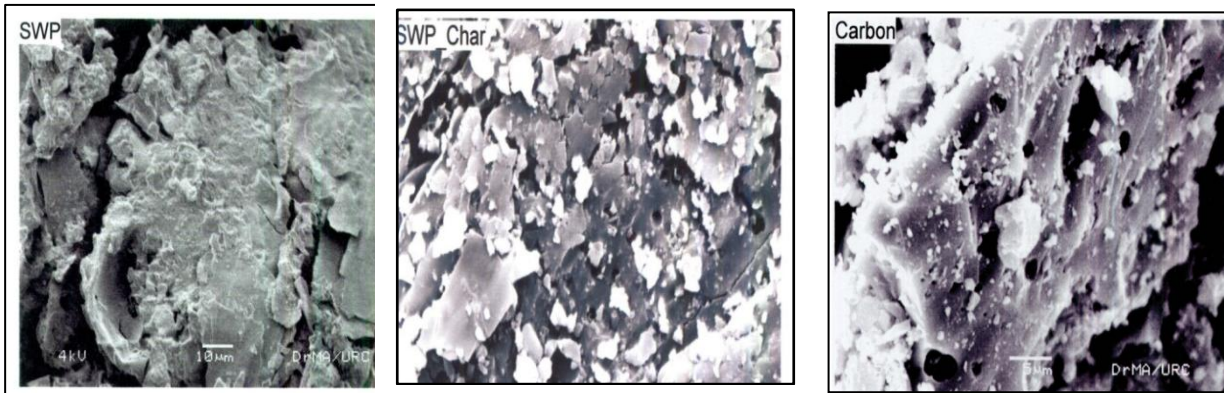
Sr. No	Physico-chemical Properties (%w/w)	Coconut Coir Pith Activated Carbon	Medicinal Charcoal (Food Grade, Germany)	Commercial AC (China Product)
1.	Moisture content	9.45	9.12	16.12
2.	Ash content	7.58	6.84	8.84
3.	Volatile matter content	5.19	4.70	7.70
4.	Fixed carbon content	77.78	79.34	67.34

Shelf-life of processed activated carbon = 3 years at (30 -33) °C

Table 4 Characteristics of Coconut Coir Pith Activated Carbon

Sr. No	Characteristics	AC-CCP	Medicinal Charcoal (Food Grade, Germany)	Commercial AC (China Product)
1.	pH	6.5	6	5.9
2.	Surface area (m^2/g)	1376	1362	1405
3.	Bulk density (g/cm^3)	0.51	0.63	0.83
4.	Iodine sorption capacity (%)	88.12	86.5	80.9
5.	Methylene blue number (mg/g)	380	395	365

AC-CCP = Coconut Coir Pith Activated Carbon



(a) **(b)** **(c)**

Figure 2 SEM Images of (a) Coconut Coir Pith Activated Carbon
(b) Commercial Activated Carbon (China Product)
(c) Medicinal Charcoal (Food grade, Germany)

Table 5 Effect of Solid to Liquid Ratio on the Abatement of Colour and Sodium Chloride Content of Fish Sauce Treated by Activated Carbon

Sr. No	Activated Carbon : Fish Sauce (% w/v)	Fish Sauce Treated by AC-CCP			
		Colour Absorbance(at 420 nm)		NaCl(% w/w)	
		After Treatment by AC-CCP	Bleaching Efficiency (%)	After Treatment by AC-CCP	Salt Reducing Efficiency (%)
1.	1:100	1.119	23.46	27.01	6.12
2.	2:100	1.012	30.78	26.52	7.82
3.	3:100*	0.959	34.40	26.28	8.65
4.	4:100	0.964	34.06	26.38	8.31
5.	5:100	0.965	33.99	26.36	8.38

* Most suitable condition

Table 6 Effect of Temperature on the Abatement of Colour and Sodium Chloride Content of Fish Sauce Treated by Activated Carbon

Sr. No	Agitation Temperature (°C)	Fish Sauce Treated by AC-CCP			
		Colour Absorbance (at 420 nm)		NaCl (% w/w)	
		After Treatment by AC-CCP	Bleaching Efficiency (%)	After Treatment by AC-CCP	Salt Reducing Efficiency (%)
1.	30*	0.959	34.40	26.28	8.65
2.	40	0.961	34.27	26.47	7.99
3.	50	0.973	33.44	27.68	3.79
4.	60	1.244	14.91	28.80	-
5.	90	1.679	-	30.93	-

* Most suitable condition

Table 7 Effect of Agitation Time on the Abatement of Colour and Sodium Chloride Content of Fish Sauce Treated by Activated Carbon

Sr. No	Agitation Time (min)	Fish Sauce Treated by AC-CCP			
		Colour Absorbance (at 420 nm)		NaCl (% w/w)	
		After Treatment by AC-CCP	Bleaching Efficiency (%)	After Treatment by AC-CCP	Salt Reducing Efficiency (%)
1.	30	1.142	21.89	27.49	4.45
2.	60	0.959	34.40	26.28	8.65
3.	90*	0.836	42.82	26.18	9.00
4.	120	0.848	42.00	26.24	8.80
5.	150	0.948	35.16	26.82	6.78

* Most suitable condition

Table 8 Physico-chemical Properties of Fish Sauce before Treatment, after Treatment and Commercial Product

Sr. No	Properties	Fish Sauce			Golden Boat Fish Sauce (Thailand product)
		Before Treatment	After Treatment by AC-CCP	After Treatment by Membrane Filtration	
1.	Ash content (% w/w)	12.38±0.57	9.43±2.1	9.76±5.12	10.13±1.01
2.	Protein content (% w/w)	20.32±3.45	19.63±4.86	15.12±2.88	13.3±4.66
3.	Total nitrogen content (% w/w)	3.25±0.42	3.14±2.88	2.42±0.32	2.12±0.03
4.	Fibre content (% w/w)	0.5	0.4	0	0
5.	Lipid content (% w/w)	0.393±1.02	0.298±1.42	0.367±0.12	0.421±0.1
6.	Salt content (% w/w)	28.77±2.98	26.18±3.89	28±3.27	27.22±4.55
7.	pH	6.5±3.41	6.7±2.56	5.8±2.81	5.7±2.61
8.	Colour (absorbance at 420 nm)	1.462±0.014	0.836±0.016	0.612±4.13	0.678±1.961

Table 9 Changes in the Physical Properties of Fish Sauce by Treating with Adsorbent and Membrane Filtration

Sr. No	Properties	Fish Sauce before Treatment	Treated Fish Sauce by Activated Carbon	Treated Fish Sauce by Membrane Filtration
1.	Turbidity (NTU)	82±2.09	31±1.34	35±2.23
2.	Total solids (mg/L)	743± 2.89	589± 2.49	628±3.89
3.	Suspended solids (mg/L)	57± 0.18	8±0.03	16±0.13
5.	Colour (at 420 nm)	1.462±0.014	0.225±0.012	0.276±0.014
6.	Colour (TCU) (Colourimeter)	43.5±1.298	21±1.561	29.5±0.871
7.	Colour (Tintometer)	Red-7, Yellow-20,	Red-5 Yellow-8	Red- 5, Yellow-12

Table (10) Microbial Analysis of Fish Sauce (before Treatment, after Treatment and Commercial Product)

Sr. No	Microorganisms	Fish Sauce			
		Before Treatment	Treated Fish Sauce by AC-CCP	Treated Fish Sauce by Membrane Filtration	Golden Boat Fish Sauce (Thailand product)
1.	Yeast and mould counts (cfu/L)	5×10^3	2×10^3	1×10^3	4×10^5
2.	Total plate count (cfu/L)	15×10^4	14×10^3	3×10^3	16×10^5
3.	*E. coli*	ND	ND	ND	ND

(a) (b) (c)

Figure3 (a) = Fish Sauce, before Treatment
(b) = Fish Sauce after Treatment by Membrane Filtration
(c) = Fish Sauce after Treatment by Adsorbent (Activated Carbon)

Discussion

Firstly, Table (1) shows that protein and total nitrogen content of processed fish sauce were higher than Thailand fish sauce. From Table (2), it could be seen that fish sauce had 2×10^3 cfu/L of yeast and mould counts and 15×10^3 cfu/L of total plate count. Table (3) shows that properties of activated carbon were similar to the medicinal charcoal (food grade).

From Table (4), it can be observed that the surface area of AC-CCP (coconut coir pith activated carbon) was 1376 m^2/g and its methylene blue number was 380 mg/g. Methylene blue numbers and iodine sorption capacity had relationship with the pore structure of adsorbents. MB number indicates the capacity of an adsorbent to adsorb large molecular size species into its macro pores, while the iodine sorption capacity is related to the degree of micro and mesopores present in the adsorbent. The methylene blue number is a measure of mesoporosity present in activated carbon and an indicator of ability of a carbon to adsorb high molecular weight substances like dye molecules. AC-CCP had higher surface area and iodine sorption capacity, indicating that it could be suitable for colour removal.

From Figure (2), it could be observed that activated carbon consisted of more canals like structure than commercial activated carbon. In AC-CCP, surfaces were pitted and fragmented.

Effect of solid to liquid ratio on the bleaching efficiency and sodium chloride content in fish sauce are shown in Table (5). When fish sauce was treated with coconut coir pith activated carbon, the colour decreased from 1.462 to 0.959 at the ratio of 3:100 activated carbon to fish sauce. Sodium chloride content of treated fish sauce (by AC-CCP) decreased at the ratio of 3:100 at 30 ºC agitation temperature. Bleaching efficiency and salt reducing efficiency increased sharply with an increase in adsorbent dose due to the availability of more adsorbent sites as well as greater availability of specific surfaces of the adsorbents. And then, beyond a certain dose, reducing efficiencies rapidly decreased with increase in adsorbent dose in all treatments.

Effect of temperature on the bleaching efficiency and sodium chloride content in fish sauces at the most suitable solid to liquid ratio (3:100) by AC-CCP at 200 rpm agitation rate for 60 min agitation time were determined and data are presented in Table (6). It was observed that bleaching efficiency and sodium chloride content decreased as the temperature was increased in fish sauces. So, 30 ºC was the most suitable agitation temperature on the bleaching efficiency and salt reducing efficiency of fish sauces.

Effect of agitation time on the bleaching efficiency and sodium chloride content of fish sauce at the most suitable solid to liquid ratio are presented in Table (7). It was found that the salt content of fish sauce decreased from 28.77 % to 26.18 % and the highest colour removal percentage, 42.82 % was observed at 90 min agitation time. In the bleaching efficiency and salt content of fish sauce treated by activated carbon, the most suitable conditions were observed at the ratio of 3:100 coconut coir pith activated carbon to fish sauce at 30 °C for 90 min agitation time.

The quality of treated fish sauces by two methods were compared to Golden Boat fish sauce (Thailand Product). The data are presented in Table (8). It can be seen that ash content of fish sauce reduced from 12.38 ± 0.57 % w/w to 9.43 ± 2.1 % w/w by the adsorption with AC-CCP. Among the fish sauces it can be seen that fish sauce treated by AC-CCP had the least amount of salt. So, it was observed that the quality of treated fish sauce had 19.63 ± 4.86 % w/w

protein content, 9.43 ± 2.1 % w/w minimum ash content, 3.14 ± 2.88 % w/w total nitrogen content and 26.18 ± 3.89 % w/w salt content, respectively.

Physical properties of treated fish sauces are presented in Table (9). After treatment, the colour of treated fish sauce changed from dark brown to golden yellow and the odour reduced significantly. Coconut coir pith activated carbon could improve the quality of fish sauce because it possessed effective micropores, mesopores and high adsorption intensity which were confirmed by SEM. Bellona (2012) stated that ultrafiltration process could not remove dissolved particles smaller than 0.1μm (pore size). Colour of processed fish sauce gradually increased due to Maillard reaction. Ultrafiltration could not reduce the dissolved sugar (0.0001 μm) in fish sauce. So, after membrane filtration, the physical properties of fish sauce were not significantly changed except turbidity. But total plate count of fish sauce was sharply reduced from 15×10^3 cfu/L to 3×10^3 cfu/L after membrane filtration. Yeast and mould counts were also decreased from 5×10^3 cfu/L to 1×10^3 cfu/L after treatment. *E. coil* was not detected in fish sauce.

Conclusion

Not only AC-CCP possessed the same characteristics of food grade activated carbon but also it occupied more micropores and mesopores and thus consequence was more advantages for abatement on colour and sodium chloride content of fish sauce. The treated fish sauce by AC-CCP had many attractive features like golden yellow colour, clear, slightly salty in flavour, and sweet aroma that was suitable for using as condiment and seasoning to complement the dish. In ultrafiltration process, colloids and microorganisms were effectively removed but it could not reduce the salt content of fish sauce. It could be concluded that the adsorption mechanism of processed activated carbon effectively improved the aroma and flavour of fish sauce to reach the standard quality.

Acknowledgements

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STUDY ON THE PREPARATION OF PAIN RELIEF BALM USING KAN-ZAW SEED OIL

Hnin Wai Lin[1], Nwe Nwe Aung[2] and Khin Thet Ni[3]

Abstract

In this research work,the dried Kan-zaw seeds were collected from Myoe Haung Village, Palaw Township, Myeik District, Tanintharyi Region. Firstly the phytochemical investigation of dried Kan-zaw seed was carried out. Secondary plant metabolites such as flavonoids, tannins, carbohydrates, saponins, phenols, glycosides, reducing sugars, terpenoids, steroids and alpha-amino acids were found to be present in Kan-zaw seed. The oil was extracted from dried Kan-zaw seed by using oil press machine. The physico-chemical characteristics of expressed Kan-zaw seed oil such as acid value, peroxide value, iodine value and free fatty acids were studied. FTIR analysis of functional groups present in the Kan-zaw seed oil was also conducted. Fatty acid profile of expressed Kan-zaw seed oil was evaluated by Gas Chromatography Flame Ionization Detection (GCFID) method. Based on the expressed Kan-zaw seed oil, pain relief balm was formulated using D-optimal mixture design of Design Expert Software trial version 11.0. Optimum formulation of pain relief balm was judged by its properties such as pH, viscosity and spreadability. Pain relief balm with pH 6.52, viscosity 11613.33 cP and spreadability 29.56 gcm/s was found as optimum composition. Skin irritation test for prepared pain relief balm was conducted in rabbit model and there were no skin rashes, inflammation and itching or redness on applied portions for 96 hours. Consumer acceptance for pain relief balm with optimum composition was studied by 9 point-hedonic scale. Total score for pain relief balm was 6.7 and it was between like slightly and like moderately.

Keywords: Kan-zaw, D-optimal mixture design, skin irritation test, hedonic scale test

Introduction

Medicinal plants have provided the modern medicine with numerous plant derived therapeutic agents (Evans, 2000). The therapeutic value of the plant depends on the active constituents present inside the different parts of the plant, which may be present in the small or large quantity. The secondary metabolites are the important substance responsible for the main medicinal properties in the crude drugs (Patel *et al*, 2018). The bioactivity of natural products is associated with the effects of various phytochemicals such as tannins, terpenoids, cardiac glycosides, saponins, flavonoids among others (Njerua *et al*, 2013).

A variety of herbs and essential oils can be used for pain and inflammation associated with sports and exercise, as well pain and inflammation associated with rheumatism, arthritis, surgery, or other medical conditions. Herbal pain relief oil is a perfect blend of oils like sesame oil, essential oils and herbs. It gives quick relief from any type of ache in the body after rubbing on the body, as a result no strains persist in the body (Chauhan *et al*, 2016).

Madhuca indica J. F. Gmel. (English Name: Indian Butter Tree, Family Sapotaceae, locally known as Kan-zaw in Myanmar. It is a tremendous therapeutic plant growing throughout the subtropical region of the Indo-Pak subcontinent and also in Tanintharyi Region, Myanmar. *Madhuca Indica* has several pharmacological activities, and potential to provide health to the

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society. It is used as anti-diabetic, antiulcer, hepatoprotective, antipyretic, antifertility, analgesic, antioxidant, swelling, inflammation, piles, emetic, dermatological, laxative, tonic, anti-burn, anti-earth worm, wound healing headache and many more problems. Madhuca oil contains fatty acids such as palmitic 21.3%, stearic 24.3%, oleic 36.7%, linoleic 15.2% and arachidic 1.3% (Dhara , 2010). Kan-zaw seed oil, obtained from plant origin, has been used as versatile oil throughout Myanmar. The present study was aimed to extract the Kan-zaw seed oil by expression method and to analyze the characteristics of the Kan-zaw seed oil and finally to formulate the pain relief balm using Kan-zaw seed oil.

Materials and Methods

Materials

Kan-zaw seeds were purchased from Myoe Haung Village, Palaw Township, Myeik District, Tanintharyi Region.

The raw materials such as beeswax, paraffin wax, vaseline, camphor and menthol were purchased from Empire Chemical Store, Pabedan Township, Yangon Region.

Methods

Extraction of Kan-zaw Seed Oil by Mechanical Expression Method

The dried Kan-zaw seeds were dehulled by hand to remove the seed-coats and then air-dried for three days and stored in air-tight plastic bag. The dried Kan-zaw seeds were expressed by using expeller (Taiwan made Best Oil Press Machine with Heater, Model No.02) between 45-50^{o}C to obtain Kan-zaw seed oil and oil cake residues separately. The expressed oil was settled and filtered to remove the residual solids.

(i) (ii) (iii)

Figure 1 (i) Kan-zaw Seed with Seed Coat (ii) Kan-zaw Seed without Seed Coat (iii) Expressed Kan-zaw Seed Oil

Identification of Kan-zaw seed and Analysis of Kan-zaw Seed Oil

Phytochemical characteristics of Kan-zaw seed powder were firstly investigated. The physico-chemical characteristics of Kan-zaw seed oil such as acid value, peroxide value, iodine value and free fatty acid were also studied. Functional group of Kan-zaw seed oil was also investigated. Fatty acid profile of Kan-zaw seed oil was evaluated by Gas Chromatography.

Preparation of Pain Relief Balm using Kan-zaw seed Oil

Basic formulation of pain relief balm was selected from the preliminary investigation of characteristics of balm before implementing the experimental design. Formulation of pain relief balm was based on the levels as shown in Table (1) and 25 samples were formulated according to the D-optimal mixture design of the Design expert software trial 11. Firstly 8.63g of beeswax, 4.76g of paraffin wax, and 54.15g of vaseline were mixed and heated with stirring in water bath at 75-80°C for 15min to get the phase I. Secondly, 11.76g of Camphor, 15.76g of menthol and 4.93g of Kan-zaw seed oil were mixed and stirred thoroughly at room temperature to obtain the phase II. The two phases were mixed at room temperature and continuously stirred until the mixture became thick. Finally, the balm formed were filled into the sterilized glass bottles during flowable condition.

Table 1 Level of Components in the Formulation of Pain Relief Balm

Sr. No.	Component	Low level (g)	High level (g)
1.	Beeswax	7	10
2.	Paraffin wax	3	7
3.	Vaseline	50	60
4.	Kan-zaw seed oil	4	6
5.	Camphor	8	16
6.	Menthol	14	18

Analysis of Prepared Pain Relief Balm

pH, viscosity, spreadability, stability, skin irritation test and consumer acceptance of prepared pain relief balm were also studied.

Results and Discussion

From the results of phytochemical investigations shown in Table(2), it can be seen that Kan-zaw seed powder contains flavonoids, glycosides, phenols, reducing sugars, tannins, saponins, carbohydrates and α - animo acids except alkaloid and cyanogenic glycoside.

Calixto *et al.,reported that* recently discovered analgesic substances include, alkaloids, flavonoids and terpenoids. According to Table (3), it was known that acid value and peroxide value of Kan-zaw seed oil were in accordance with Codex Standard. Table (4) and Figure (2) show the functional groups present in the Kan-zaw seed oil which could be assigned. According to these results, Kan-zaw seed oil may contain alkane groups, carbonyl groups, methyl groups and carboxylic acid groups. The fatty acids, retention times (RT) and peak area (%) of Kan-zaw seed oil were analysed by GCFID and the respective chromatogram and fatty acid profile are illustrated in Figures (3) , (4) and Table (5). From these results, it was known that Kan-zaw seed oil contained 56.375% oleic acid, 23.137% palmitic acid, 12.205% stearic acid and 7.299% linoleic acid as major components and trace amount of other acids such as arachidic, palmitoleic and linolenic acid. Myristoleic, palmitoleic, oleic, linolenic, linoleic acids are polyunsaturated omega fatty acids. Because of their beneficial properties on the skin it is used for anti-inflammatory, acne reductive, skin ligthtening and moisture retentive properties of the skin. Table (6) shows the formulae of 6 components and responses of pain relief balm. The properties

of pain relief balm such as pH, viscosity and spreadability were judged for the optimum formulation. Table (7) shows the optimum composition of pain relief balm predicted by design expert. This composition was judged by pH, 6.52±0.03, viscosity, 11613.33±32 and spreadability 29.56±0.65 of prepared pain relief balm. Table (8) represents the summary of ANOVA (analysis of variance) for pH, viscosity and spreadability. In fit summary of response pH, design expert suggested for quadratic model. The sequential F-test by design expert indicated that the quadratic model and F value were significant. In this case, p-value was significant and lack of fit was not significant. Similarly, for responses, viscosity and spreadability, the sequential F-test by design expert indicated that the quadratic model and F value were significant and p-value was significant and lack of fit was not significant respectively. Figures (5), (6) and (7) indicate the residual plots, contour graphs and 3D plots for pH, viscosity and spreadability of pain relief balm. From the results, it can be seen that, viscosity and spreadability were affected by beeswax, paraffin wax and vaseline. On studying the stability of pain relief balm, there was no distinct change in pH, viscosity and colour of the products during two months and the results are shown in Table (9). Skin irritation test for pain relief balm was conducted with rabbit model. There were no skin rashes, inflammation and itching or redness on applied portions for 96 hr. Investigation of consumer acceptance for pain relief balm with optimum composition was carried out by 9-point hedonic scale and results are shown in Table (10). Total score for pain relief balm was 6.7 and it was between like slightly and like moderately.

Table 2 Phytochemical Screening of Kan-zaw Seed

Sr. No.	Phytoconstituent	Test	Results
1	Alkaloid	Mayer's reagent	-
		Wagner's reagent	
		Dragendorff's reagent	
		Hager's reagent	
2	Carbohydrate	10% α naphthol &	+
		conc:H_2SO_4	
3	Glycoside	10% lead acetate solution	+
4	Phenols	1% $FeCl_3$ solution	+
5	α- amino acid	Ninhydrin reagent	+
6	Saponin	Frothing test	+
7	Tannin	1% gelatin & 10% NaCl solution	+
8	Flavonoid	Mg ribbon & conc:HCl	+
9	Steroid	Acetic anhydride & conc: H_2SO_4	+
10	Terpenoid	Acetic anhydride & conc: H_2SO_4	+
11	Reducing sugar	Fehling solution	+
12	Starch	Iodine solution	+
13	Cyanogenic glycoside	H_2O, conc: H_2SO_4	-

(+) present (-) absent

Table 3 Physico-chemical Properties of Kan-zaw Seed Oil

Sr. No.	Properties	Kan-zaw Seed Oil	Literature value*	Sr. No.	Properties	Kan-zaw Seed Oil (oil press machine)	Literature value*
1.	Relative density (at 20˚C)	0.910	0.856-0.870	6.	Acid value (mg KOH/g)	3.565	3.5
2.	Refractive index	1.464	1.452-1.462	7.	Peroxide value (mleq/peroxide oxygen/kg)	2.641	2.78
3.	Saponification value (mg KOH/g)	190.569	187-196	8.	Moisture (%w/w)	0.155	-
4.	Unsaponifiable matter (%)	1.016	1.00-3.00	9.	Aflatoxin	-	not detected
5.	Iodine value (mgI_2/g)	63.294	58.00-70.00	10.	Yield of oil content (%w/w)	32.7	-
				11.	Yield of residual oil (%w/w)	2.7	-

*R.S.Kureel and et-al (2009)

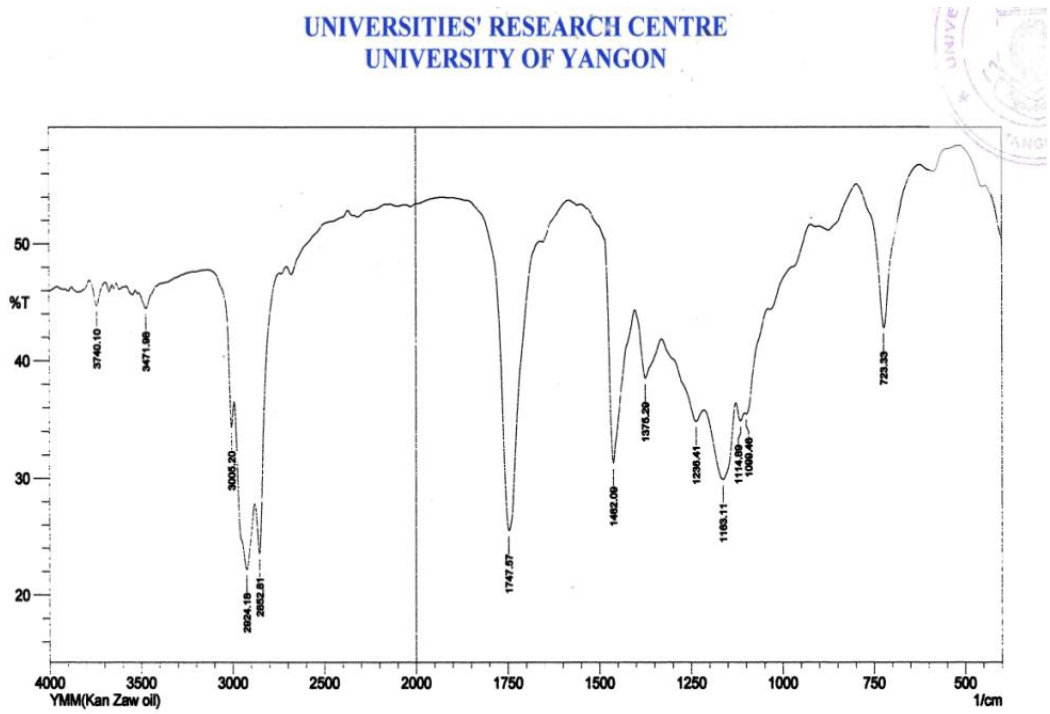


Figure 2 FTIR Spectrum of Kan-zaw Seed Oil

Table 4 Functional Group of Kan-zaw Seed Oil Analysed by FTIR

Wave number, cm^{-1}		Functional group	
Kan-zaw Seed Oil	**Literature***		
3740.10	3650	ν-OH	Stretching vibration of hydroxyl compound
3471.96	3550-3450	ν -OH	Stretching vibration of hydroxyl compound
3005.20	3100-3020	ν-CH	Stretching vibration of olefinic C-H
2924.18	2930	ν -CH	Stretching vibration of alkane
2852.81	2850	ν-CH	Stretching vibration of alkane
1747.57	1870-1650	ν-C=O	Stretching vibration of carbonyl group
1462.09	1460	δ- CH	Asymmetric bending vibration of methyl group
1375.29	1380	δ-CH	Symmetric bending vibration of methyl group
1236.41	1255-1210	δ-OH	OH in plane bending vibration
1163.11 1114.89	1200 and 1410-1310	δ-OH	OH in plane bending vibration
1099.46	1180-1080	ν -C-O	Stretching vibration of monomeric carboxylic acid
723.33	750-650	δ-OH	OH out of plane bending vibration

*Mohan, (2000)

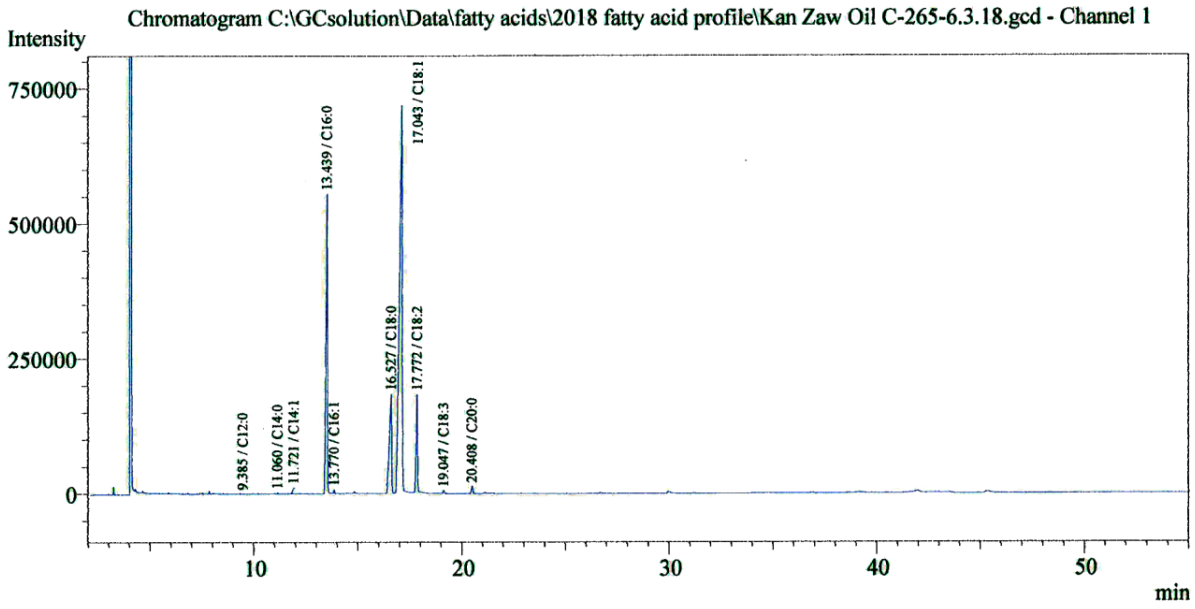


Figure 3 GC Chromatogram of Kan-zaw Seed Oil

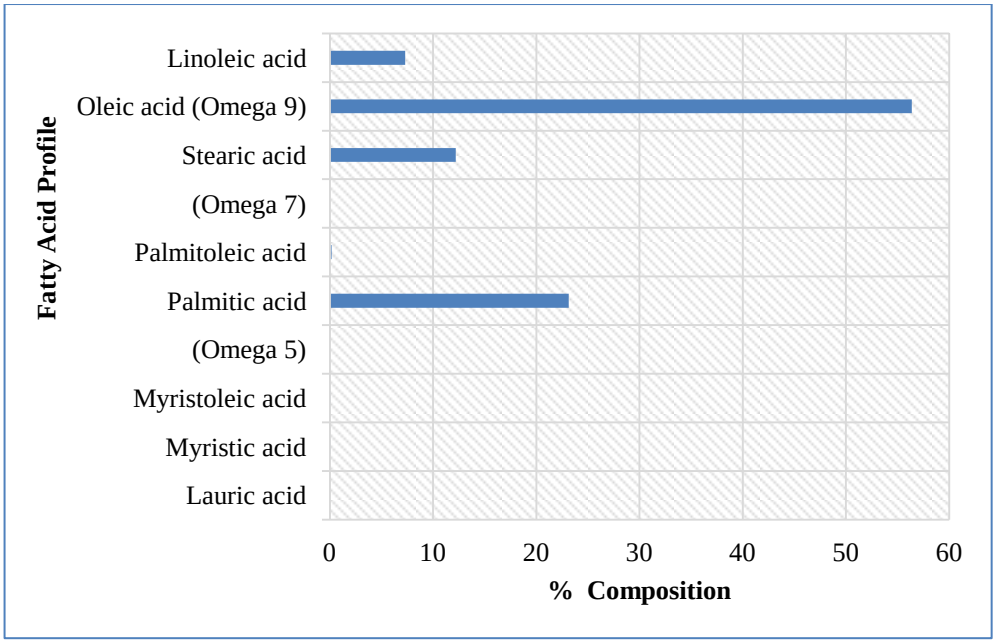


Figure 4 Fatty Acid Profile of Kan-zaw seed oil

Table 5 Fatty Acid Profile of Kan-zaw Seed Oil Analysed by GC

Sr. No.	Fatty Acid Profile	Molecular weight (g/mol)	Molecular formula*	Retention Time (min)	% Composition
1.	Lauric acid	200.322	$C_{12}H_{24}O_2$	9.385	0.01
2.	Myristic acid	228.37	$C_{14}H_{28}O_2$	11.060	0.047
3.	Myristoleic acid (Omega 5)	226.36	$C_{14}H_{26}O_2$	11.721	0.025
4.	Palmitic acid	256.43	$C_{16}H_{32}O_2$	23.137	23.137
5.	Palmitoleic acid (Omega 7)	254.41	$C_{16}H_{30}O_2$	13.439	0.162
6.	Stearic acid	284.48	$C_{18}H_{36}O_2$	13.770	12.205
7.	Oleic acid (Omega 9)	282.47	$C_{18}H_{34}O_2$	16.527	56.375
8.	Linoleic acid (Omega 6)	280.45	$C_{18}H_{32}O_2$	17.043	7.299
9.	Linolenic acid (Omega 3)	278.436	$C_{18}H_{30}O_2$	19.047	0.204
10.	Arachidic acid	312.54	$C_{20}H_{40}O_2$	20.408	0.607

*https://pubchem.ncbi.nlm.nih.gov>…

Table 6 Formulation of Pain Relief Balm

Run	Variable						Response		
	Beeswax (g)	Paraffinwax (g)	Vaseline (g)	Kan-zaw oil (g)	Camphor (g)	Menthol (g)	pH	Viscosity (cP)	Spreadability (gcm/s)
1	10.00	7.00	50.00	4.00	15.00	14.00	6.50	12560	27.77
2	10.00	3.00	51.00	6.00	16.00	14.00	6.20	11675	28.40
3	10.00	3.00	60.00	4.00	9.00	14.00	6.40	10975	27.85
4	7.00	7.00	50.00	4.00	14.00	18.00	5.90	11854	28.50
5	7.00	3.00	60.00	4.00	8.00	18.00	6.30	10650	29.20
6	10.00	4.87	56.23	4.94	8.00	15.96	6.10	11980	28.12
7	10.00	7.00	50.00	5.28	9.72	18.00	6.70	12780	27.30
8	7.97	3.00	55.28	4.79	10.96	18.00	6.60	10956	29.50
9	8.69	7.00	50.00	6.00	12.06	16.25	5.90	10675	30.45
10	7.00	3.00	56.00	4.00	16.00	14.00	6.30	12120	27.85
11	7.00	7.00	54.15	4.00	13.13	14.73	5.80	12050	27.00
12	7.00	7.00	59.56	4.44	8.00	14.00	6.40	11950	27.95
13	10.00	7.00	55.00	6.00	8.00	14.00	6.10	12750	26.25
14	7.00	6.38	54.62	6.00	8.00	18.00	5.70	12560	25.59
15	9.01	7.00	53.99	4.00	8.00	18.00	6.10	12655	26.65
16	7.00	3.00	57.36	6.00	11.52	15.12	6.50	11876	26.41
17	10.00	7.00	55.00	6.00	8.00	14.00	6.20	12945	25.75
18	7.00	3.00	50.00	6.00	16.00	18.00	5.50	10395	30.56
19	8.53	3.48	59.99	6.00	8.00	14.00	6.80	10655	30.23
20	10.00	3.21	51.55	6.00	11.24	18.00	5.60	11765	27.40
21	7.00	5.00	58.59	4.00	11.41	14.00	6.70	11654	27.90
22	10.00	3.00	50.00	4.00	15.00	18.00	6.40	10565	31.30
23	7.00	7.00	50.00	6.00	16.00	14.00	5.20	11786	28.25
24	10.00	3.00	60.00	4.00	9.00	14.00	6.10	10987	27.95
25	10.00	3.00	55.00	6.00	8.00	18.00	5.50	11905	28.20

Table 7 Predicted Formula of Pain Relief Balm

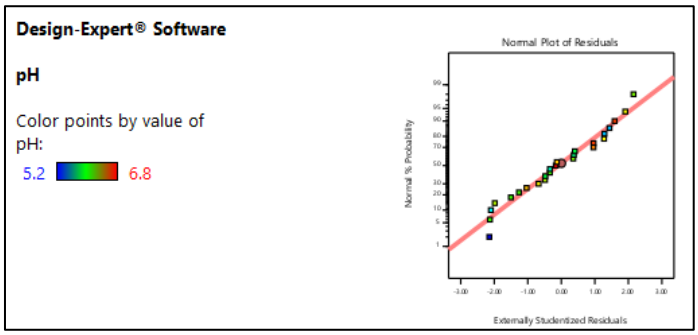
Sr. No.	Component	Composition (g)	pH	Viscosity (cP)	Spreadability (gcm/s)
1	B	8.63	6.52 ± 0.03	11613.33 ± 32	29.56 ± 0.65
2	P	4.76			
3	V	54.15			
4	KZO	4.93			
5	C	11.76			
6	M	15.76			

B = Beeswax, P = paraffin wax, V = Vaseline, KZO = Kan-zaw seed oil, C = camphor, M = menthol

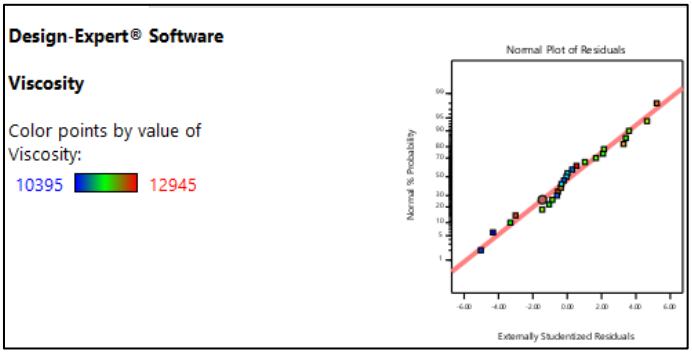
Table 8 Analysis of Variance (ANOVA) for Pain Relief Balm

Response	pH	Viscosity (cP)	Spreadability (gcm/s)
R-squared	0.9695	0.9852	0.9686
Mean squared	0.2046	2.054	2.55
P-value	0.042	0.011	0.044
F-value	6.35	11708	6.17
Pred R-squared	-8.2849	-5.6935	-13.0448

Figure 5 Pain Relief Balm



(a)



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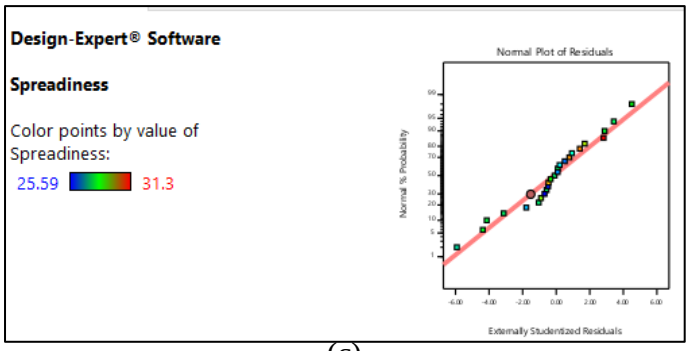
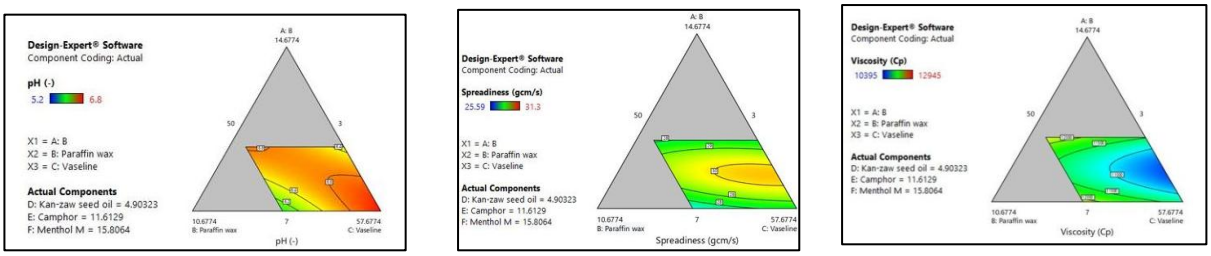


(c)

Figure 6 Residual Plots of (a) pH (b) Viscosity (c) Spreadability of Pain Relief Balm



(a) (b) (c)

Figure 7 Contour Graph of (a) pH (b) Viscosity (c) Spreadability of Pain Relief Balm

Table 9 Physico-chemical Properties of Pain Relief Balm (after 2 months)

Sr. No.	Formulation	pH	Viscosity (cP)	Colour & Appearance
1.	Pain relief balm	6.5±0.03	11578.67±17	pale yellow & non greasy

Table 10 Investigation of Consumer Acceptance for Pain Relief Balm by 9- Hedonic Scale

Panelist	1	2	3	4	5	6	7	8	9	10	Total score	Average	Overall acceptability
Odour	1	6	6	7	5	6	6	6	8	8	59	5.9	6.7
Sensation	5	7	6	8	7	7	7	8	8	8	71	7.1	
Relief of pain/aches	5	7	6	8	7	7	7	8	8	8	71	7.1	

9 =like extremely 6 = like slightly 3 = dislike moderately
8 = like very much 5 = neither like nor dislike 2 = dislike very much
7 = like moderately 4 = dislike slightly 1 = dislike extremely

Conclusion

In this research, Kan-zaw seed oil expressed by oil press machine was used to prepare the pain relief balm. The pain relief balm was formulated using D-optimal mixture design from Design Expert Software Trial version 11.0. By using design expert software, the robustness of the quality products can be obtained. The prepared pain relief balm showed significant effects in anti-inflammable activities. From this research, it can be concluded that Kan-zaw seed oil can be used safely in indigenous medicinal products and it can be a valuable potential source of new drugs due to the presence of many bioactive secondary metabolites in it.

Acknowledgements

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OPTIMIZATION OF BIOETHANOL PRODUCTION FROM WASTE MANGO USING RESPONSE SURFACE METHODOLOGY

War War Thin[1], Khin Swe Oo[2], Soe Soe Than[3]

Abstract

In the present study, waste fruit of mango (*Mangifera indica* L.); Yin Kwe`, was used as raw material for the production of bioethanol. Baker's yeast (*Saccharomyces cerevisiae*) was used for fermentation of waste mango. Response Surface methodology (RSM) based on Box-Behnken Design (BBD) was applied to optimize the strength of ethanol during bioethanol production. The process variables for the maximum strength of ethanol were 0.73 % (w/w) amount of yeast, 76.08 % (w/v) of substrate concentration and pH of 4.6, respectively. Specific gravity (sp.gr) and Gas Chromatography (GC) methods were used to measure and identify the strength of ethanol. The observed strength of ethanol 25.05±1 % (v/v) was found to be very close to the predicted value 24.11 % (v/v). The coefficient of determination, R^2 value was 0.9867 that indicates the goodness of fit for regression model. The insignificance lack of fit (p=0.118) also proved that the model fitted well to the experimental data.

Keywords: waste fruit, *Saccharomyces cerevisiae*, optimization, RSM, BBD

Introduction

The excessive consumption of non-renewable energy has greatly resulted environmental deterioration and public health problems (Kahia *et al*., 2016). This in turn has resulted in the need to find a source of renewable energy. Ethanol is an alcoholic compound that has considered a renewable bio-energy source; it is clear-colorless liquid and eco-friendly potential fuel to power automotive engines (Hossain, 2015). The fermentation of sugar and starch containing crops or byproducts from industries based on such crops could be produced approximately 80% of world supply of alcohol. However, the use of food sources such as corn, sugarcane, wheat and sugar beet as raw material has been continuously debated. Therefore, other low-cost and abundant raw materials such as rice, sugarcane baggase, agricultural and kitchen residues have been investigated as alternative substrates (Uncu and Cekmecelioglu, 2011). The cheapest and easily available source of sugary material such as waste fruits was considered for the production of bioethanol. Among the fruit crops, mango is at the fifth rank of the most significant foodstuffs after rice, corn and milk. According to a report published by Reddy and Reddy in 2005, mango contains a high concentration of sugar 16-18 % (w/v) and acids with organoleptic properties and also contains antioxidants. Sucrose, glucose and fructose are the principal sugars in ripe mango with small amount of cellulose, hemicellulose and pectin. These pulpy fruits are more prone to spoilage due to their nature and this spoilage occurs at the time of harvesting, storage, marketing and processing resulting as wastes. The production of bioethanol from these food processing wastes could be an alternative and attractive disposal of the polluting residues. In the present study, the whole waste mangoes (pulp and peel) was used as the basic raw materials for the production of bioethanol by *Saccharomyces cerevisiae*.

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Materials and Methods

Materials

The waste mangoes were collected from Mawbi Township, Yangon Region. Hydrochloric acid, sodium hydroxide (Analar grade, BDH, England), and *Saccharomyces cerevisiae* (baker's yeast) were purchased from Super Shell (Chemical Store), 27th street, Pabedan Township, Yangon Region.

Preparation of Bioethanol

For the preparation of bioethanol from waste mango, the process flow diagram was shown in Figure (1). Firstly, waste fruits were thoroughly washed with water and seeds were removed. Flesh and peels of waste fruits were sliced into small pieces and pulped using a household blender. And then, pulps were sterilized in an autoclave at 121ºC for 15 min. The fermentation of waste pulp was carried out according to Box-Behnken Design (BBD) by *Saccharomyces cerevisiae* under anaerobic condition. Different ethanol fermentation conditions such as amount of yeast, substrate concentration and pH were used as process variables for experimental design. The initial pH of substrate was adjusted by applying (1 M) hydrochloric acid and (1 M) sodium hydroxide solution. The fermentation period and temperature were limited to 4 days and room temperature of 32ºC. Ethanol was then separated from the fermented broth by simple distillation at 78 ± 1ºC. The distillate was further purified by fractional distillation at 78 ± 1ºC using fractionating column for about 3 hours.

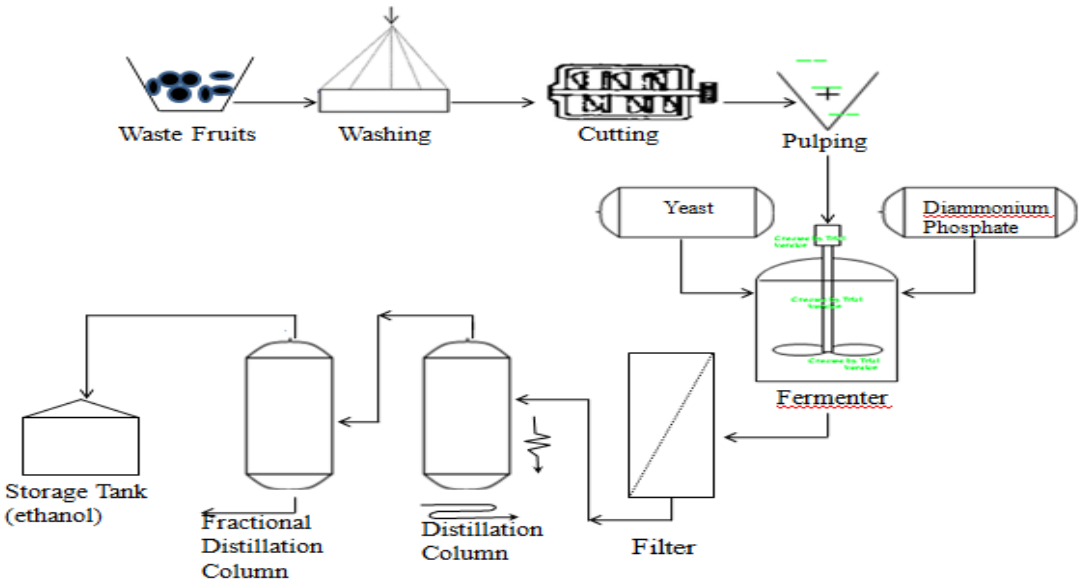


Figure 1 Process Flow Diagram for the Preparation of Bioethanol from fermentation of Waste Mango

Experimental Design

The response surface methodology (RSM) based on Box-Behnken Design was applied to estimate the number of runs and optimum conditions for three independent variables (amount of yeast, substrate concentration and pH) that effecting fermentation process. Table (1) shows the process parameters and levels for fermentation of waste mango.

Table 1 Process Parameters and Levels for Fermentation of Waste Mango

Sr. No.	Parameters	Coded	Levels	
			Low	High
1	Amount of Yeast %(w/w)	X_1	0.4	1.2
2	Substrate Concentration % (w/v)	X_2	50	100
3	pH	X_3	3.5	5.5

Determination of Ethanol Strength

Determination of Strength of Ethanol by Gas Chromatography (GC)

Ethanol strength was determined by gas chromatography by using GC 2010 SHIMADZU equipped with flame ionization detector (FID) at the laboratory of Amtt Co., Ltd. The sample was taken and gathered into a syringe and then injected into an injector port on the device. The temperature of the injector port must be in excess of the boiling point for the sample to obtain accurate readings. This allowed ethanol to convert into gas, which was then pushed into the filters by nitrogen carrier gas. As the gases were passed through the filters, the compound were identified by electronic detector and the alcohol content was then determined.

Analysis of Physicochemical Properties of Bioethanol

Determination of Specific Gravity (sp.gr)

The strength of ethanol was measured by specific gravity. The prepared ethanol sample 100 ml was weighed and filled into a 500 g round bottom flask. 50 ml of distilled water was added into it. The liquid was distilled until approximately 100 ml of solution was obtained. The ethanol sample was then cooled to 15ºC and the specific gravity of the ethanol was measured at 20ºC using a specific gravity bottle. A clean and previously weighed specific gravity bottle was used for this purpose. The specific gravity of the distilled water was also measured using a specific gravity bottle. After the density of the ethanol was determined from the ratio of the weight of liquid held in specific gravity bottle and the weight of water held in specific gravity bottle, ethanol content by volume from specific gravity at 20ºC was read from the table that tabulates the ethanol by volume at 15.56ºC from apparent specific gravity at 20ºC (Lees, 1975).

Determination of pH

The pH of the prepared sample was determined by using a digital pH meter (pH 300, HANNA, China). The glass electrode assembly was first calibrated by using buffer solutions of pH 4 and pH 7 and the electrode was adjusted to those values. After that, pH of the sample

Determination of Total Acidity

10 ml of bioethanol was put in a conical flask with a pipette and two drops of phenolphthalein was then added. It was titrated with 0.1 N sodium hydroxide solution from a burette until the end point was reached. The above procedure was carried out in triplicate. The acidity was calculated as follows:

$$\text{Total acidity as acetic acid } (\%) = \frac{\text{Titre} \times \text{Normality of NaOH} \times 0.006005}{\text{Volume of sample taken}} \times 100$$

1 cm^3 of 0.1 N of NaOH = 0.006005 g of acetic acid

Determination of Refractive Index

The refractive index of the bioethanol was measured by refractometer (Shibuya Optical Co., Ltd, Tokyo, Japan) at the Food Industries Development Supporting Laboratory (FIDSL), UMFCCI Tower, Lanmadaw Township, Yangon Region.

Results and Discussion

The experimental design and statistical analysis for fermentation of waste mango were performed according to the Box-Behnken Design of RSM using MINITAB Software (Version 18.1). (15) experimental runs were conducted according to Box-Behnken Design as tabulated in Table (2). All experiments were carried out in a randomized order to minimize the effect of unexpected variability in the observed response due to extraneous factors. Significance of each coefficient was determined by ANOVA using the resulting experimental data.

Table 2 The Observed values for Fermentation of Waste Mango

Run No.	Amount of Yeast % (w/w)	Substrate Concentration % (w/v)	pH	Strength of Ethanol % (v/v) Measured by Sp.gr
1	0.8	50	5.5	21.98
2	0.8	100	5.5	25.05
3	1.2	100	4.5	26.72
4	1.2	75	3.5	21.89
5	0.8	75	4.5	24.05
6	0.4	75	5.5	20.12
7	0.8	75	4.5	24.05
8	0.8	100	3.5	23.39
9	0.4	75	3.5	27.79
10	1.2	75	5.5	28.35
11	1.2	50	4.5	23.12
12	0.4	50	4.5	27.21
13	0.8	75	4.5	24.42
14	0.4	100	4.5	21.76
15	0.8	50	3.5	26.35

Based on the ANOVA results in Table (3), the statistically significance of coefficient were determined with a confidence interval greater than 95% ($p<0.05$). Hafid *et al*., (2011) stated that the smaller the p-value, the higher the significance of each variable because p-value represents the significance of variables. The results of ANOVA table revealed that the model was highly reliable with significant linear and interaction effects ($p<0.05$). Puligundla Pradeep *et al*., (2012) reported that the insignificance of the model terms ($p>0.05$) implies the factors have a more influence on the production of alcohol and changes in those variables will significantly affect the process. Moreover, the fitting of the experimental data to the regression model were checked by the coefficient of determination, R^2. Since the coefficient of determination, R^2 value was 0.9867, it is indicated that at least 98% of the total variation could

be explained by the model (Grahovac *et al.*, 2012) and revealed the good agreement between experimental and predicted values. Haaland (1989) and Chauha, *et al*., (2004) have explained on the acceptance of any model with $R^2 > 0.75$. The insignificance lack of fit (p=0.118) also proved that the model fitted well to the experimental data.

Table 3 Analysis of Variance (ANOVA) for Fermentation of Waste Mango

Source	Sum of Squares (SS)	Degree of Freedom (DF)	Mean Square (MS)	F-value	P-value
Model	84.2957	9	9.3662	41.35	0.000
Linear	3.6924	3	1.2308	5.43	0.050
Square	0.7139	3	0.2380	1.05	0.447
2-Way Interaction	79.8894	3	26.6298	117.58	0.000
Error	1.1324	5	0.2265		
Lack-of-fit	1.0412	3	0.3471	7.61	0.118
Pure Error	0.0913	2	0.0456		
Total	85.4281	14			
R-Squared	0.9867				
Adjusted R-Squared	0.9629				

To determine the model satisfies the assumptions of the analysis of variance (ANOVA), the normal plot with residue was analyzed. In the normal probability plot of the raw data, the analysis of variance shows more effective (straightforward) relationship with the residuals. The quadratic polynomial model satisfies the assumptions analysis of variance (ANOVA) i.e. the error distribution is approximately normal (Figure 2).

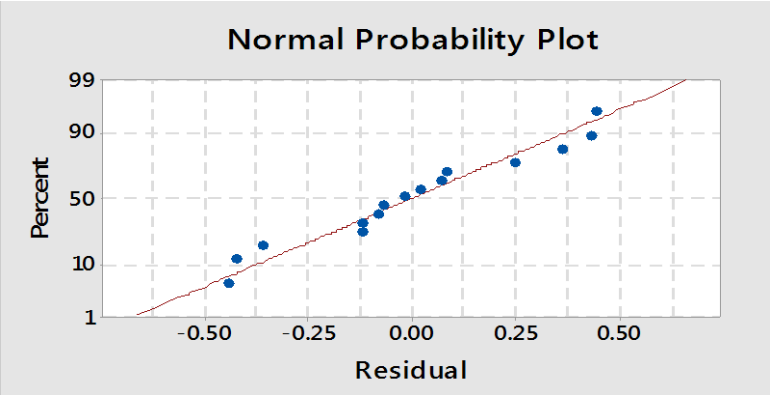
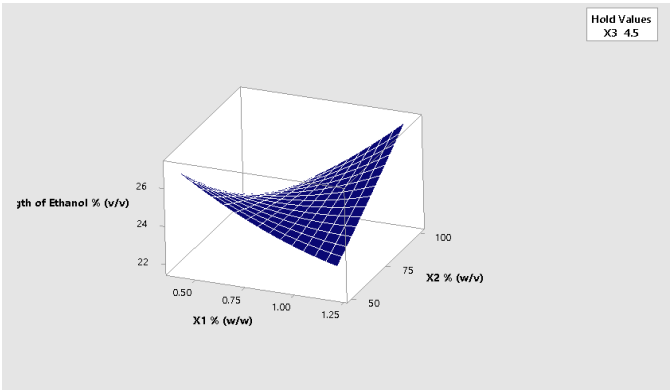


Figure 2 Normal Plot of Residual for Waste Mango

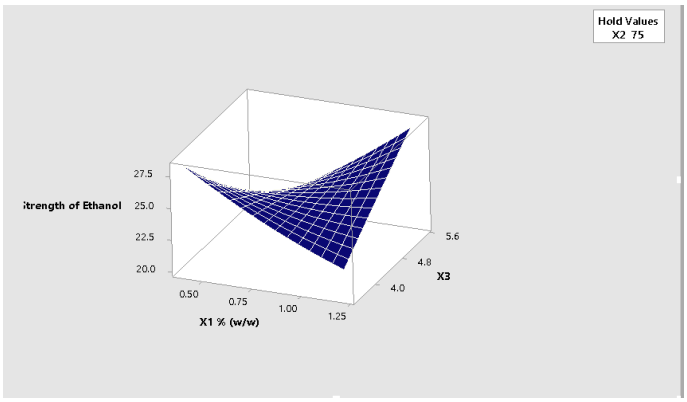
Using the results of experiments, the regression model of strength of ethanol for waste mango is given in equation (1).

$$\text{Strength of ethanol \% (v/v)} = 24.173 + 0.411X_1 - 0.229X_2 - 0.490X_3 + 0.426X_1^2 + 0.081X_2^2 - 0.062X_3^2 + 2.285\ X_1\ X_2 + 3.533\ X_1X_3 + 1.508X_2X_3 \quad \text{.....Eq. (1)}$$

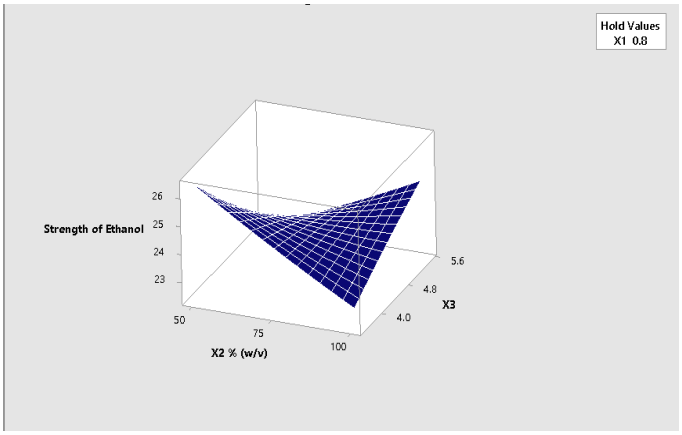
Where X_1, X_2 and X_3 are the coded values of the process variables; amount of yeast, substrate concentration and pH. The sign and magnitude of the coefficients indicate the effect of the variable on the response. The interactive terms of all variables in equation (1) indicated that positive effect on the strength of ethanol. Positive sign of the coefficient means increase in response when the level of the variable is increased while negative sign indicated decrease in the response (Montgomery, 2004). Similarly, the quadratic terms X_1^2 and X_2^2 have positive effect but X_3^2 has negative effect on the response. By using response surface 3D plots, the interaction between two variable factors and their optimum levels could be easily understood. Figure 3 (a, b and c) shows the maximum positive contribution of amount of yeast, substrate concentration and pH on the strength of ethanol during fermentation. Figures (a and b) revealed that the strength of ethanol decreased with increasing amount of yeast, independent of substrate concentration and pH. This may be due to high amount of yeast can adversely affect ethanol production because high increase of yeast level decreases the viability of yeast population and causes inadequate development of ethanol production (Powchinda *et al*., 1999). By increasing the level of pH from 4.0 to 5.6 in Figures (b and c) the strength of ethanol increased gradually. According to the results, the response surface suggests that pH and amount of yeast was a dominance interaction factor on the strength of yeast during bioethanol production from waste mango.



(a)



(b)



(c)

Figure 3 Response Surface Plots for fermentation of Waste Mango (a) Substrate Concentration and Amount of Yeast (b) pH and Amount of Yeast (c) pH and Substrate Concentration

The optimum values of the selected process variables and strength of ethanol were calculated from equation (1) by using MATLAB software. The observed experimental values and values predicted by the equations of the model are presented in Table (4).When compared the strength of ethanol, the predicted value 24.11 % (v/v) was closely agreed with the experimental value of 25.05 ± 1 % (v/v). Similar results were reported by Karuppaiya *et al*., (2009). Under optimum conditions, the variables substrate composition of 62 %(v/v), pH 5.5, incubation temperature 32ºC and fermentation time 37 hrs were utilized to obtain the maximum ethanol concentration of (12.64 g/l) from waste cashew apple juice by *Zymomonas mobilis.* Sasikumar *et al*., (2010) also reported that the maximum response for ethanol production was achieved under the optimum conditions at temperature 32ºC, pH 5.6 and fermentation time 110 hrs. The strength of ethanol identified by gas chromatography (GC) was shown in Table (5) and Figure (4). According to the GC analysis, 86.27% (v/v) strength of ethanol was obtained from second distillate. Some physicochemical properties of bioethanol such as specific gravity, pH, total acidity, refractive index and physical appearance were shown in Table (5). The results revealed that the properties of bioethanol from waste mango met some of the properties of standard bioethanol except the strength of ethanol (global biofuels, 2014).

Table 4 Optimum Process Conditions for Fermentation of Waste Mango

Sr. No.	Parameters	Values	Strength of Ethanol % (v/v) Analyzed by Sp.gr	
			Predicted Value	Experimental Value
1	Amount of Yeast % (w/w)	0.73	24.11	25.05±1
2	Substrate Concentration % (w/v)	76.08		
3	pH	4.6		

Table 5 Physicochemical Properties of Bioethanol after Fractional Distillation

Sr. No.	**Properties**	**Ethanol** (from waste mango)	**Literature Value*** **(**Anhydrous Ethanol**)**
1	Ethanol strength (% v/v)	86.27	99.3 (min.)
2	Specific gravity	0.8304	0.789
3	pH	7.0	6.5-9.0
4	Total acidity %(w/v)	0.0072	0.007
5	Refractive index	1.363	1.36
6	Physical Appearance	Clear and Colourless	Clear and Colourless `

*__Source of data:__ global biofuel, 2014

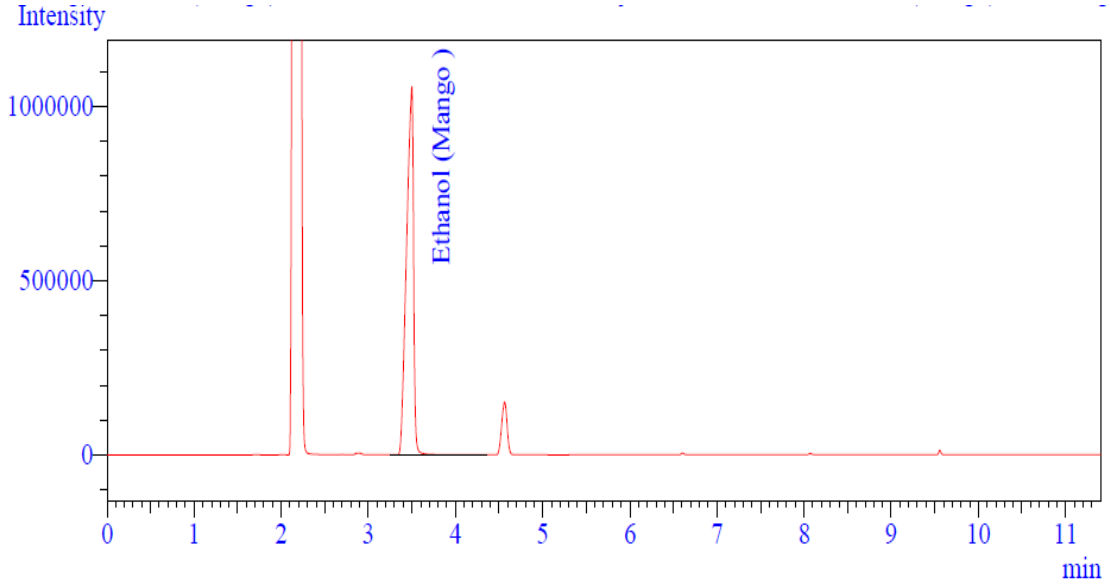


Figure 4 Gas Chromatogram of Bioethanol obtained by Fermentation of Waste Mango

Conclusion

The present study was employed RSM based BBD for the optimization of the strength of ethanol from waste mango using *Saccharomyces cerevisiae*. The results illustrated that the maximum strength of ethanol 25.05±1 % (v/v) was obtained at 0.73 % (w/w) amount of yeast, 76.08 % (w/v) of substrate concentration and pH of 4.6 at a fixed temperature and fermentation time. The slight discrepancies between the experimental and predicted strength of ethanol proved that the RSM was an accurate and applicable tool to optimize the ethanol production from waste mango. Besides, the high reducing sugar content 303 ± 30 (mg/g) of waste mango with small amount of cellulose, hemicelluloses and pectin showed the potential of waste mango as a good feedstock for bioethanol production, also it can be used as an alternative fuel to reduce the load on conventional fossil fuel resources.

Acknowledgement

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STUDY ON PRODUCTION OF BIOGAS FROM FISH PROCESSING WASTEWATER

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Abstract

In this work, the physico-chemical characteristics of wastewater effluent from Makro Co, Ltd, fish processing industry, Tharketa Township, were systematically analyzed. The effluent from draining steps of Platu Fish processing unit was collected. It was observed that the characteristics of effluent highly depend on the type of processing undertaken. One of the main task of anaerobic digestion is the conversion of organic matter to biogas. Therefore, bench scale anaerobic filter reactor was fabricated to treat fish processing wastewater for production of biogas. Biogas consisted of CH_4 (50 %-75 %), CO_2 (25 %-45 %) and few by-products such as H_2S (<1%) analyzed by the experiments were conducted at various the Hydraulic Retention Time (HRT) (6 hr,12 hr, 24 hr, 36hr and 48 hr) , pH (6,7,8)and different influent COD concentration (COD = 6000 ± 200 mg/L ,10000± 200mg/L,16000 ± 200mg/L and 20000 ± 200mg/L). The maximum gas-production from a given amount of raw material depends on the type of substrate and other parameters such as changes in ambient temperature. The methane content also depends on the influent COD concentration. The maximum amount of biogas has been yielded for the reactor with influent COD concentration of 20000 ± 200 mg/L operating for 48 hr in the neutral pH.

Keywords: influent, biogas, methane

Introduction

There is a growing interest in alternative energy sources as a result of increase demand for energy coupled with a rise in the cost of available fuels. Rapid Industrialization has resulted in the generation of a large quantity of effluents with high organic matter contents. It is treated suitably a perpetual source of energy can be trapped. In spite of the fact that there is a negative environmental impact associated with industrialization, the effect can be minimized and energy can be tapped by means of anaerobic digestion of wastewater.

In recent years, considerable attentions have been paid towards the development of reactors of anaerobic treatment of wastes leading to the conversion of organic molecules into biogas (Lettinga, 1995).Additional benefits of anaerobic digestion are the conservation of fertilized value of the feed material, pathogen reduction, odor reduction, resource recovery, and mitigation of greenhouse gases of environmental concern. The way in which the digestion is carried out depends mainly on the type of feed. It can be carried out as a batch process, a continuous process or a multi-stage process (Harris, 1999).

Biogas originates from bacteria in the process of biodegradation of organic material under anaerobic (absence of air) conditions. The natural generation of biogas is an important part of the biogeochemical carbon cycle. Raw material for a biogas plant may be obtained from a variety of sources; animal waste such as human excreta, cow dung, pig dung, chicken drops; agricultural waste such as rice straw, cane-trash, corn stubble and bagasse; plant waste such as aquatic weeds, fallen leaves, water hyacinth and filamentous algae, such as brewery waste, cannery waste, dairy

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waste and distillery waste, industrial food waste and municipal solid waste; residential commercial and institutional waste. Organic waste can be divided into two groups; carbon-rich such as plant waste, agricultural waste and municipal solid waste and nitrogen-rich such as animal waste and kitchen waste. The carbon-rich wastes contain a lot of carbon cellulose, which promotes biogas production and the nitrogen-rich wastes provide nutrients which promote the growth and reproduction of anaerobic bacteria (Environmental Protection Agency, 1996).

The maximum gas-production from a given amount of raw material depends on the type of substrate and other parameters such as changes in ambient temperature that can have a negative effect on bacterial activity (Bastien, Y, 2003).

Materials and Methodology

Materials

Fish processing wastewaters were collected from Marko Co. Ltd., Tharketa Industrial Zone,Yangon Region. The wastewater samples were maintained at 4°C in a refrigerator before analysis, treatment and production of biogas.

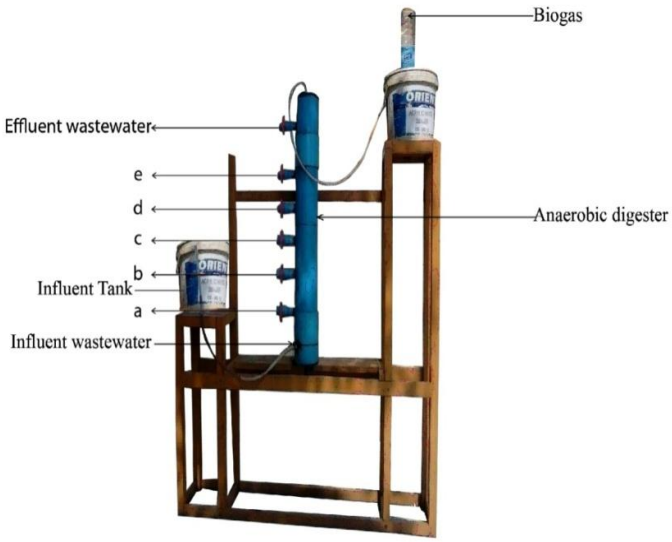
Methodology

Analysis of COD of Fish Processing Wastewater

Sample 500 mL was homogenized using the prepared sample (2.5 mL) was mixed with potassium dichromate solution (1.5 mL) and sulfuric acid (3.5 mL), and digested in COD digester (DRB200) at 150°C for 2 hours.The COD concentration of digested samples was read using Digit Logging Colorimeter (DR/890, HACH). (Closed Reflux Colorimeter method)

Composition of Starter-sludge

The composition of Starter-sludge used in upflow anaerobic filter reactor was 100 g of straw, 500 g of cow manure, 15 L of water and 50 mL of effective microorganisms (EM) (Singh, 1960).



1 = Influent Tank, 2 = Submersible Pump, 3 = Packing Media, 4= Sludge Outlet, 5= Different Levels (a,b,c,d,e) , 6= Effluent Wastewater,

Figure 1 Upflow Anaerobic Filter Reactor

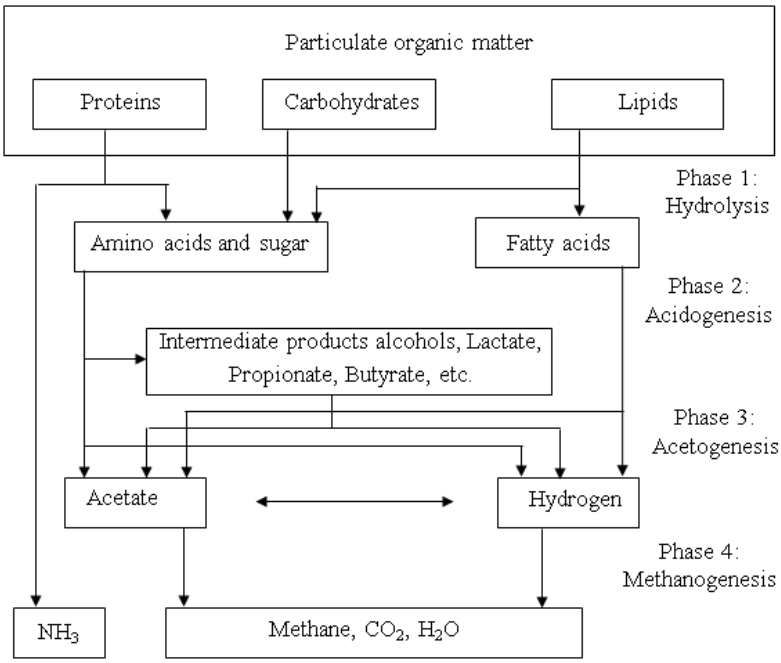


Figure 2 Process Flow Sheet for Biogas Production

Table 1 Operational Parameters for Upflow Anaerobic Filter Reactor Based on the Upflow Anaerobic Filter Reactor Obtained from Internet

Sr. No.	Parameters	Value
1	Reactor working volume (L)	5.5
2	Cross-sectional area (m^2)	8.11×10^{-3}
3	Upflow velocity (m/h)	0.113,0.056,0.028,0.019,0.014
4	Hydraulic retention time (hr)	6, 12, 24, 36 and 48
5	Flow rate (m^3/d)	0.022,0.011, 5.5×10^{-3}, 3.67×10^{-3} , 2.75×10^{-3}
6	Influent COD concentration (mg/L)	6000, 10000, 16000, 20000
7	pH	6, 7, 8

Production of Biogas from Fish Processing Wastewater by Varying Operational Parameters

pH of diluted fish processing wastewater with different COD concentration (COD = 6000 ± 200 mg/L ,10000± 200mg/L,16000 ± 200mg/L, 20000 ± 200mg/L) and its pH was adjusted to (pH : 6,7,8 ± 0.1). Then this prepared wastewater was pumped continuously down the bottom of the reactor and the flow rate($0.022 m^3$/d,$0.011 m^3$/d, $5.5 \times 10^{-3} m^3$/d, 3.67×10^{-3} m^3/d,$2.75 \times 10^{-3} m^3$/d) was controlled to obtain the various hydraulic retention times (6 hr,12 hr, 24 hr, 36 hr and 48 hr). It flowed upward through the filter medium (PVDF) and came out from the effluent point near the top of the reactor. The effluent pipe was connected to a water-sealed arrangement to prevent the escape of the gas through the effluent. A periodic cleaning of the submersible pump was also carried out. The height of the influent wastewater tank and the biogas collector were kept constant throughout the entire experiment. Biogas was collected by water displacement method. The operating temperature of the reactor was in the mesophilic range (29-35°C).

Table 2 Total Amount of Biogas Evolved for (influent COD = 6000±200 mg/L) at Different pH

Sr. No.	Hydraulic Retention Time (hr)	Total amount of Biogas (L)		
		pH 6	pH 7	pH 8
1	6	14.5	15.5	15
2	12	15	16.3	15.4
3	24	15.3	17.5	16
4	36	15.5	17.7	16
5	48	16	18.3	17

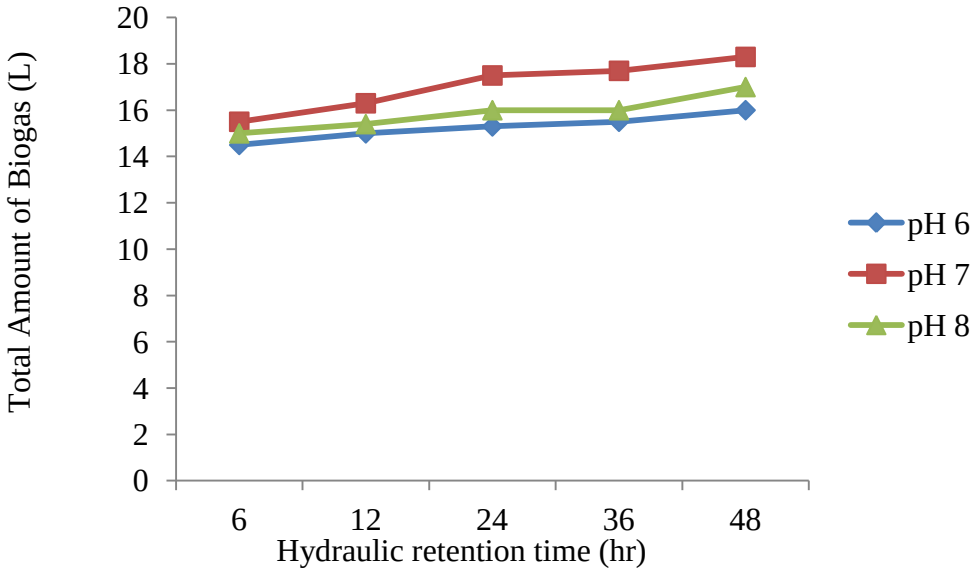


Figure 3 Total Amount of Biogas Evolved for (influent COD = 6000±200 mg/L) at Different pH

Table 3 Total Amount of Biogas Evolved for (influent COD =10000 ±200 mg/L) at Different pH

Sr. No.	Hydraulic Retention Time (hr)	Total amount of Biogas (L)		
		pH 6	pH 7	pH 8
1	6	18.6	21.5	20
2	12	19.2	27.7	20
3	24	20	24	21.8
4	36	23	25.5	23.5
5	48	23.2	27.3	25.7

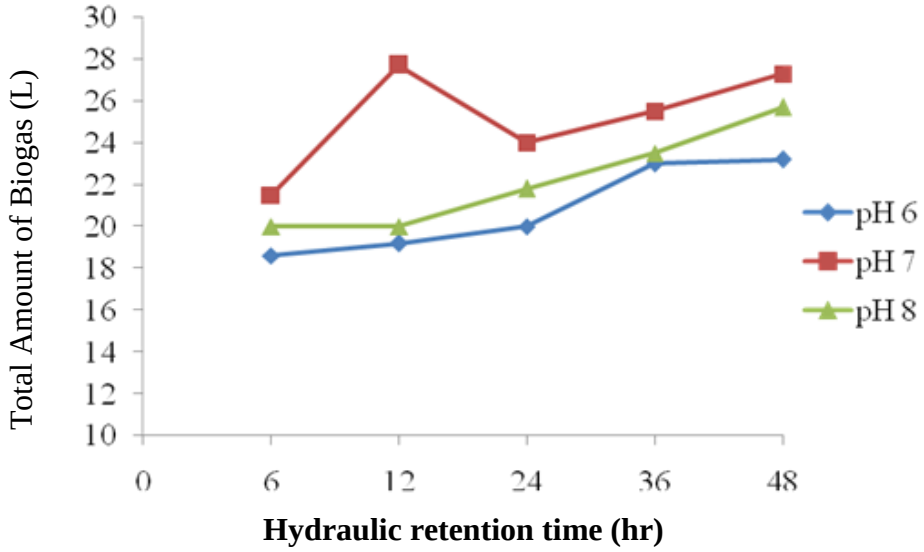


Figure 4 Total Amount of Biogas Evolved for(influent COD = 10000±200mg/L) at Different pH

Table 4 Total Amount of Biogas Evolved for(influent COD =16000±200 mg/L)at Different pH

Sr. No.	Hydraulic Retention Time (hr)	Total amount of Biogas (L)		
		pH 6	pH 7	pH 8
1	6	25.4	29	27
2	12	27	32	30
3	24	28	36	32.8
4	36	29.2	37	33
5	48	30	38.5	34

.

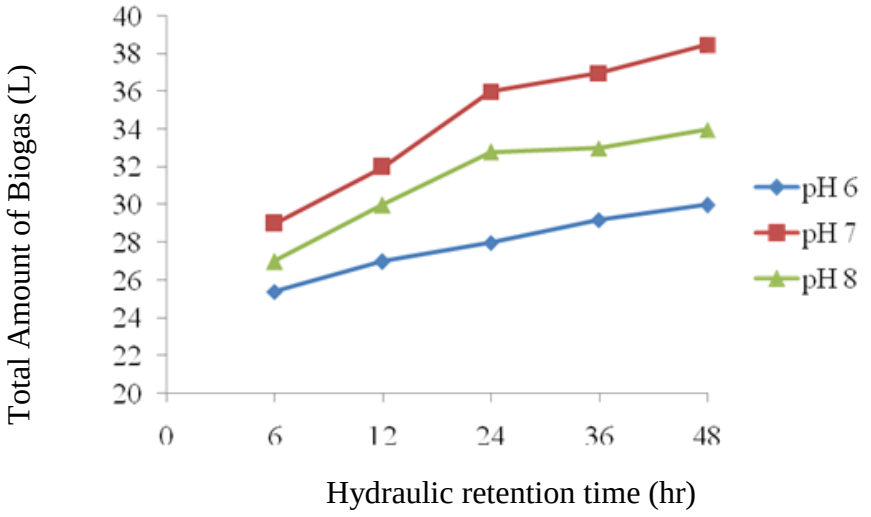


Figure 5 Total Amount of Biogas Evolved for(influent COD = 16000±200mg/L) at Different pH

Table 5 Total Amount of Biogas Evolved for (influent COD =20000± 200mg/L at Different pH

Sr. No.	Hydraulic Retention Time (hr)	Total amount of Biogas (L)		
		pH 6	pH 7	pH 8
1	6	21	31	30
2	12	27.5	32.5	32
3	24	32	35	33
4	36	32.5	36	35
5	48	35	38	37.2

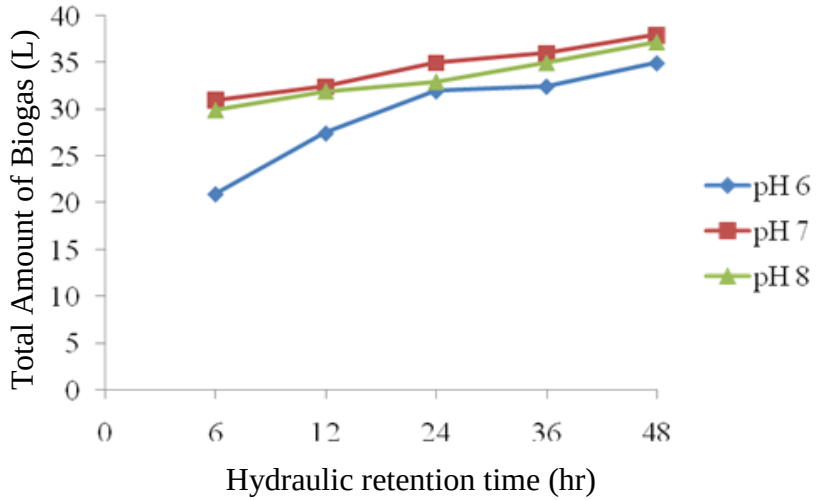


Figure 6 Total Amount of Biogas Evolved for (influent COD = 20000±200 mg/L) at Different pH

Results and Discussion

Production of Biogas from fish processing wastewater was conducted with various COD concentration at different pH. The results of the total amount of Biogas evolved for various influent COD concentrations are shown in Tables (2) - (5) and figures (3) - (6).According to the results, the higher the hydraulic retention time, the higher the amount of biogas evolved. If the COD concentration was the most increased, the amount of biogas was increased than it. Fish processing wastewater was diluted with tap water to obtain the desired COD concentration and pH was adjusted by using NaOH, and HCl. It was shown in Tables (2) to (5).while other operating parameters (Hydraulic Retention Time and influent COD concentration) were kept constant. The removal efficiencies were better in neutral (i.e. pH 7) than slightly acidic (pH 6) and slightly alkaline (pH 8) conditions, while other operating parameters (Hydraulic Retention Time and influent COD concentration) were kept constant. By varying the influent COD concentration, it was clearly observed that the removal efficiencies decreased with increase in influent COD concentration, while other operating conditions were kept constant. In general, COD of a waste is higher than BOD because more compounds can be chemically oxidized than can be biologically oxidized. For many types of wastes, it is required to correlate BOD with COD to obtain the estimate BOD value without determination. This can be very useful because the COD can be determined in three hours, compared with five days for the BOD.

According to the COD and BOD data obtained from the treatment process, the amount of BOD and COD was highly correlated. Therefore the Up flow Anaerobic Filter Reactor has good performance in chemical and biological degradation.

In this study, wastewaters from fish processing industry were collected and characterized. According to the results, it was found that the characteristics of wastewater were highly depended upon the type of fish being processed and the type of processing undertaken. In general, the wastewater contained relatively higher amount of organic pollutants and it should not be disposed of without treatment.

According to the results obtained, In case of biogas; higher influent COD concentration produced more biogas. The methane content also depends on the influent COD concentration. The maximum amount of biogas was obtained when the reactor was fed with wastewater of influent COD concentration of 20000 ± 200 mg/L operating at 48 hr in neutral pH. pH influent and pH effluent was always within the optimal ranges; however, pH effluent was always higher than the pH influent. pH in the reactor varied between 6.69 and 7.68, so the reactor was always optimally self-buffered.

Conclusion

In general, at the longer Hydraulic Retention Time and the lower influent COD concentration, better removal efficiencies were obtained. The removal performance of the anaerobic reactor in terms of COD depends on HRT, organic loading rat. In case of biogas, higher influent COD concentration produced more biogas.

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OPTIMIZATION OF PROCESS CONDITIONS FOR THE PREPARATION OF CHITOSAN FROM SHRIMP SHELL

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Abstract

Natural polymers viz., chitin and its deacetylated derivative form, chitosan consist of β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). Chitin and chitosan were prepared from shrimp shell by demineralization, deproteinization and deacetylation. Effects of demineralization, deproteinization and deacetylation conditions on their preparations were studied. The X-ray Diffraction (XRD) method was used to study the correlation between degree of deacetylation (DD) and crystallinity of the extracted chitin and chitosan. The functional groups and the effect of deacetylation of the extracted samples were studied by Fourier Transform Infrared (FTIR) spectroscopy. The viscosity average molecular weight of chitosan was determined depending on the different deacetylation time. The changes in surface morphology between the extracted chitin and deacetylated chitosan were studied by SEM techniques.

Keywords: chitosan, degree of deacetylation, crystallinity, functional groups, SEM

Introduction

After cellulose, chitin and chitosan are the second most available biopolymer. They are sourced mainly from exoskeleton of crustaceans such as crab and shrimp, but are also available from other sources such as fungi and some insect's wing (Al-Sagheer *et al.*, 2009). Shrimp shells are necessary first to be converted into chitin. Generally, extraction of chitin from shrimp shells consists of three steps, including demineralization, deproteinization and decolorization. After that, chitin can be converted into chitosan by deacetylation process which partially removed the acetyl group from the molecular chain of chitin. Chitin is widely distributed natural biopolymer composed of N-acetyl-D-glucosamine (GluNAc) subunits linked with β-1,4-glucosidic linkage. Chitosan is a nontoxic, deacetylated product of chitin, which is a linear heteropolysaccharide and contains N-acetyl-D-glucosamine, same linkages as in chitin (Shahidi & Abuzaytoun, 2005).

Chitosan contains the reactive amino group at C-2 and the hydroxyl group at C-3 and C-6. This chitosan is widely used in various industries such as pharmaceutical, biochemistry, biotechnology, cosmetics, biomedicine and paper industry. Beside that, chitosan can be used as an emulsifier, coagulant, chelating agent, and thickener emulsion. In addition, chitosan can be used as a substitute for formalin to preserve food that serves a relatively safe for consumption (Muzzarelli, 1985).

The use of chitin and chitosan in various fields depends on quality characteristics such as degree of deacetylation, solubility, viscosity and molecular weight. Quality of chitosan is mainly determined by the degree of deacetylation, where the deacetylation degree is dependent on materials and conditions process such as concentration of alkali solution, temperature, and time (Suhardi, 1993).

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In Myanmar, crustacean shell wastes are abundantly produced annually and these wastes create the environmental pollution problem in the form of bad odor and aesthetically damage the environment as well as health problem. By utilizing these wastes as value added products, the problems could be reduced. The objective of the present study was to obtain the processing conditions of demineralization, deproteinization and deacetylation for chitin and chitosan from shrimp shell waste.

Materials and Methods

Materials

Shrimp

Family : Penaeidae
Genus : *Penaeus*
Scientific Name : *Penaeus Monodon* (Fabricius, 1978)
Common Name : Puzun Kyar
Local Name : Giant Tiger Prawn (or) Asian Tiger Shrimp

(Giant Tiger Prawn)

Shrimp shells were collected from Yuzana Fishery Co., Ltd. Kwin Kyaung Street, Ahlone Township, Yangon Region. Required chemicals such as hydrochloric acid (specific gravity 1.16), sodium hydroxide and ethanol (Analar Grade) were purchased from Super Shell Chemical Shop, 27th Street, Pabedan Township, Yangon Region.

Methods

Extraction of Chitin from Shrimp Shell

About (100) g of shrimp shell powder was demineralized with 1 M of hydrochloric acid solution with shell powder to hydrochloric acid solution ratio (1:20 w/v) at ambient temperature (30 ± 4ºC). The mixture was continuously stirred by a 1.5-inch oblong stir-bar on a magnetic stirrer for 18 hr. Then, the solid and liquid portions were separated by filtration and the solid portion was washed with distilled water until neutral pH was achieved. The solid portion was dried at 60ºC for 6 hr. The dried solid portion was deproteinized with 2 M sodium hydroxide solution with shell powder to sodium hydroxide solution ratio (1:10 w/v) at 95ºC for 2 hr with constant stirring. Then, the deproteinized shell was washed with distilled water until neutral pH was obtained. The alkaline treatment was repeated four times. After neutralization of deproteinized shell, they were dried at 60ºC for 4 hr. Then, the crude chitin was purified with ethanol (1:10 w/v) for 30 min and dried at 50ºC for 3 hr. Then, the chitin was obtained.

Preparation of Chitosan

The chitin (100) g was deacetylated with 50 % (w/v) NaOH solution at 90ºC with a chitin to NaOH solution ratio (1:10 w/v) for 11 hr by refluxing. Then, the residue was washed with distilled water (70 ºC) until neutral pH was achieved. Finally, the obtained chitosan was dried at 60ºC for 3 hr.

Figure 1 Chitin from Shrimp Shell

Figure 2 Chitosan from Shrimp Shell

Characterization of Chitin and Chitosan

Determination of Moisture Content (AOAC 2011, 930.15)

About (3) g of sample was weighed accurately in a clean, dry and previously weighed moisture dish. The sample was dried at 105ºC until constant weight was obtained. After the drying was completed, sample was removed from the oven and placed in a desiccator for about 30 min and weighed accurately and the moisture content was calculated by equation (1).

$$\text{Moisture}(\%) = \frac{(B - C)}{A} \times 100 \qquad (1)$$

Where, A = sample weight in gram, B = weight of dish + sample prior to drying, C = weight of dish + sample after drying.

Determination of Ash Content (AOAC 2011, 942.05)

About (3) g of sample was weighed in a clean and previously weighed porcelain crucible and burnt in a muffle furnace at 600ºC for 6 hr. The crucible containing the ash was cooled in a desiccator and weighed. Heating, cooling and weighing were repeated until a constant weight was obtained. According to the equation (2), the total ash content was calculated as follows:

$$\text{Ash}\,(\%) = \frac{\text{weight of ash}}{\text{weight of sample}} \times 100 \qquad (2)$$

Determination of Degree of Deacetylation of Chitosan (Sabins and Block, 2000)

2 g of chitosan sample was completely dissolved in 100 cm^3 of freshly prepared 0.2 M HCl solution and 100 cm^3 of concentrated HCl was then added to the homogeneous chitosan solution with vigorous stirring to precipitate the hydrochloride salt. The resultant solution was centrifuged for 15 min and the supernatant was discarded. The chitosan hydrochloride salt was then filtered off and washed several times with methanol. Residual moisture in the chitosan hydrochloride salt was removed by stirring for 6 hr in acetone. Then, the precipitate was dried in a vacuum desiccator for 12 hr to obtain white chitosan chloride salt.

The resulting salt was divided into two portions; one portion was used for the determination of moisture content, while the other was used for titration. An accurately weighed (approximately 0.2 g) chitosan chloride salt was dissolved in distilled water, volume made up to 100 cm^3 in the volumetric flask. The resulting solution 25 cm^3 was titrated against 0.1 M standard NaOH solution by using phenolphthalein as an indicator. The degree of deacetylation of chitosan products were calculated by equation (3).

$$DD(\%) = \frac{M_1 V_1}{1000} \times \frac{V_0}{V_2} \times MW \times \frac{100}{W(1 - \%MC/100)} \quad (3)$$

Where,
M_1 = molarity of NaOH solution
V_1 = volume of titrated NaOH
V_0 = total volume of chitosan solution
V_2 = volume of titrated chitosan solution
MW = molecular weight of one monomer unit of chitosan
W = weight of chitosan
%MC = moisture content

Determination of Molecular Weight of Chitosan (Sabins and Block, 2000)

2 g of chitosan sample was dissolved in 0.1 M acetic acid solution containing 0.2 M sodium hydroxide solution and stirred. Dilution was made using this homogeneous solution to obtain different concentrations of chitosan solution (0.002 to 0.01 %). Different concentrations of chitosan solution were filled in the viscometer by placing pipetting bulb over the capillary arm, the chitosan solutions were drawn up to a point above the upper mark. The bulb was removed and the time taken for the lowest point of the meniscus to pass from the upper to the lower mark was recorded. The procedure was repeated three times for each chitosan sample. The efflux times of the solvent and prepared chitosan solutions were also determined by stop watch. Molecular weight of chitosan was calculated by using Mark-Houwink-Sakurada equation.

$$[\eta] = K M_v^{\ a} \quad (4)$$

Where,
$[\eta]$ = intrinsic viscosity
K = constant (1.81×10^{-3} dLg^{-1})
a = constant (0.93)
M_v = viscosity average molecular weight (Da)
t_0 = flow time of solvent
t = flow time of solution

* K and a are constants for given solute-solvent system and temperature.

Determination of Solubility of Chitosan **(Fernandez-Kim, 1991)**

The solubility of the chitosan sample was determined according to the reported procedure. 0.1 g of chitosan powder in triplicate was placed in a centrifuge tube and then dissolved with 10 ml of 1% (v/v) acetic acid for 30 min using an incubator shaker operating at 240 rpm and 25ºC. The chitosan solution was then immersed in a boiling water bath for 10 min, cooled down to room temperature and centrifuged at 10,000 rpm for 10 min and the supernatant was decanted. The undissolved particles were washed with distilled water (25 ml) and the centrifugation process was repeated. The supernatant liquid was removed and undissolved particles were dried at 60ºC for 24 hr. Then, the particles were weighed and the percentage solubility was determined using following equation.

$$\text{Solubility}(\%) = (A - B) \times (A - C) \times 100 \quad (5)$$

Where,
A = Initial weight of the centrifuge tube with chitosan (g)
B = Final weight of the centrifuge tube with chitosan (g)
C = Initial weight of the centrifuge tube (g)

X-ray Diffraction (XRD)

X-ray diffraction is a very powerful, nondestructive tool for analyzing materials and a variety of information can be deduced from the obtained diffraction pattern. The X-ray diffraction analysis was applied to detect the crystallinity of the extracted chitin and chitosan and their patterns were recorded in the 2θ range of 5º to 70º using RIGAKU, Smart Lab X-ray Diffractometer with CuK_α radiation, λ = 1.54056 Å at the Universities' Research Center, University of Yangon. The crystallinity index (CrI) was obtained by using the following equation:

$$\text{CrI}\ (\%) = \frac{I_{max} - I_{am}}{I_{max}} \times 100\% \qquad (6)$$

where I_{max} is the maximum intensity of the (110) diffraction peak at $2\theta \approx 20°$ and I_{am} is that of the amorphous diffraction signal at $2\theta \approx 16°$.

Fourier-Transform Infrared (FTIR) Spectroscopy

To study the effect of deacetylation and the various functional groups contained in chitin and chitosan, the FTIR spectra were recorded at room temperature using PC-controlled SHIMADZU FTIR-8400 Spectrophotometer at the Universities' Research Center, University of Yangon.

Morphological Analysis by SEM

The change in morphology from chitin to chitosan was investigated by using JEOL JSM-5610LV SEM at Universities' Research Centre, University of Yangon.

Results and Discussion

The physico-chemical characteristics of shrimp shell used in this research are shown in Table (1). The calcium carbonate content of shrimp shell was 47.15 % (w/w) which closely matched with the literature value. The protein content of shrimp shell was 25.25 % (w/w) while the literature value was 30-40 %. The results in Table (2) indicate that 1 M hydrochloric acid concentration was adequate for demineralization of shrimp shell. It was noted that if hydrochloric acid concentration was higher than 1 M, the ash content was slightly decreased. The ash content indicates the effectiveness of demineralization process. The deproteinization ability of shrimp shell was studied by using different concentrations of sodium hydroxide and it is shown in Table (3). The total nitrogen of chitin from shrimp shell by using different alkali treatment varied from 6.30 - 6.36 % (w/w), respectively. According to AOAC 1975, protein nitrogen is the difference of total nitrogen and chitin nitrogen. Then, it was found that the more alkali concentration was used, the less protein content was found. For the deacetylation process, it was observed that the less protein content in chitin was more effective than the more protein content. The less protein contents in chitin obtained from 2 M and 2.5 M were the 0.56 and 0.50 % (w/w), respectively. From the economic point of view, 2 M was chosen as the suitable deproteinization concentration because the two values were not significantly different and matched with the literature ("marine-bio-resources / CHITIN & CHITOSAN", n.d.).

Table 1 Physico-chemical Characteristics of Shrimp Shell Powder (- 32 mesh)

Sr. No	Composition	Shrimp Shell	Literature Value # (Crustacean Shells)
1	Moisture (% w/w)	10.97 ± 0.01	-
2	Ash (% w/w)	48.9 ± 0.1	-
3	Protein (% w/w)	25.25	30-40
4	$CaCO_3$ (% w/w)	47.15 ± 0.01	30-50
5	Nitrogen Content (% w/w)	4.04	-

(Knorr, 1984)

Table 2 Effect of Hydrochloric Acid Concentration on the Demineralization of Shrimp Shell Powder (- 32 mesh)

Weight of Sample = 100 g
Demineralization Time = 24 hr
Demineralization Temperature = Ambient Temperature (30 ± 4ºC)
Weight of Shell and HCl Solution Ratio = 1:20 (w/v)

Sr. No	HCl (Conc:) (M)	Ash (% w/w)	$CaCO_3$ (% w/w)
1	0.5	20.38±0.02	17.73±0.1
2	1.0*	0.98±0.01	ND
3	1.5	0.97±0.02	ND
4	2.0	0.97±0.01	ND
5	2.5	0.97±0.02	ND

*Most suitable condition, ND = Not Detected

Table (4) shows the effect of deacetylation time on the physico-chemical properties of chitin. It was observed that 11 hr and 13 hr gave the higher deacetylation degree with 50 % (w/v) sodium hydroxide solution at 90ºC. In addition, it can be seen that the increasing of the deacetylation degree decreases the molecular weight.

Table 3 Effect of Sodium Hydroxide Concentration on the Deproteinization of Demineralized Shrimp Shell Powder (-32 mesh)

Weight of Sample = 100 g
Deproteinization Temperature = 65ºC
Deproteinization Time = 2 hr
Weight of Shell and NaOH Solution Ratio = 1:10 (w/v)

Sr. No	NaOH (Conc:) (M)	Total Nitrogen (% w/w)	Chitin Nitrogen (% w/w)	Residual Protein (%w/w)
1	0.5	6.36	6.18	1.13
2	1.0	6.33	6.20	0.81
3	1.5	6.31	6.21	0.63
4	2.0*	6.30	6.21	0.56
5	2.5	6.30	6.22	0.50

*Most suitable condition
Protein Nitrogen = Total Nitrogen – Chitin Nitrogen
Protein Content = Protein Nitrogen × 6.25

Table 4 Effect of Deacetylation Time on the Properties of Chitosan

Weight of Chitin = 100 g Deacetylation Temperature = 90ºC
NaOH Concentration = 50 % (w/v) Chitin and 50 % (w/v) NaOH Solution Ratio = 1:10 (w/v)

Sr. No	Time (hr)	Properties	
		DD (%)	M_v (Da)
1	5	83.94 ±0.4	1.01×10^6
2	7	88.4 ± 0.3	6.04×10^5
3	9	93.05 ± 0	3.31×10^5
4	11*	96.67 ± 0.09	1.04×10^5
5	13	97.66 ± 0	1.04×10^5

*Most suitable condition
DD = Deacetylation Degree
M_v = Molecular Weight

The solubility of chitosan increases with decreases in molecular weight. In this research, the deacetylation degree in shrimp shell chitosan for 11 hr and 13 hr are 96.67 % and 97.66 %, respectively. Deacetylation degree and molecular weight are the critical factors for chitosan's quality. The chitosan's quality was increased with increasing deacetylation degree and solubility of chitosan in 1 % (w/v) acetic acid. In this research, the values of deacetylation degree and molecular weight obtained from deacetylation times (11 hr and 13 hr) were not significantly different. According to deacetylation degree and molecular weight, 11 hr was suitable deacetylation time. Table (5) shows the physico-chemical characteristics of prepared chitosan and commercial standard chitosan. It was found that the prepared chitosan was compared well with standard chitosan.

X-ray Diffraction Analysis

The XRD stacked pattern of the chitin and chitosan extracted form shrimp shells are shown in figure (3). The chitosan behaves the semi-crystalline structure because the characteristic peaks appeared at around 2θ values 10º and 20º corresponding to the crystallographic planes (020) and (110) and minor reflections at higher 2θ values. When compared with chitin, the diffraction angle's intensity of chitosan was weakened significantly. The crystallinity of the chitin was 80.24 % and this value was reduced to 60.88 % when the chitin was treated with strong alkali to prepare chitosan. The crystalline structure of chitin was destroyed and the crystallinity was decreased as a result of strong alkali treatment during deacetylation process.

Table 5 Physico-chemical Properties of Prepared Chitosan

Sr. No	Characteristics	Experimental Value (Shrimp Shell)	Literature Value# (Shrimp Shell)
1	Colour	White	White or Buff
2	Appearance	Powder	Powder
3	Moisture (% w/w)	2.97 ± 0.03	< 10
4	Ash (% w/w)	0.32 ± 0.05	< 0.5
5	Deacetylation Degree (%)	96.67 ± 0.09	-
6	Molecular Weight (Da)	1.04×10^5	-
7	Crystallinity Index (%)	60.88	-
8	Solubility in 1 % (w/v) acetic acid solution	100 ± 0	-
9	Yield (%)	14.5	20-30

(ZHANG *et al*., 2011)

FITR Spectroscopic Analysis

Figure (4) shows FTIR stacked spectra of chitin and chitosan extracted from shrimp shell wastes. In the FTIR spectrum of extracted chitin, absorption peaks at 3443.05 and 3261.74 cm^{-1} represent the stretching vibrations of the –OH and $–NH_2$ groups. The most important signals in the spectrum of chitin, peaks of amide bands I, II, III were observed at 1662.69, 1558.54, 1315.50 cm^{-1} in shrimp chitin which compared well with the amide peaks at 1668.66, 1558.51, 1314.40 cm^{-1} reported by Varun *et al*., 2017. NH– stretching peak was also found in the extracted chitin at 3105.50 cm^{-1}. The absorption peaks appeared at 1074.39 and 1020.38 cm^{-1} in both spectra are due to the stretching vibrations of –C–O–C– of the glucosamine ring. The peak appeared at 898.86 cm^{-1} is ring stretching a characteristic band for β-1, 4 glycosidic bonds.

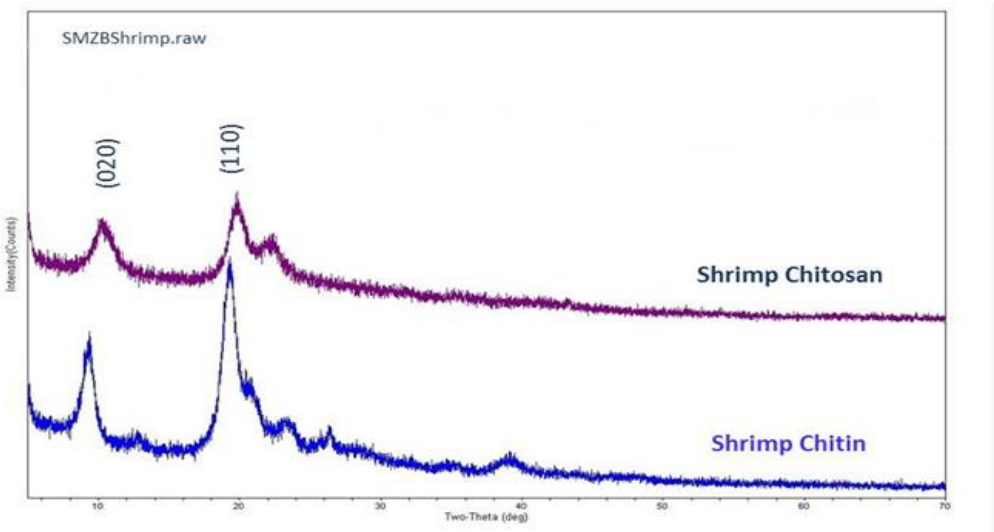


Figure 3 XRD Stacked Pattern of Prepared Chitin and Chitosan Extracted from Shrimp Shell

In the FTIR spectrum of extracted chitosan, stretching vibration of –OH group, $–NH_2$ group and hydrogen bonding showed peak at 3385.18 cm^{-1} and peak of amide I bond was indicated at 1656.91 cm^{-1}. Peak at 1591.33 cm^{-1} in extracted chitosan showed the presence of bending vibration of amide band II (N–H). The vibrational characteristics of CH_3 group of $NHCOCH_3$ (amide bond) was pointed at 1383.01 cm^{-1}. The absorption peaks appeared at 1151.54 cm^{-1} and 895.00 cm^{-1} were due to the stretching vibrations of oxygen in glycosidic linkage and Pyranose ring, respectively. Dahmane *et al*., (2014) reported that after the deacetylation of chitin, disappearance of peak 1556 cm^{-1} and appearance of new peak occurred at 1595 cm^{-1} which represents $–NH_2$ bending. Compared with the present research, the same process was found with disappearance of absorption peak at 1558.54 cm^{-1} (as in chitin) and appearance of new peak at 1591.33 cm^{-1} in chitosan and it can be seen in stacked FTIR spectra of chitin and chitosan. So, it can be said that the process of deacetylation was successfully performed in the present study. The characteristic of the asymmetric bridge O_2 stretching of the glycosidic linkage was indicated by the absorption peak appeared at 1151.54 cm^{-1}. The existence of CH_3, CH_2 and CH groups as well as the primary and secondary –OH groups attached to the Pyranose ring, is represented by the spectra between 1422 and 603 cm^{-1}.

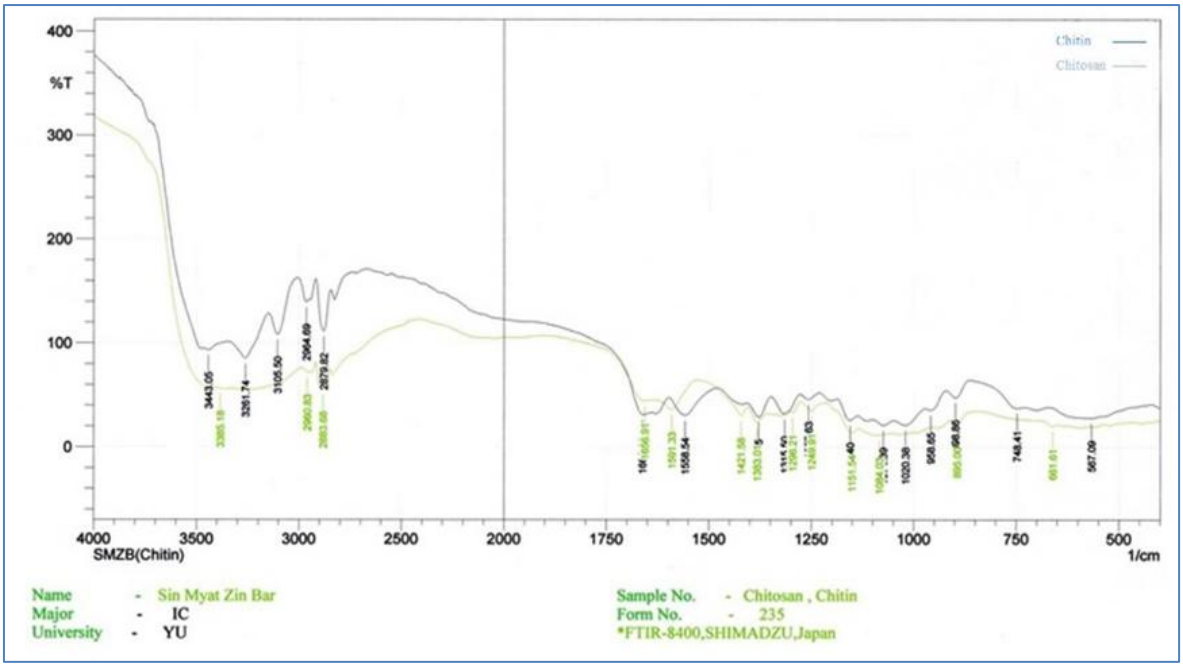
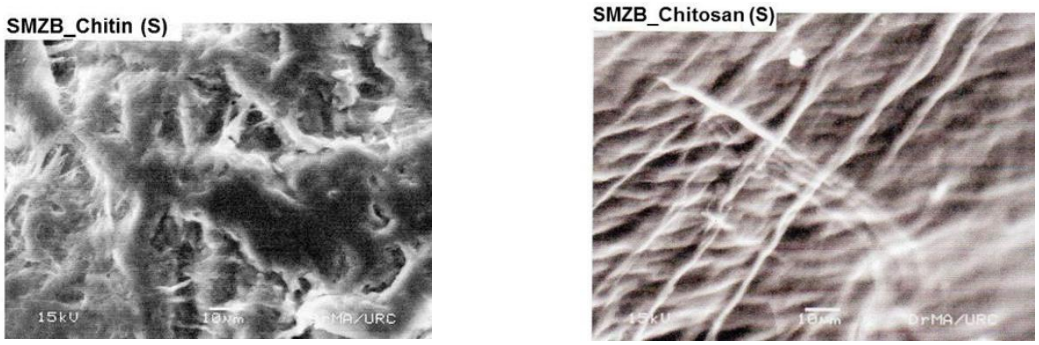


Figure 4 FTIR Stacked Spectra of Chitin and Chitosan Extracted from Shrimp Shell

Scanning Electron Microscopic Analysis

The surface morphology of demineralized chitin characterized by SEM is shown in Figure 5(a). The SEM micrograph of the chitin extracted from shrimp shell showed the smooth surface with some straps and shrinkages. The smoother microanalysis showed the disappearance of calcium carbonate after the process of demineralization with hydrochloric acid. Figure 5(b) showed the SEM micrograph of chitosan prepared from shrimp shells. In this microstructure, it was clearly seen the lesser microfibrillar structure with rough surface morphology than in chitin. Moreover, the microstructure of shrimp chitosan behaves no smoothing surface with straps and shrinkages. Rough microcrystalline surface of chitosan was due to the treatment of chitin with a stronger sodium hydroxide concentration on deacetylation reaction. So, it can be assumed that the deformation of the acetylated structure is associated with the differences in the morphology between chitin and chitosan of the obtained microstructures.



(a) (b)

Figure 5 SEM micrographs of (a) Shrimp Chitin (b) Shrimp Chitosan

Conclusion

In the present research, chitosan was successfully extracted from shrimp shell as a white powder with adequate physico-chemical properties such as ash content 0.32 % and completely soluble in 1 % (w/v) acetic acid. The deacetylation degree obtained was about 96.67 % and the crystallinity index was 60.88 %. The molecular weight and yield percent of the extracted chitosan were 1.04×10^5 Da and 14.5 % (w/w), respectively. Optimum values for chitin production were found to be 1 M HCl for demineralization and 2 M NaOH for deproteinization processes. 11 hr deacetylation time was used as optimum condition for the deacetylation of the chitosan preparation.

Acknowledgements

I wish to express my sincere appreciation to Professor Dr Soe Soe Than, Head of Department of Industrial Chemistry, University of Yangon, for her valuable suggestions and comments for this research.

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CHARACTERIZATION OF EXTRACTED NATURAL COLOUR FROM CAPSICUM AND ITS APPLICATION IN FOOD PRODUCT

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Abstract

Natural colour, a kind of high-quality natural dye, has a great interest as an alternative colourant to use in food products. This work aims to design a strategy for the preparation of free-flowing colour powder from dried capsicum by encapsulating the capsanthin (colour compound) with the coating material. In this work, firstly, 72.63% of capsaicin (piquancy) was eliminated using 0.1 M sodium hydroxide solution. The piquancy-eliminated capsanthin colour compound was extracted under various conditions to optimize the extraction process. The colour value and capsanthin content were calculated by the ASTA analytical method and Beer-Lambert Law respectively, using the absorbance measured at the wavelength 460 nm throughout this research. Additionally, the microencapsulation process of oily-capsicum paste was implemented by coating with gum arabic (GA) on the oily-capsicum particles to improve the appearance and ease of handling. The colour value and capsanthin content of encapsulated powder are 143.17±3.46 and 22.89±0.44 mg g^{-1}. The solubility of encapsulated powder was found 100% in water. The resultant data have proved that this colour could have a natural colouring material giving an added value to the various food products. Therefore, this capsicum colour can be used in the food industry as an alternative to synthetic dye.

Keywords: capsicum, food colour, capsaicin, capsanthin, encapsulation, gum arabic.

Introduction

The development of products with attractive colour and appearance is an essential sensory characteristic in food and cosmetic industry since the colour and appearance impart the first impression to consumers for a rapid judgment of quality (Chan, 2015). Natural dyes have many properties such as little side effects, high safety, biodegradable, green environmental protection. Besides, food colourants can be classified as natural colours, synthetic colours (Rodriguez-Amaya, 2016), there are two types of food colourants approved for industrial use: certified and uncertified. Certified food colourants are synthetic dyes for which testing and approval are processed by the Food and Drug Administration (FDA), whereas uncertified food colourants are natural colours that do not need batch approval by the FDA (Chan, 2015).

Capsicum is the round-shaped chilli that has the non- or mild-pungency. Capsicum colour extracted from the dry capsicum is a high-quality natural dye that has anticancer food additives and colouring properties. Sweet and hot capsicum are widely consumed as vegetables and are used as food colourants because they are a good source of the red carotenoid pigment: capsanthin ($C_{40}H_{56}O_3$) and capsorubin ($C_{40}H_{56}O_4$). As carotenoid compounds are lipophilic compounds, they are usually extracted with acetone or ethanol (Tanaka, 2009; Arimboor, 2015). The capsicum extracts involve bioactive compounds, such as polyphenols, carotenoids, capsaicinoids, and ascorbic acid.

The pungency of capsicum depends on the concentration of capsaicinoids, particularly capsaicin ($C_{18}H_{27}NO_3$). Despite the pungency could be attractive food ingredients in spicy, it is

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unavailable for use in food industry. To address this limitation, the capsaicin content should be eliminated to give a higher attractive ingredient as colouring of various products (Zhao & Chen, 2018). Capsicum extract contains colouring carotenoids predominantly capsanthin and capsaicinoids. Besides the pigments, chemical entities such as flavours, essences, vitamins and fatty oil are also present in the capsicum extract (Rafajlovska, 2011).

Microencapsulation is a process by which solid, liquid or gas is enclosed in microscopic particles of wall material thin coatings around the substances (Ribeiro, 2010). For example, the entrapment of oily colour paste within coating material can protect them from environmental factors such as moisture, air or light. The structure, formed by the microencapsulating agent around the substance to be encapsulated (core), is called the wall (Gupta, 2015). The wall protects the core against deterioration and releases under desired conditions that is a key functionality provided by microencapsulation (Aguiar, 2016).

Materials and Methods

Raw Materials

The dried capsicum was collected from Sin Phyu Kyoon Township, Magwe Region. 95% ethanol, acetone, sodium hydroxide, gum arabic were purchased from Academy Chemical group, 28th Street, Pabedan Township, Yangon Region. All chemicals are of analytical grades and gum arabic is of food grade.

Methods

Preparation of capsicum colour extract from capsicum powder

Firstly, as shown in Figure 1, dried capsicum (50 g) was washed, dried and ground into fine powder (about -30+60 mesh). To eliminate the capsaicin (piquancy) content, the capsicum powder was immersed into NaOH solution at R.T for 12 h. After elimination of capsaicin, the capsaicin-eliminated capsicum was washed with water and dried again. Since the oily capsicum red colour was soluble only in the solvent, the capsicum red colour was extracted with the solvent (acetone and ethanol) for different time (0.5, 1, 2, 4, 6, 8 h) at the temperature range (15, 20, 25, 30, 40, 50 ºC). After the solvent was recovered by distillation, the capsicum colour extract was evaporated by sun-drying to remove the residual solvent. The dried capsicum colour extract is an oily phase so that it cannot be dried into the powder form.

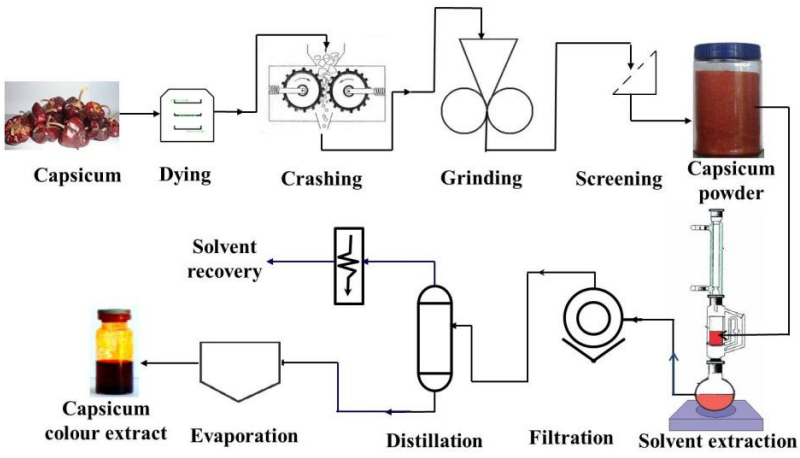

Figure 1 Schematic diagram of the extraction of capsicum colour from dried capsicum.

Preparation of microencapsulated capsicum colour powder

Since the capsicum colour extract is an oily and insoluble in water, oil-in-water emulsion containing capsicum extract as dispersed oil droplet was prepared to carry out the microencapsulation process. For preparing capsicum extract-loaded emulsion (Figure 2), the dried capsicum paste was dispersed with 10% gum arabic (GA) solution to give it in the forms of fine droplets. The GA solution was prepared by vigorous shaking with distilled water before the capsicum extract was added into it. Stirring was continued until the good emulsion was obtained. During emulsification, the oily capsicum extract was surrounded by GA molecules as the coating material. After this encapsulated or entrapment was successfully done, the water was removed by sun-drying and the resultant encapsulated capsicum was ground into fine powder.

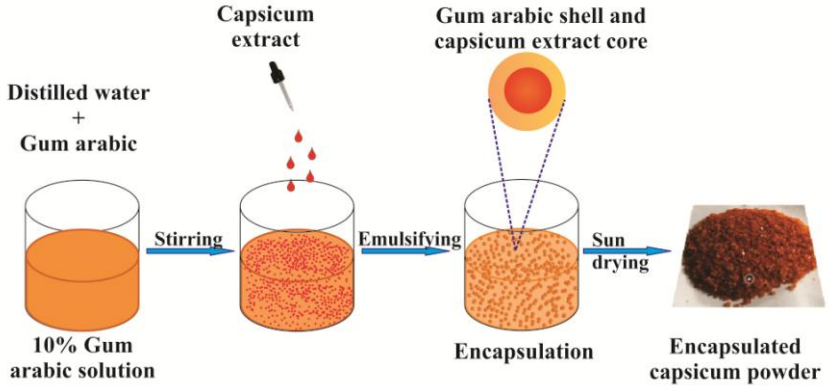

Figure 2 Schematic diagram of capsicum colour powder encapsulated with gum arabic solution.

Characterization of capsicum powder and capsicum colour extract

The characteristics of capsicum powder were studied by phytochemical investigations: polyphenol, flavonoid, glycoside, phenolic, sugar, tannin and saponin (Harborne, 1973). For capsicum powder, colour extract and encapsulated colour powder, physicochemical properties in terms of colour value, capsaicin content and capsanthin content were calculated from resultant absorbance values determined by UV-Vis spectrophotometer (UVmini-1240), respectively. From each sample, a mean value of three readings was recorded to avoid any miscalculation. The possible functional groups for characteristic absorption peak were examined with Fourier Transform Infrared (FT-IR) spectroscopy (Kopec, 2012).

Determination of capsaicin (piquancy) content

The capsaicin content of capsicum was investigated to eliminate the piquancy of capsicum. The concentration of capsaicin was estimated from the standard calibration curve for capsaicin given as follows (Rafajlovska, 2011):

$$y = 9.64\ x + 0.005 \qquad R^2 = 0.9909$$

where x = µg capsaicin/mL of extract, y = absorbance at 281 nm.

Determination of colour value

According to ASTA (American Spice Trade Association) analytical method (Method 20.1), colour values were evaluated throughout this research by the absorbance at 460 nm (AOAC official method 971.26) (Frick, 2003). The colour value was calculated as follow:

ASTA colour value for capsicum= {($A_{extract}$ at 460 nm} x (16.4 *I_f)}/g of sample

where, 16.4 is the conversion factor, $I_f = 0.0600/A_s$ (A_s is the absorbance of standard solution) and $A_{extract}$ is the absorbance values of extract determined by UV-Vis spectrophotometer (UVmini-1240) (Mı´nguez-Mosquera, 2001).

Determination of capsanthin content

The capsanthin content of capsicum colour was calculated by Beer-Lambert Law in this study. The extinction coefficient of the major pigment capsanthin ($1\%E^{460nm} = 2300$) in acetone (Hornero-Méndez et al., 2000).

$$A = \varepsilon cl$$

where, A = Absorbance at 460 nm, c = concentration of capsanthin, mmol mL^{-1}, l = length of cuvette, cm and ε = molar extinction coefficient, L mol^{-1} cm^{-1}

Determination of pH stability

The capsicum colour powder was dissolved into the different pH solution which is prepared using HCl or NaOH. And then absorbance values at 460 nm were measured.

Results and Discussion

To prepare the capsaicin-eliminated capsicum colour extract that can use in food and cosmetics, we have developed the capsicum free-flowing powder prepared by the encapsulation process. In this study, the mild-heat capsicum was chosen because it can give a high intensity of colour. The physicochemical characteristic of capsicum powder: the contents of moisture, ash, crude fiber, total solids; percentages of capsaicin and capsanthin; and then pH and colour value were determined and compared with the literature values. It was observed that the resultant data meets with the literature values as shown in Table 1.

Table 1 Physicochemical characteristics of raw capsicum powder

Sr. No.	Characteristics	Experimental values	Literature values (Krithika, 2014)
1	Moisture content, (%w/w)	8.46	8-14
2	Ash content, (%w/w)	4.19	4-10
3	Crude fibre content, (%w/w)	25.74	15-40
4	Total solids content, (%w/w)	91.54	-
5	pH	4.0	3.00 -5.00
6	Colour value	104.09	>100
7	Capsaicin content, (%w/w)	0.036	0.01-0.5
8	Capsanthin content, (%w/w)	0.28	>0.1

In the phytochemical examination by AOAC method (AOAC, 1990), it has colouring compounds such as polyphenol, flavanoid, phenolic and sugar in capsicum powder and no glycoside, tannin and saponin compounds as presented in Table 2.

Table 2 Phytochemical characteristics of raw capsicum powder

Sr. No.	Tests	Extract	Reagents	Observation	Inference
1	Polyphenols	EtOH	1% $FeCl_3$+1%K_4{Fe(CN)	Greenish blue colour	+
2	Flavonoids	EtOH	H_2SO_4(conc:) + Mg turning	Pink colour	+
3	Glycosides	H_2O	10% $FeCl_3$	Purple colour	-
4	Phenolics	H_2O	10% $FeCl_3$	Blue-black colour	+
5	Sugar	H_2O	Benedict's solution	Red ppt	+
6	Tannins	H_2O	2% NaCl+ 1% $FeCl_3$	Deep blue ppt	-
7	Saponin	H_2O	1% $NaHCO_3$	Froth	-

+ = present, - = absent

Table 3 Effect of sodium hydroxide concentration on the percent removal of capsaicin (piquency) in capsicum extract

Immersion temperature = 28-32 ºC
Immersion time = 12 h
Ratio of capsicum to NaOH solution(w/v) = 1:10

Sr. No.	Concentration of NaOH solution (M)	Capsicum extract			
		Absorbance (281 nm)	Residual capsaicin (μg g^{-1})	Residual capsaicin (%w/w)	Eliminated capsaicin (%w/w)
1	0	1.81	36.19	100.00	0.00
2	0.01	1.57	31.38	86.70	13.30
3	0.05	0.83	16.67	46.07	53.93
4	0.1*	0.50	9.91	27.37	72.63
5	0.2	0.48	9.65	26.65	73.35
6	0.4	0.48	9.56	26.41	73.59

* The most suitable NaOH concentration

Although the capsaicin (piquancy) is one of the active compounds in capsicum and it has benefits as an antioxidant and anti-fungus actions, it is unsuitable as a colourant (dye) for food and cosmetics. The capsaicin content can be removed by the treatment with suitable NaOH concentration at room temperature for 12 h while stirring. To eliminate the capsaicin present in capsicum powder, different concentrations of NaOH (0.01, 0.05, 0.1, 0.2 and 0.4 M) were used. The capsaicin content was calculated by using the absorbance at the wavelength of 281 nm (Chen, 2009). It was found that 0.1 M NaOH is the minimum concentration to eliminate the maximum amount of capsaicin (72.62%) as shown in Table 3 and Figure 3a. The small absorbance peak at 281 nm is observed in Figure 3b.

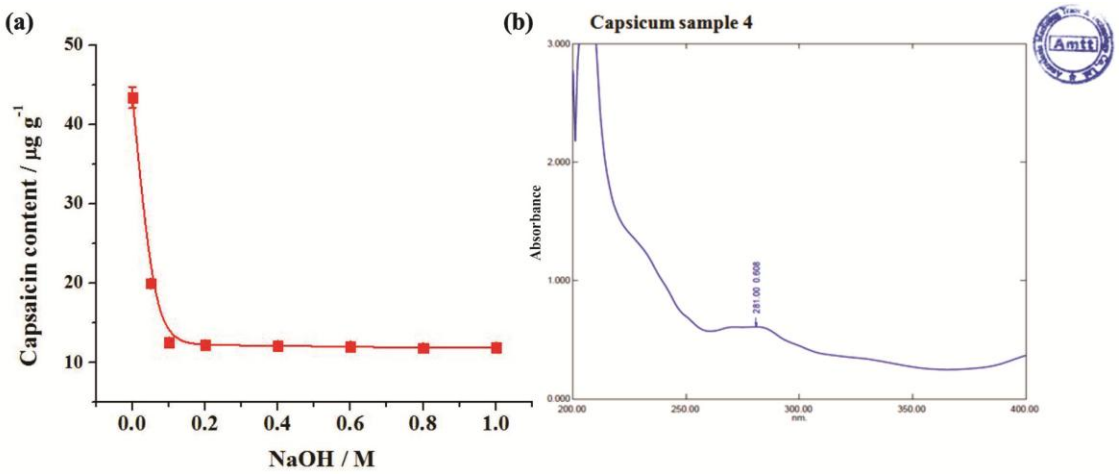

Figure 3 Capsaicin content analysis (a) changes in capsaicin content with the concentrations of NaOH and (b) UV-Vis spectrum of capsaicin (72.62%) eliminated capsicum colour extract treated with 0.1 M NaOH solution.

To explore the optimal extraction and purification process of capsicum colour extract from dry capsicum, the effect of material to solvent ratio, extraction temperature, and extraction time on the yield percent, colour value and capsanthin content in extracted capsicum colour were investigated. The effect of capsicum powder to solvent (acetone and ethanol) ratio on the yield percent, colour value and capsanthin content of extracted colour are presented in Table 4. It was observed that the highest yield percent (4.2% w/w), colour value (159.04) and capsanthin content (25.42 mg g^{-1}) of extracted capsicum colour was obtained from the 1:15 ratio of capsicum powder to acetone solvent when the extraction temperature at 28-32 ºC for 6 h.

Table 4 Effect of capsicum-solvent ratio on the yield percent, colour value and capsanthin content

Capsicum colour extraction temperature = 28-32 ºC
Capsicum colour extraction time = 6 h

Sr. No.	Capsicum : solvent	Capsicum colour extract (Acetone)				Capsicum colour extract (Ethanol)			
		Yield (%w/w)	Abs	Colour value	Capsanthin (mg g^{-1})	Yield (%w/w)	Abs	Colour value	Capsanthin (mg g^{-1})
1	1:5	2.2	0.79	125.28	20.02	2.2	0.70	111.14	17.76
2	1:10	3.8	0.99	157.71	25.21	2.1	0.70	111.00	17.74
3	1:15*	4.2	1.00	159.04	25.42	2.5	0.71	112.59	18.00
4	1:20	4.1	0.97	154.53	24.70	2.8	0.72	114.58	18.32
5	1:25	3.8	0.98	155.85	24.91	2.7	0.79	125.32	20.03

* The most suitable solvent is acetone and capsicum to acetone ratio is 1:15.

* Abs = absorbance

The effect of extraction temperature and time on the yield percent, colour value and capsanthin content of extracted capsicum colour was examined based on the different extraction temperature (15, 20, 25, 30, 40, 50 ºC) and different extraction times (0.5, 1, 2, 4, 6, 8 h) in capsicum colour extraction. The absorbance was measured both for different extraction temperatures and extraction times and then the colour value and capsanthin content were

calculated with the absorbance at the wavelength 460 nm (Arimboor, 2015). As shown in Table 5, the highest yield, colour value and capsanthin content of extracted capsicum colour are indicated when the extraction temperature was 30 ºC. The extraction time that gave the maximum yield, colour value and capsanthin content is 4 h, as expressed in Table 6.

Table 5 Effect of extraction temperature on the yield percent, colour value and capsanthin content

Capsaicin-eliminated capsicum powder = 50 g
Volume of acetone = 750 mL
Capsicum colour extraction time = 6 h

Sr. No.	Temperature (ºC)	Capsicum colour extract			
		Yield (%w/w)	Absorbance	Colour value	Capsanthin (mg g^{-1})
1	15	2.0	0.59	107.66	14.90
2	20	2.3	0.67	123.07	17.04
3	25	2.7	0.82	151.03	20.91
4	30*	3.9	1.02	187.05	25.89
5	40	4.2	1.00	183.83	25.45
6	50	4.1	0.97	178.78	24.75

* The most favourable temperature

Table 6 Effect of extraction time on the yield percent, colour value and capsanthin content

Capsaicin-eliminated capsicum powder = 50 g
Volume of acetone = 750 mL
Capsicum colour extraction temperature = 28-32 ºC

Sr. No.	Time (h)	Capsicum colour extract			
		Yield (%w/w)	Absorbance	Colour value	Capsanthin (mg g^{-1})
1	0.5	1.5	0.46	85.21	11.80
2	1	1.8	0.58	105.62	14.62
3	2	2.3	0.61	112.86	15.62
4	4*	4.1	1.02	187.56	25.97
5	6	4.1	1.01	184.75	25.58
6	8	4.1	0.99	182.15	25.22

* The most favourable time

The optimal capsicum colour extract was characterized by UV-Vis spectroscopy at 460 nm and Fourier Transform Infrared (FT-IR) spectroscopy. Figure 4a indicates the large colour peak in the UV-Vis spectrum at 460 nm. In Figure 4b, the characteristic absorption peak of capsicum extract is as follows: the peak of cyclopentane and cyclohexane is at 2950 -2800cm^{-1}, the stretching absorption peak of carbonyl (C=O) is at 1720-1710 cm^{-1}, absorption peaks of methylene (CH_2) and methyl (CH_3) are at 1465 cm^{-1},and the stretching absorption peak of methoxy (C-O) is at 1170-1150 cm^{-1} (Sun, 2008).

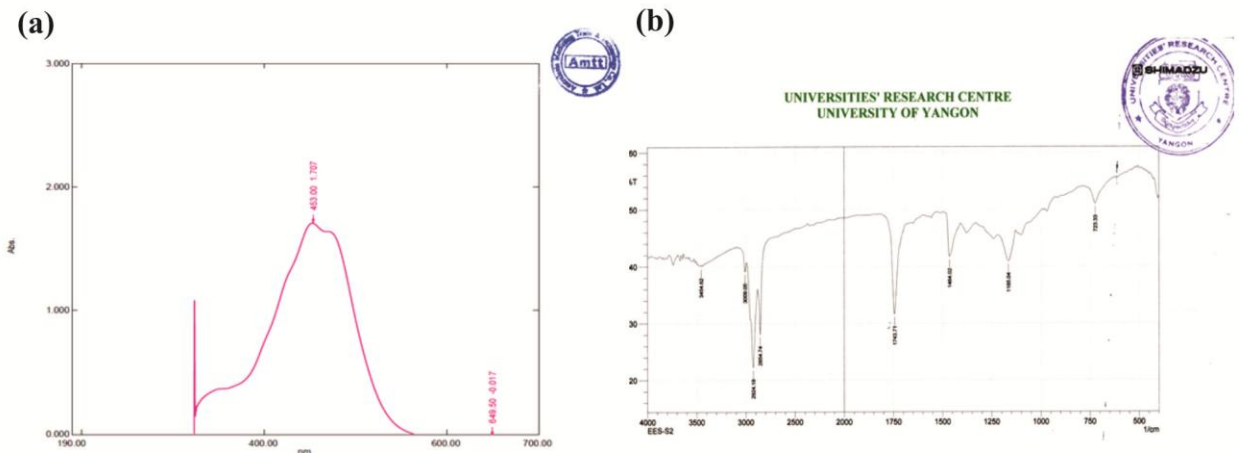

Figure 4 Characterization of capsicum colour extract by (a) UV-Vis spectrum at 460 nm and (b) FT-IR spectrum.

Table 7 Effect of ratio of capsicum extract to gum arabic (GA) on the colour value and capsanthin content of encapsulated capsicum extract

Gum arabic solution = 10% in distilled water
Temperature of sun-drying = 30-40 ºC

Sr. No.	Extract : GA	Encapsulated capsicum colour powder					
		Yield (%w/w)	Absorbance	Colour value	Capsanthin (mg g^{-1})	Organoleptic properties	
						Colour	Phase
1	1:1	2.2	1.01	160.85	25.71	chilli red	oily paste
2	1:5	3.6	0.99	156.91	25.08	chilli red	oily soft film
3	1:10*	4.1	0.9	143.17	22.89	chilli red	non-oily red powder
4	1:25	5.0	0.68	107.78	17.23	yellowish red	slightly hard powder
5	1:50	6.5	0.57	89.88	14.37	yellowish brown	hard powder

* The most suitable ratio of capsicum extract to gum arabic solution.

To develop the encapsulated colour powder, the dispersion of capsicum extract in 10% gum arabic solution was done by shaking to be thoroughly homogeneous phase. The microencapsulated capsicum colour powder was obtained by a cost-effective sun-drying method instead of spray-drying and freeze-drying. The effect of different ratios of capsicum extract to gum arabic (1:1, 1:5, 1:10, 1:25, 1:50) on the characteristics of capsicum powder were studied. As shown in Table 7, 1:1 and 1:5 ratios were found to be oily film manner but 1:25 and 1:50 ratios impart the hard texture and the brownish colour. The 1:10 ratio gave a good appearance and colour as well as free-moving manner on the colour powder.

The stability test of encapsulated capsicum colour powder was conducted in different pH solutions in Table 8. The results show that the colour was not stable in pH 1 because the absorbance value is declined to 0.29. At the pH range of 2-10, it was found that absorbance

values slightly decreased when the pH is lower or greater than 7. It was revealed that capsicum colour can be stable in food products.

As regards the colour values were calculated, the colour value of raw capsicum powder was found to be 104.09±7.06, capsaicin-eliminated capsicum extract was 187.56±2.53 and encapsulated capsicum colour powder was 143.17±3.46 (Table 9). It was found that the capsanthin, one of the carotenoids, content is about 2.6 % of capsicum extract and 2.3 % of encapsulated capsicum powder. These results indicate that it has anticancer properties and free-radical scavenging.

Table 8 The pH stability test for encapsulated capsicum colour powder

pH	pH 1	pH 2	pH 3	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9	pH 10
Absorbance	0.29	0.62	0.81	0.84	0.83	0.88	0.92	0.90	0.89	0.85

Table 9 Physicochemical characteristics of capsaicin-eliminated capsicum extract and encapsulated capsicum colour powder

Sr. No.	Characteristics	Raw capsicum powder	Capsaicin-eliminated capsicum extract	Encapsulated capsicum colour powder
1	Absorbance	0.57±0.12	1.02±0.3	0.90±0.05
2	ASTA Colour value	104.09±7.06	187.56±2.53	143.17±3.46
3	Capsaicin content, µg g^{-1}	36.19±1.34	9.91±0.15	5.14±0.12
4	Capsanthin content, mg g^{-1}	2.88±0.90	25.97±0.32	22.89±0.44

* For the absorbance measurement, all samples were diluted 100 times with acetone. These errors indicate the standard deviation of triplicates.

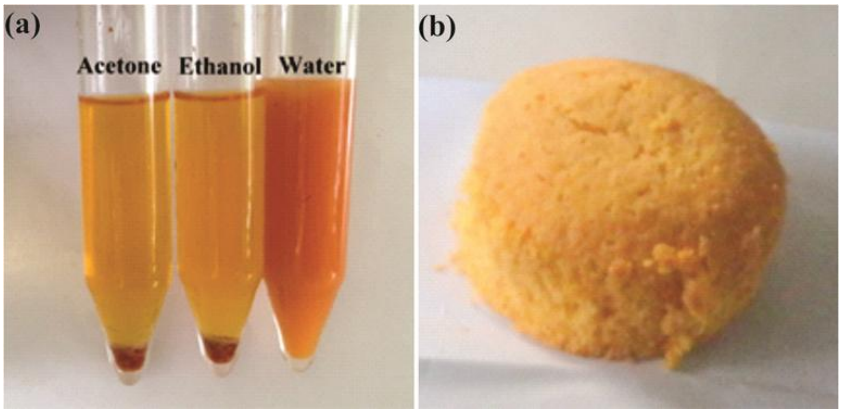

Figure 5 (a) The solubility test of encapsulated capsicum powder diluted with 20 times each of acetone, ethanol and water. It was found 100% solubility in water. (b) Cake with encapsulated capsicum colour powder.

The solubility of encapsulated capsicum colour powder was examined with different solvent; acetone, ethanol and water. Although the capsicum extract is insoluble in water, the encapsulated capsicum powder was observed that it is absolutely soluble in water (100%). It has about 50% solubility in acetone and ethanol as observed in Figure 5a. The encapsulated capsicum colour was applied in the cake baking as shown in Figure 5b. The encapsulated colour

solution (1%, 1 mL) as the colour additive and the other common ingredients in cake baking were used as presented in Table 10. The cake photo reveals that encapsulated capsicum powder gives attractive colour used in confectionery. In a consequence of these results, this encapsulated capsicum colour powder will give many benefits such as appearance, stability, antioxidant, etc., in the food products.

Table 10 Composition of the ingredients used in the cake baking

Sr.No	Ingredients	Amount
1	Flour (g)	150
2	Sugar (g)	120
3	Butter (g)	120
4	Egg (no.)	2
5	Baking powder (g)	0.5
6	Vanilla essence (mL)	0.1
7	Natural colour solution (mL)	1

* 1% colour solution in distilled water

Conclusion

To improve the cost-effective method, the extracted capsicum colour was encapsulated with the optimum ratio of extract to wall material (1:10) by sun-drying method. The capsaicin (pungency) in capsicum powder was eliminated using 0.1 M NaOH solution. The suitable condition for the extraction of colour from capsicum was found to be 1:15 ratios of capsicum to acetone at 28-32 ^{o}C for 4 h. The entrapment of oily-capsicum particles within 10% gum arabic coating not only can protect them from environmental factors such as moisture, air and light but also can transform the oily-capsicum paste into the free-moving particles. Moreover, the lipophilic oily-capsicum extract was changed into the hydrophilicity after microencapsulation. The resultant values in terms of colour value and capsanthin content of capsicum might reveal to use as an alternative colour in food industry. Thus, microencapsulation enhanced the solubility and bioavailability of capsicum colour extract as well as the ease of handling in food and cosmetic industry.

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STUDY ON DRYING CHARACTERISTICS AND NUTRITIONAL COMPOSITION OF WHITE RADISH BY SOLAR CABINET DRYERS, TRAY DRYER AND OPEN SUN DRYING METHODS

Yin Mar Naing[1], Thin Thin Khaing[2], Pansy Kyaw Hla[3]

Abstract

Solar energy, a form of sustainable energy, has been claimed as a great potential for drying of fruits and vegetables since it is naturally accessible. Solar dryers using natural convection or forced convection are used for drying of agricultural products. In this research, white radishes were dried by distributed solar cabinet dryer (DSCD), forced convection solar cabinet dryer (FCSCD), Tray Dryer (TD) and by sun drying (SD). The dryers were fabricated by locally available materials such as plywood, corrugated galvanized iron sheet and glass. The dry bulb and wet bulb temperature (^{o}C), relative humidity (%) and rate of drying (g/hr. sq.cm) were determined as drying characteristics during the drying process. A rather effective of solar radiation in lowest tray of DSCD and FCSCD was observed. The effects of four drying method on the physico-chemical properties and nutritional values, minerals and heavy metals constituents and rehydration ratios of dried white radishes were investigated and compared with fresh and commercial products. The determination of water activity and microbial load were examined to extend the shelf-life of dried products. The organoleptic properties of rehydrated white radish were also determined by 9-point Hedonic Scale Rating Test. The findings suggested that the commercial scale use of DSCD for drying of vegetables during off-season.

Keywords: white radish, drying characteristics, 9-point Hedonic Scale Rating

Introduction

Solar drying comprises the application of heat to vapourise moisture and some means of removing water vapour after its separation from the food products. It is thus a combined and simultaneous heat and mass transfer operation for which energy must be supplied. It brings about substantial reduction in weight and minimise packaging, storage and transportation costs and enables storability of the product under ambient temperatures (Hii & Mujumdar, 2012).

Solar drying is different from "open sun drying". In solar drying, equipment is used to collect the sun's radiation in order to harness the radiated energy for drying applications. (Prakash & Kumar, 2013) Open sun drying has some disadvantages, of which are, unnecessary exposure of products to weather elements such as rain, ultraviolet rays, and contamination by wind-borne dirt and dust. Others include infestation of insects, rodents, pests and other animals as well as degradation by bacteria. (Adelaja & Babatope, 2013)

Solar drying is advantageous over normal convective dryers like hot air dryer, which requires enormous fuel and energy cost (Hii & Mujumdar, 2012). Solar dryers can process the vegetables and fruits in clean, disinfected and hygienic conditions. This can be used also to promote renewable energy sources. (Prakash & Kumar, 2013) There has been a steady evolution of a solar dryer technology from natural convection types to forced convection types. (Adelaja & Babatope, 2013)

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In Myanmar, people enjoy consuming vegetables in daily diet. However, there is almost no post-harvest technology to prevent the nutritional and economic losses during seasons. In this regard, solar drying technology seems to be one of the most promising alternatives to reduce the post-harvest losses. (Mohanraj & Chandrasekar, 2009).

White radish (*Raphanus sativus* L.), Myanmar name Mone-lar-u is an edible root vegetable belonging to family Brassicaceae. Radish is originally native to Southeast or continental East Asia. They are eaten raw and have a mild flavour, or they can be sliced and cooked (Daikon, The Free Encyclopedia, 2014). Radish is a rich source of carbohydrates, potassium, magnesium, copper, calcium, vitamins and antioxidants like ascorbic acid and folic acid etc. These nutrients make it a very effective cancer-fighting food (Dr. Nishadh & Mathai, 2014). In Myanmar, white radishes are extensively grown in Shan State, Delta Region and Yangon Region.

The objective of this work is to utilize solar energy in drying of fruits and vegetables such as white radish under hygienic condition and, at the same time, to compare the various drying methods so that the results can be reliable by the small- and medium-scale enterprises.

Materials and Methods

Raw Materials

White radishes (*Raphanussativus* L.) were purchased from Hmawbi Township, Yangon Region. Sodium metabisulphite and ascorbic acid were purchased from Shwema Chemical Shop, Pabeden Township, Yangon Region. All are analytical grade.

Methods

Preparation of Dried White Radish

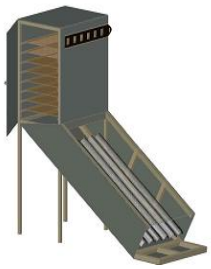
The cleaned radishes were peeled and cut into slices of 2mm thickness and the sliced radishes were weighed 200g. The radish slices were evenly spread on the trays of distributed solar cabinet dryer (DSCD) with temperature ranging from 35-65^{o}C, in forced convection solar dryer (FCSCD) with temperature ranging from 35-60^{o}C and in tray dryer (TD), the temperature was calibrated at 50^{o}C±4. For sun drying, 200g of radish slices were spread on the plastic sieve trays and sun dried. At equal time interval of 30 minutes, the samples were withdrawn and were weighed using a digital weighing machine to monitor weight reduction due to loss of moisture in the samples. After drying processes have taken place, dried white radishes were put into the air-sealed plastic boxes and stored in a dry place away from direct sunlight. The organoleptic properties, physico-chemical properties, nutritional value, minerals and heavy metals composition, water activity and microbiological analysis of dried white radishes were determined.

Effect of Food Additives on Drying of White Radish

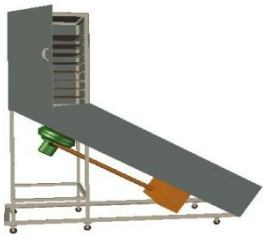
The effects of concentration of sodium metabisulphite solution, ascorbic acid solution and dipping time on the drying of white radishes were determined by organoleptic properties and shelf-life.

Determination of Drying Conditions of Distributed Solar Cabinet Dryer

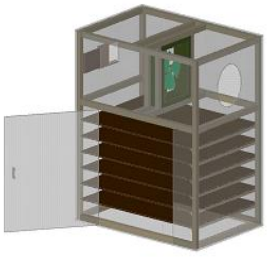
The drying conditions of Distributed Solar Cabinet Dryer (DSCD) such as solar radiation intensity, temperature, air velocity and humidity were measured and the efficiencies of solar dryers such as system drying efficiency, collection efficiency and pick-up efficiency were calculated.



Isometric view of DSCD



Isometric view of FCSCD



Isometric view of TD

Results and Discussion

The changes in moisture content of fresh white radish with respect to time using DSCD was measured for tray one (lowest tray) to four. The data obtained were mentioned in Tables (1a) to (1d). It was pointed out that the longer the drying time the smaller the moisture content in the drying sample and lesser the rate of drying in DSCD. According to Table (2), the pick-up efficiency and system drying efficiency were higher in first tray than the upper trays. Therefore, the first tray could remove more moisture than upper trays. The bottom tray exhibited the fastest drying rate as expected since evaporated moisture from this tray accumulated on the upper trays. Thus, the upper trays exhibited slower drying rates as their ambient condition was surrounded by humidified air. It can be deduced that the drying efficiency of tray one in DSCD was chosen as the best tray among the others. The test was also done in tray one of FCSCD, TD and SD. The drying rate curve and drying curve of white radish in DSCD, FCSCD, TD and by sun drying are illustrated in Figures (1), (2), (3) and (4) respectively.

To choose the optimum food additive in solar cabinet dryers, experiments were done by varying the ratio (% w/v) of sodium metabisulphite and ascorbic acid based on immersing time variation and the effect of those additives on organoleptic properties were determined. Tables (3) and (4) showed that 0.1 (% w/v) of sodium metabisulphite and 0.04 (% w/v) of ascorbic acid with six minutes immersing time gave the most suitable condition for pretreatment of the samples. From those results, it was found to choose 0.04 (% w/v) of ascorbic acid for the pretreatment, the consideration was based on the white colour and fluffy texture of the white radish samples.

The comparison of the organoleptic properties of white radish with respect to different types of dryers was carried out (Table 6) and it was clear that the use of DSCD gave desirable colour and texture among others. Similarly the rehydration ratios for dried white radish (Table 7) with 50 minutes rehydration time based on 3g of dried samples were tested and the results showed that the rehydration ratio used by DSCD gave the highest values. The rehydration ratios of dried samples were gradually high with respect to time.

The determination of the physico-chemical properties and nutritive values of dried white radish were carried out and the results obtained were mentioned in Table (8). From the results of Table (9), by comparing fresh and dried white radish, except manganese and zinc, other minerals decreased after drying. There are no heavy metals present such as arsenic, lead and cadmium in fresh and dried white radishes in all drying modes. The minerals compositions of dried samples are less than fresh because of pretreatments before drying such as washing and slicing operation and may be due to drying.

In this research work, the determination of water activity also played an important role to estimate the shelf-life of the samples of white radish (Table 10). It was found that the results using DSCD were a_w=0.27 for dried white radish. At the room temperature (35^{o}C), most bacteria require a water activity in the range of about 0.90 to 1.00. Some yeasts and moulds grow slowly at a water activity down to as low as about 0.65. Water activity of the dried samples was 0.49 which is lower than 0.65 therefore yeast, mould and bacteria could not grow in this condition.

For the preparation of dried food, determination of microorganisms also is very significant for health. The results obtained were mentioned in Table (11) and the amounts were acceptable limit. According to the analysis of residual pesticides in fresh white radish, the results showed that there are no residual pesticides in all samples. The rehydrated white radishes of DSCD were prepared as salad and Table (12) showed the sensory evaluation results for white radish salad. The appearance, colour and texture were obtained higher average scores and 7.6 of overall acceptability were achieved.

Table 1(a) Changes in Moisture Content of Fresh White Radish on varying the Drying Time using Distributed Solar Cabinet Dryer (1st Tray)

Sr. No.	Drying Time (hr.)	Moisture Content (% w/w)	Drying Rate (g/hr. sq.cm)	Temperature (^{o}C)		RH (%)
				D.B	W.B	
1	0.5	53.39	0.1000	45	29	40
2	1.0	9.23	0.0677	45	28	37
3	1.5	14.77	0.0562	47	28	34
4	2.0	8.01	0.0478	47	27.5	33
5	2.5	5.83	0.0399	47	27.5	33
6	3.0	1.74	0.0339	44	27	25

D.B: Dry Bulb, W.B: Wet Bulb, RH: Relative Humidity

Table 1(b) Changes in Moisture Content of Fresh White Radish on varying the Drying Time using Distributed Solar Cabinet Dryer (2nd Tray)

Sr. No.	Drying Time (hr.)	Moisture Content (% w/w)	Drying Rate (g/hr. sq.cm)	Temperature (^{o}C)		RH (%)
				D.B	W.B	
1	0.5	28.73	0.0617	46	29	38
2	1.0	21.38	0.0538	47	28	34
3	1.5	19.61	0.0499	47	28	34
4	2.0	10.59	0.0431	47	27.5	33
5	2.5	7.97	0.0379	45	27.5	33
6	3.0	2.38	0.0324	44	27	25

Table 1(c) Changes in Moisture Content of Fresh White Radish on varying the Drying Time using Distributed Solar Cabinet Dryer (3rd Tray)

Sr. No.	Drying Time (hr.)	Moisture Content (% w/w)	Drying Rate (g/hr. sq.cm)	Temperature (°C)		RH (%)
				D.B	W.B	
1	0.5	26.77	0.0574	44	28	46
2	1.0	20.30	0.0504	46	28	45
3	1.5	14.13	0.0438	46	27.5	40
4	2.0	15.55	0.0412	46	27.5	36
5	2.5	6.36	0.0358	45	27	30
6	3.0	4.74	0.0315	45	27	28

Table 1(d) Changes in Moisture Content of Fresh White Radish on varying the Drying Time using Distributed Solar Cabinet Dryer (4th Tray)

Sr. No.	Drying Time (hr.)	Moisture Content (% w/w)	Drying Rate (g/hr. sq.cm)	Temperature (°C)		RH (%)
				D.B	W.B	
1	0.5	29.02	0.0623	43	28	42
2	1.0	23.06	0.0559	44.5	28	39
3	1.5	16.95	0.0494	45	27.5	33
4	2.0	12.02	0.0435	46	27.5	30
5	2.5	5.86	0.0372	45	27	28
6	3.0	3.98	0.0324	45	27	25

Table 2 Drying Condition of Distributed Solar Cabinet Dryer for White Radish

Location		University of Yangon			
Crop		White Radish			
Drying Period		March 2015			
Maximum Drying Temperature in Dryer (°C)		~ 60			
Drying Time (hr.)		5			
Total Insolation (kWh/m^2/day)*		6.02			
Items		1st Tray	2nd Tray	3rd Tray	4th Tray
Ambient Air Temperature (°C)	High	35	35	35	35
	Low	21.7	21.7	21.7	21.7
	Mean	28.35	28.35	28.35	28.35
Ambient Air Relative Humidity	High	76	76	76	76
	Low	69	69	69	69
	Mean	73	73	73	73
Air Flow (ms^{-1})	High	0.3	0.3	0.3	0.3
	Low	0.1	0.1	0.1	0.1
	Mean	0.2	0.2	0.2	0.2
Weight or Moisture Content of Batch (g)	Start	300	300	300	300
	End	15.28	28.00	35.63	27.85
Pick up Efficiency (%)		3.68	3.55	3.41	3.55
Collection Efficiency (%)		0.2	0.2	0.2	0.2
System Drying Efficiency (%)		3.73	3.60	3.47	3.60

* (Boxwell, 2015)

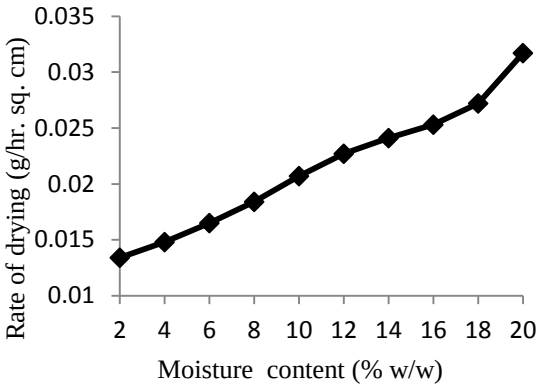


Figure 1 Drying Rate Curve of Fresh White Radish using Distributed Solar Cabinet Dryer

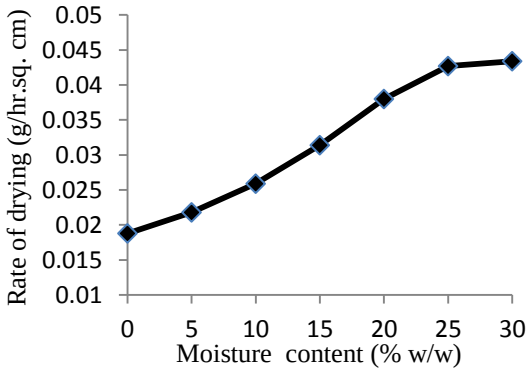


Figure 2 Drying Rate Curve of Fresh White Radish using Forced Convection Solar Cabinet Dryer

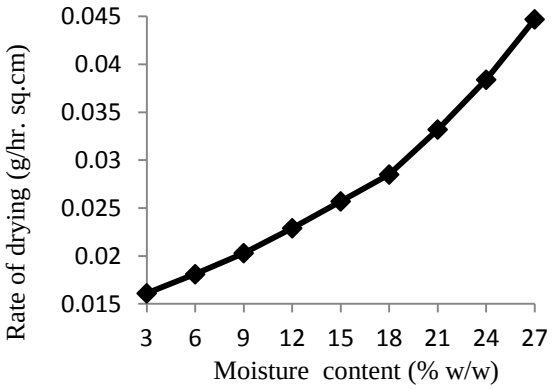


Figure 3 Drying Rate Curve of Fresh White Radish using Tray Dryer

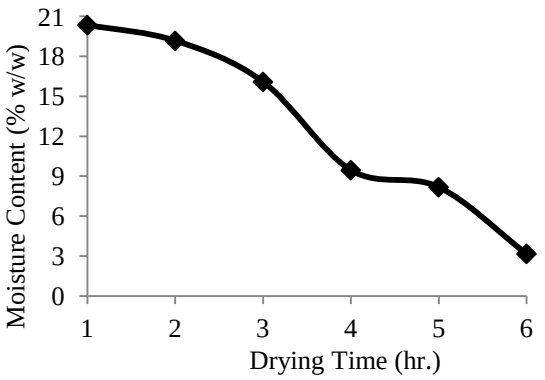


Figure 4 Drying Curve of Fresh White Radish by Sun Drying

Table 3 Effect of Sodium Metabisulphite on Organoleptic Properties of Dried White Radish using Solar Cabinet Dryer

Sample No.	Sodium metabisulphite (% w/v)	Organoleptic Properties		Remarks
		Colour	Texture	
1	0.05	light cream	Crisp	Up to 30 days changes in colour and texture noted
2*	0.1	light cream	Fluffy	Up to 30 days no changes in colour and texture noted
3	0.15	Cream	Crisp	Up to 30 days no changes in colour and texture noted
4	0.2	pale yellow	Hard and crisp	Up to 30 days changes in colour and texture noted

* Most suitable condition

Table 4 Effect of Ascorbic Acid on Organoleptic Properties of Dried White Radish using Solar Cabinet Dryer

Sample No.	Ascorbic acid (% w/v)	Organoleptic Properties		Remarks
		Colour	Texture	
1	0.02	Light cream	Crisp	Up to 30 days changes in colour and texture noted
2	0.03	Light cream	Crisp	Up to 30 days changes in colour and texture noted
3*	0.04	Light cream	Fluffy	Up to 30 days no changes in colour and texture noted
4	0.05	Cream	Crisp	Up to 30 days no changes in colour and texture noted
5	0.06	Cream	Crisp	Up to 30 days no changes in colour and texture noted

* Most suitable condition

Table 5 Effect of Dipping Time of Ascorbic Acid on Organoleptic Properties of Dried White Radish using Solar Cabinet Dryer

Sample No.	Dipping time (min.)	Organoleptic Properties		Remarks
		Colour	Texture	
1	2	light cream	Crisp	Up to 30 days changes in colour and texture noted
2	4	light cream	Crisp	Up to 30 days changes in colour and texture
3*	6	light cream	Fluffy	Up to 30 days no changes in colour and texture noted
4	8	Cream	Crisp	Up to 30 days no changes in colour and texture noted
5	10	Cream	Crisp	Up to 30 days no changes in colour and texture noted

* Most suitable condition

Table 6 Effect of Dryer Types on Organoleptic Properties of Dried White Radish

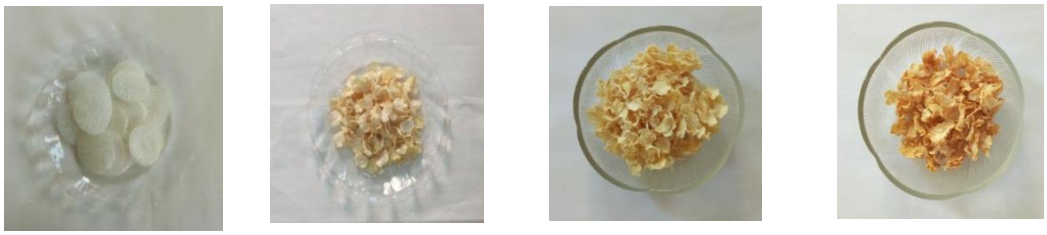
Sample No.	Drying Modes	Organoleptic Properties		Remarks
		Colour	Texture	
1	FCSCD	White	Crisp	Change in colour but no change in texture within three months
2*	DSCD	White	Fluffy	Change in colour but no change in texture within three months
3	TD	White	Crisp	Change in colour but no change in texture within three months
4	SD	Yellowish White	Fluffy	Change in colour and texture within 30 days

* Most suitable condition

Table 7 Effect of Dryer Types on Rehydration Ratio of Dried White Radish

Weight of dried white radish - 3g
Volume of water - 100 ml

Sr. No.	Drying Modes	Rehydration Ratios				
		Rehydration Time (min.)				
		10	20	30	40	50
1	FCSCD	5.33	6.41	6.72	7.4	7.68
2	DSCD	5.92	6.45	7.22	7.56	8.12
3	TD	5.80	6.36	6.86	7.74	7.61
4	SD	4.55	4.95	5.07	5.35	5.63



(a) Fresh White Radish **(b)** Dried by DSCD **(c)** Dried by FCSCD **(d)** Dried by TD

(e) Dried by SD **(f)** Rehydrated **(g)** Salad

Figure 5 White Radish

Table 8 Physico-chemical Properties and Nutritional Value of White Radish

Sr. No.	Properties	Fresh White Radish		Dried White Radish			
				Types of Dryer			
		Literature value*	Experimental value	DSCD	FCSCD	TD	SD
1	Moisture content (%w/w)	95.69	94.84	18.41	12.56	16.09	29.85
2	Ash content (%w/w)	0.52	0.29	8.48	8.12	6.16	5.62
3	Crude fibre content (%w/w)	1.64	0.55	7.57	6.25	8.31	6.97
4	Crude protein content (%w/w)	0.69	0.76	8.06	9.67	9.58	8.01
5	Crude fat content (%w/w)	0.09	0.01	0.18	0.15	0.32	0.11
6	Carbohydrate (%w/w)	3.45	3.55	57.30	63.85	59.54	49.44
7	Energy value (Kcal)	18.6	20	262	295	283	229
8	pH	-	5.6	5.1	5.8	5.8	5.6
9	Colour intensity	-	0.43	0.51	0.50	0.51	0.56

* Daikon, The Free Encyclopedia, 2014

Table 9 Minerals and Heavy Metals Composition in White Radish

Sr. No.	Constituents	Fresh (ppm)	Dried White Radish (ppm)				Limit of Detection (ppm)
			Types of Dryer				
			DSCD	FCSCD	TD	SD	
1	Sodium	7.12	5.67	5.58	6.09	5.54	0.002
2	Potassium	6.18	4.48	4.66	4.94	4.21	0.002
3	Calcium	12.54	7.25	7.51	7.84	7.15	0.002
4	Manganese	< LOD	< LOD	< LOD	< LOD	< LOD	0.009
5	Magnesium	9.14	6.39	6.38	6.91	6.29	0.002
6	Zinc	< LOD	< LOD	< LOD	< LOD	< LOD	0.013
7	Iron	8.29	5.64	5.61	6.12	5.29	0.006
8	Arsenic	< LOD	< LOD	< LOD	< LOD	< LOD	0.026
9	Lead	< LOD	< LOD	< LOD	< LOD	< LOD	0.015
10	Cadmium	< LOD	< LOD	< LOD	< LOD	< LOD	0.007

LOD: Limit of Detection

Table 10 Water Activity of White Radish

Sr. No.	Items	Dried White Radish	
		Equilibrium Relative Humidity (%)	Water activity (a_w)
1	Fresh	87	0.87
2	DSCD	27	0.27
3	FCSCD	25	0.25
3	TD	44	0.44
4	SD	41	0.41

The sample obtained in different days.

Table 11 Microorganisms in Dried White Radish and Dried Fermented White Radish

Sr. No.	Microorganisms	Dried White Radish			
		DSCD	FCSCD	TD	SD
1	Yeasts and moulds (cfu)	1.0×10^6	1.0×10^4	1.0×10^3	2.3×10^4
2	Salmonella (cfu)	ND	ND	ND	ND
3	Coliform (cfu)	ND	ND	ND	ND

cfu: Colony Forming Unit, ND : Not Detected

Table 12 Sensory Evaluation for Dried White Radish

Sr. No.	Organoleptic Properties	Average Scores
1	Appearance	7.6
2	Colour	7.7
3	Texture	7.6
4	Overall Acceptability	7.6

Conclusion

The dried white radish can maintain its appearance and organoleptic properties nearly one month without using additives. By using ascorbic acid, it was found that the shelf life extended to three months whereas use of sodium metabisulphite gave unattractive smell and colour of finished product. It can be concluded that the effect of additive (ascorbic acid) on white radish can attain longer shelf life and attractive colour and smell than that of sodium metabisulphite. The most suitable condition for dried white radish in DSCD chosen were 0.06 (% w/v) of ascorbic acid as food additive, immersing time 6 minutes, drying time 5 hr. and drying temperature 55^{o}C.

It was observed that the rehydration ratio was increased with the increased soaking time in all four drying methods. Compared with other three methods, Sun dried white radish showed the lowest rehydration ratio. Since sun drying took longer time, it affects the texture and reduces the transport properties of water during rehydration.

The water activity of dried white radish was nearly the same by using DSCD, FCSCD, TD and SD. It was less than 0.65 therefore yeast, mould and bacteria could not grow. From the view point of physico-chemical properties and nutritional values, it is clear that solar dried products using solar cabinet dryer have higher nutritional value in comparison to open sun dried products. The quality of product depended upon the removal of moisture during drying process.

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I would like to acknowledge Professor Dr Daw Khin Hla Mon, Head of Department of Industrial Chemistry, Dagon University, for her generous consent to submit this paper. I extend my sincere gratitude to supervisor Dr Daw Pansy Kyaw Hla, Professor (Retired), Department of Industrial Chemistry, University of Yangon, for her invaluable guidance, suggestions and encouragement during this research work. I am deeply grateful to my co-supervisor Dr Daw Thin Thin Khaing, Professor and Head, Department of Industrial Chemistry, West Yangon University, for her close supervision and advice given to me while conducting this research work.

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STUDY ON PREPARATION AND CHARACTERIZATION OF WHITE BAMBOO CHARCOAL

Zar Che Win[1], Khin Thet Ni[2], Khin Hnin Aye[3]

Abstract

In this research work, the physico-chemical characteristics (moisture, ash, volatile matter, lignin content, etc,.) of two types of bamboo Kyakhatwa *(Bambusa arundinacea)*, Kyathaungwa *(Bambusa Polymorpha)* were determined and they were used to prepare white charcoal by forced air type updraft kiln and by makeshift furnace. The physico-chemical properties of prepared white charcoal samples were compared with those of the commercial white charcoals from Japan and Myanmar. The morphological analysis of prepared charcoals and commercial white charcoals was conducted by SEM (Scanning Electron Microscopy) method. The decolourization property of prepared white charcoals and commercial white charcoals was determined by using 0.01M potassium permanganate solution. According to the results, charcoal made by makeshift furnace from Kyathaungwa *(Bambusa polymorpha*) was found to have the highest fixed carbon content (86.24 %w/w) among the charcoals samples made by different methods and also higher than that of two commercial white charcoals. All white charcoal samples prepared from bamboo had a greater number of pores than commercial white charcoal. Therefore, the absorption capacity for decolourization of the prepared white charcoal was higher than that of both commercial white charcoals.

Keywords: white charcoal, decolourization, makeshift furnace, updraft kiln

Introduction

Charcoal is a light, black, porous material resembling coal, with about 85 per cent carbon. It is produced by heating biomass under a system of controlled supply of air. This can be done by the conventional pit method, in brick or metal kilns, or in drums. Wood, sugarcane waste, rice husk and bamboo are commonly used for making charcoal. Charcoal made from bamboo has good properties, similar to wood and other ligno-cellulosic material in terms of high carbon content and calorific value (Jian, 2004).

Two types of charcoal exist; black charcoal and white charcoal. These charcoals are produced using different burning methods. Although they are produced in a substantially similar way, the quality of the charcoals is totally different depending on how the flame is extinguished (Guan, 2004). Compared to ordinary black charcoal, the technology of making white charcoal is difficult. The technology is mostly used in Japan and parts of China and Korea. White charcoals are now being used in the scientific world for industrial purposes such as electronic components and medical applications. Black charcoal renders the hands black when holding, but white charcoal does not make it because it is hard like cast iron (Jian, 2004).

White charcoal is created by carbonizing the wood at a moderately low temperature, then the kiln temperature is increased to around 1000 ° C near the end of the process to make the wood red hot. It is important to be quite skilled in removing the charcoals, which have turned deep red, from the kiln when making white charcoal and smother it quickly with a powder covering to cool it. The powder is a mixture of sand, ash, and soil. This will give the charcoal

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layer a whitish hue. This is where it originated the name "white charcoal." The rapid temperature rise, followed by rapid cooling, burns the outer layer of the wood, leaving a smooth hard surface. It is also regarded as a "hard charcoal". Mangrove (an evergreen tree from tropical coasts) can be used in the manufacture of charcoal. Bamboo is producing an excellent charcoal. It not only provides a new way of using bamboo, but by reducing pollutant contamination, it also promotes environmental protection. Bamboo charcoal is a material of outstanding absorption properties that is environmentally friendly (Oya, 2002).

High-temperature carbonized bamboo charcoal has such properties as being dense and porous, with a large specific surface area of micro-hole structures; being rich with natural minerals; having good release of far-infrared ray; having good electric conductivity, etc. Bamboo charcoal is a functional resource for the protection of the environment and has developed rapidly in recent years because (1) the wood that can be used as high-grade charcoal decreased rapidly and almost exhausted; (2) the bamboo harvest period is short because it grows very quickly. As a result, producing bamboo charcoal does not harm forest and environment; (3) bamboo charcoals are similar in characteristics to and can substitute high-quality hardwood charcoal; (4) bamboo charcoal is strong in strength and easy to produce into different shapes. Although wood charcoal makes a good fuel, bamboo charcoal is almost three times as porous as wood and bamboo charcoal contains a large amount of minerals, such as iron, manganese and potassium (Guan, 2004).

Myanmar is one of the bamboo-rich countries in the world. Millions of bamboo poles are harvested each year, most of which go to paper making industry and used as a major building material in rural area. Only less than 1% of it is used for bamboo charcoal (Takaya, 1999). Therefore, preparation of bamboo white charcoal using white charcoal process would be one useful value added application of bamboo in Myanmar.

In this research, two types of bamboo were carbonized to produce white charcoal using two types of furnaces; forced air type updraft kiln and makeshift furnace.

Materials and Methods

Raw Materials

Two types of bamboo Kyakhatwa *(Bambusa arundinacea)* and Kyathaungwa *(Bambusa Polymorpha*) were collected from Hlaing Township, Yangon Region and Kan Myint village, Phayargyi Township, Bago Region. They were collected at the age of over 3 years. They were cut into required size and air dried for one day. Forced air type updraft kiln and makeshift furnace were constructed for the preparation of white bamboo charcoal and the schematic diagrams were shown in Figures (1) and (2).

Preparation of White Charcoal

White bamboo charcoal was made by carbonizing the bamboo at a moderately low temperature in forced air type updraft kiln with and without sagger and in makeshift furnace according to the process as shown in Figure (3). As shown, the air dried bamboo were loaded into the furnace and ignition was started. For ignition, firewood and wood charcoal was used for forced air type updraft kiln and electricity was used for makeshift furnace to start heating. For 2 hours, carbonization was conducted in both forms of furnaces at a relatively low temperature

(800-900 °C). Once the end of the cycle was near, to make the bamboo red hot, the kiln temperature was increased to around 1000 °C -1100 °C. The air blower was used to increase the heating temperature of forced air type updraft kiln and temperature controller was used to control the temperature of makeshift furnace. The temperature of the updraft kiln during carbonization could be noted by using pyrometer. Deep red charcoal was extracted from the kiln at the end of the carbonization process and immediately smothered it with a powder coating and cooled it. The powder is a sand, soil and ash mixture. This would then give the surface of the charcoal a whitish hue. The rapid increase in temperature followed by rapid cooling resulted in a smooth hard surface. After cooling for 2 hours, charcoal samples were taken out from the mixture, packed in the plastic bags and stored for further analysis. The steps for the preparation of white charcoal using forced air type updraft kiln and makeshift furnace were shown in Figures (4,5, and 6).

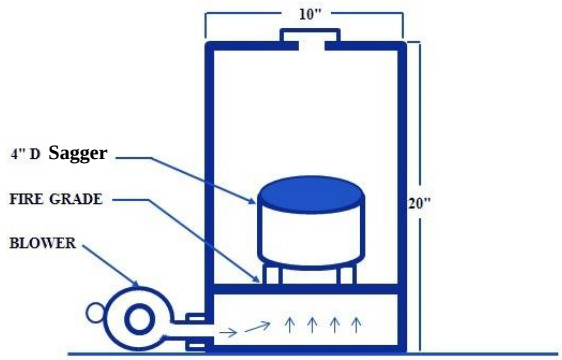

Figure 1 Schematic Diagram of Forced Air Type Updraft Kiln

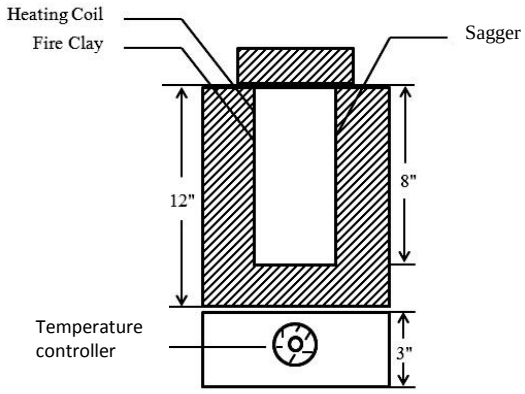

Figure 2 Schematic Diagram of Makeshift Furnace

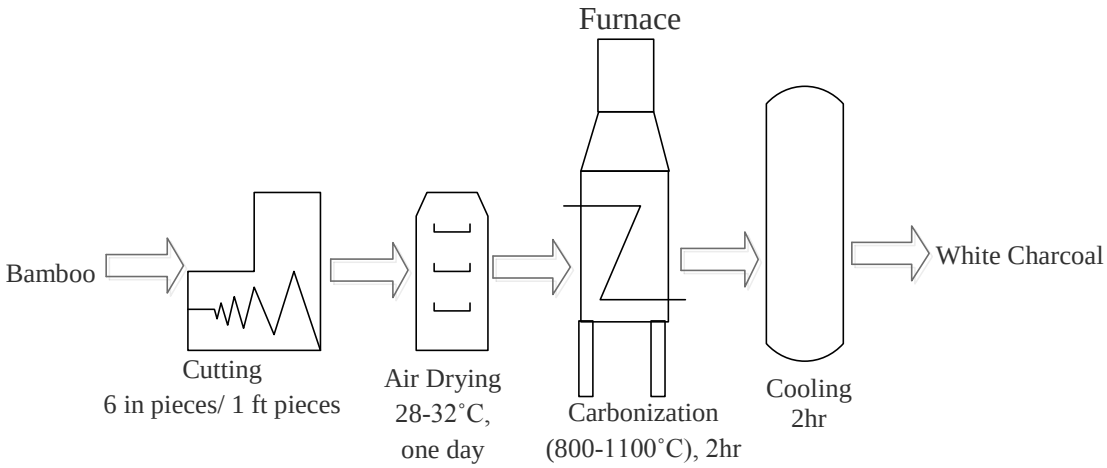

Figure 3 Process Flow Diagram for the Preparation of White Charcoal

Figure 4 Processing of White Charcoal by Forced Air Type Updraft Kiln with Sagger

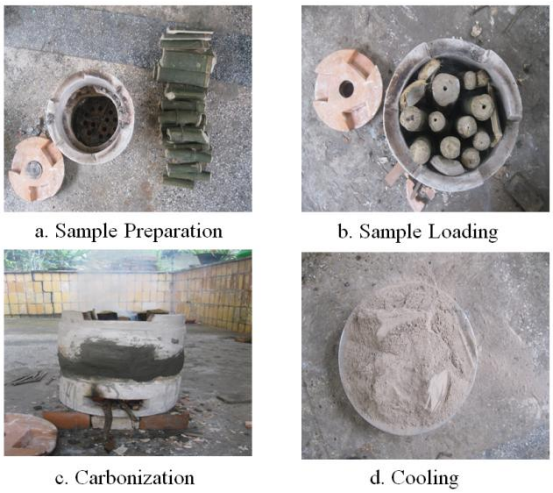

Figure 5 Processing of White Charcoal by Forced Air Type Updraft Kiln without Sagger

Figure 6 Processing of White Charcoal by Makeshift Furnace

Analysis of Samples

For the determination of decolourization property of charcoal, 2 grams of charcoal was soaked in 100 ml of 0.01 M Potassium Permanganate solution for 3 days. Absorbance was determined using Spectrophotometer.

Before carbonization, the analysis of raw bamboo was carried out according to ASTM and TAPPI standard methods. After carbonization, the analysis of charcoal was also carried out using the same procedure. The standard methods used for the analysis are listed as follows:

Determination of Moisture Content (ASTM D 2867-04)

Determination of Volatile Matter Content (ASTM D 5832-98)

Determination of Ash Content (ASTM D 2866-94)

Determination of Fixed Carbon Content (ASTM D 3172)

Determination of Acid Insoluble Lignin (T 222 om-02)

Determination of 1% Sodium Hydroxide Solubility (T 212- om 02)

Determination of Alcohol-benzene Extractives (T 204 cm-97)

Determination of Moisture Content (ASTM D 2867-04)

About (1) g of charcoal sample was weighed in a clean, dry , and weighed watch glass and it was added into a dry and tared porcelain crucible and heated at 105 ºC in an oven for (1) hour. After that, the sample was cooled in a desiccator and recorded the weight. The process was repeated until the constant weight could be obtained. The percentage loss in weight has been calculated as follows:

$$M_{,\%} = B - C/B - A \times 100$$

Where:

A = mass of porcelain crucible (g)

B = mass of porcelain crucible plus sample before drying (g)

C = mass of porcelain crucible plus sample after drying (g)

M = percentage of moisture in the analysis of sample

Determination of Volatile Matter Content (ASTM D 5832-98)

About (1) g of charcoal sample was weighed in a clean, dry , and weighed watch glass and added it into a dry and tared porcelain crucible. The crucible with lid containing the sample was kept inside the muffle furnace and heated to 950℃ for 7 minutes. Then, the sample was cooled in a desiccator for 1 h and weighed. The percentage of volatile matter in the sample has been calculated as follows:

$$\text{Volatile matter, \%} = [(A - B) / A] \times 100$$

Where: A = grams of sample before heating

B = grams of sample after heating at 950℃

Determination of Ash Content (ASTM D 2866-94)

About (1) g of charcoal sample was weighed in a clean, dry , and weighed watch glass and added it into a dry and tared porcelain crucible. The crucible with lid containing the sample was kept inside the muffle furnace and heated to 750℃ for 4 hours. The crucible with lid in place was cooled in a desiccator for 1 hour and weighed. The percentage of ash in the sample could be calculated as follows:

$$\text{Ash, \%} = (D / C) \times 100$$

Where: C = grams of sample before heating

D = grams of residue

Determination of Fixed Carbon Content (ASTM D 3172)

If the moisture content, the ash content and volatile matter content are known, then the content of fixed carbon could be calculated as follow:

$$C, \% = 100 - (M + A + VM)$$

Where: C - Fixed carbon content (%)

M - Moisture content (%)

VM - Volatile matter content (%)

A - Ash content (%)

Determination of Acid Insoluble Lignin (T 222 om-02)

The carbohydrates in wood and pulp are hydrolyzed and solubilized by sulfuric acid; the acid-insoluble lignin is filtered off, dried, and weighed. For each determination, the lignin content in the test specimen was calculated as follows:

$$\text{Lignin \%} = A/W \times 100$$

Where : A= weight of lignin, g

W= oven-dry weight of test specimen, g

Determination of Alcohol-benzene Extractives (T 204 cm-97)

The extractive content was calculated as follows:

$$\text{Extractables, \%} = [(W_e - W_b)/W_p] \times 100$$

where, W_e = oven-dry weight of extract, g

W_p = oven-dry weight of wood or pulp, g

W_b = oven-dry weight of blank residue, g

Determination of 1% Sodium Hydroxide Solubility (T 212- om 02)

Wood or pulp is extracted with hot 1% sodium hydroxide solution for 1 h. The loss in weight is determined and calculated as percent solubility. The percent solubility (*S*) was calculated as follows:

$$S = \frac{A-B}{A} \times 100$$

Where, *A* = oven-dry weight of the test specimen before extraction, g

B = oven-dry weight of the test specimen after extraction, g

Results and Discussion

The quality expressed by moisture, ash, volatile matter and fixed carbon content for bamboo, prepared charcoal samples and commercial samples from Myanmar and Japan are given in Tables (1) and (2). The colour (absorbance) of decolourized Potassium Permanganate solution by prepared charcoal samples are shown in Table (3).

Table 1 Characteristics of Two Bamboo Samples

Sr. No.	**Characteristics**	**Kyakhatwa (*Bambusa arundinacea*)**	**Kyathaungwa *(Bambusa polymorpha)***
1.	Moisture	7.21	
2.	Ash	2.00	4.00
3.	Volatile Matter	82.40	79.21
4.	Fixed Carbon	8.7	8.58
5.	Lignin	25.24	25.60
6.	Alcohol- benzene Extractives	3.2	4.29
7.	1% NaOH Extractives	20.24	24.92

Table 2 Comparison of the Physico-chemical Properties of Prepared Charcoal Samples and Commercial White Charcoals

Sr. No.	**Types of Charcoal**	**Types of Kiln**	**Physico-chemical Properties**			
			Moisture	**Ash**	**Volatile Matter**	**Fixed Carbon**
			(%w/w)			
1.	*White Charcoal	Brick Kiln	9.4	5.28	10.34	74.98
2.	**White Charcoal		9.04	1.19	3.87	85.9
3.	Kyakhatwa	Forced Air Type Updraft Kiln (Using Sagger)	5	9	28	58
4.	Kyathaungwa		**10**	**3**	11	76
5.	Kyakhatwa	Forced Air Type Updraft Kiln (without Sagger)	5	**11**	**29**	**55**
6.	Kyathaungwa		9	**11**	22	58
7.	Kyakhatwa	Makeshift Furnace	**3.33**	5.57	7.79	**83.31**
8.	Kyathaungwa		5.26	4.13	**4.37**	**86.24**

*White charcoal from Myanmar Yoshida Co.Ltd.
**White Charcoal from Japan (http://www.mokutanya.jp)

Table 3 Colour (Absorbance) Values of Decolourized Potassium Permanganate Solution by Prepared Charcoal Samples

Sr. No.	Types of Charcoal (Decolourizing Agents)	Types of Kiln	Colour (Absorbance Values)	
			Potassium Permanganate Solution	Decolourized Potassium Permanganate Solution
1.	*White Charcoal	Brick Kiln	1.832	0.139
2.	**White Charcoal			1.289
3.	Kyakhatwa	Force Air Type Updraft Kiln (Using Sagger)		**0.027**
4.	Kyathaungwa			0.103
5.	Kyakhatwa	Force Air Type Updraft Kiln (without Sagger)		**0.021**
6.	Kyathaungwa			**0.027**
7.	Kyakhatwa	Makeshift Furnace		**0.023**
8.	Kyathaungwa			0.141

Colour was determined using Spectrophotometer (TRSP-722).

*White charcoal from Myanmar Yoshida Co.Ltd.
**White Charcoal from Japan (http://www.mokutanya.jp)

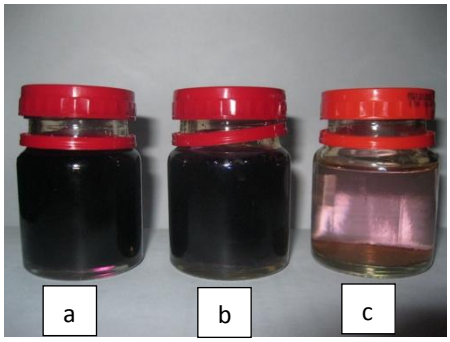

Figure 7 (a) $KMnO_4$ before Decolourization Decolourized $KMnO_4$ by Using Commercial White Charcoals from: (b) Japan (c) Myanmar

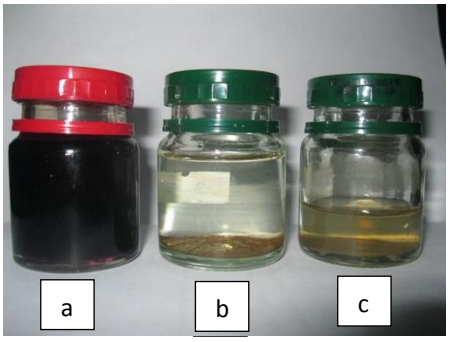

Figure 8 (a) $KMnO_4$ before Decolourization Decolourized $KMnO_4$ Solution by Using White Charcoals from: (b) Kyakhatwa (c) Kyathaungwa Prepared by Forced Air Type Updraft Kiln with Sagger

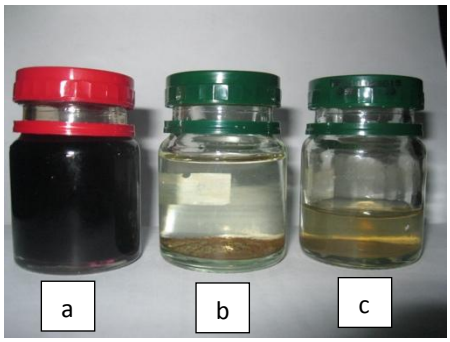

Figure 9 (a) $KMnO_4$ before Decolourization Decolourized $KMnO_4$ Solution by Using White Charcoals from: (b) Kyakhatwa (c) Kyathaungwa Prepared by Forced Air Type Updraft Kiln without Sagger

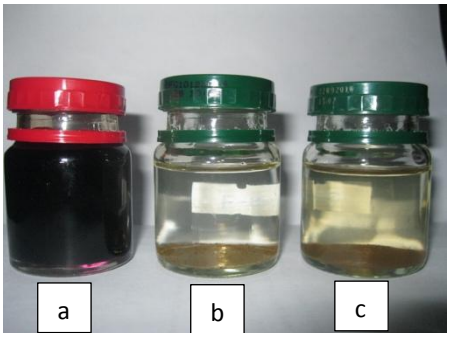

Figure 10 (a) $KMnO_4$ Solution before Decolourization Decolourized Potassium Permanganate Solution by Using White Charcoals from: (b) Kyakhatwa (c) Kyathaungwa Prepared by Makeshift Furnace

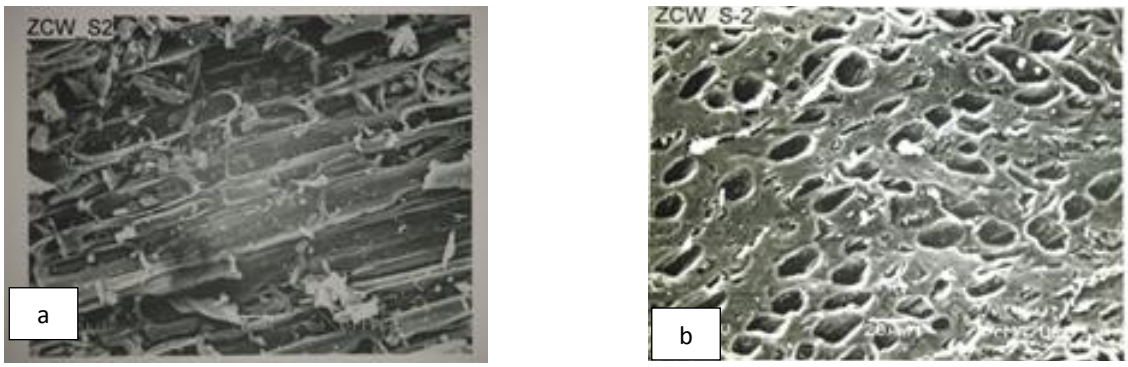

Figure 11 SEM Micrograph of (a) White Charcoal Produced by Myanmar Yoshida Company Ltd. (b) White Charcoal from Japan

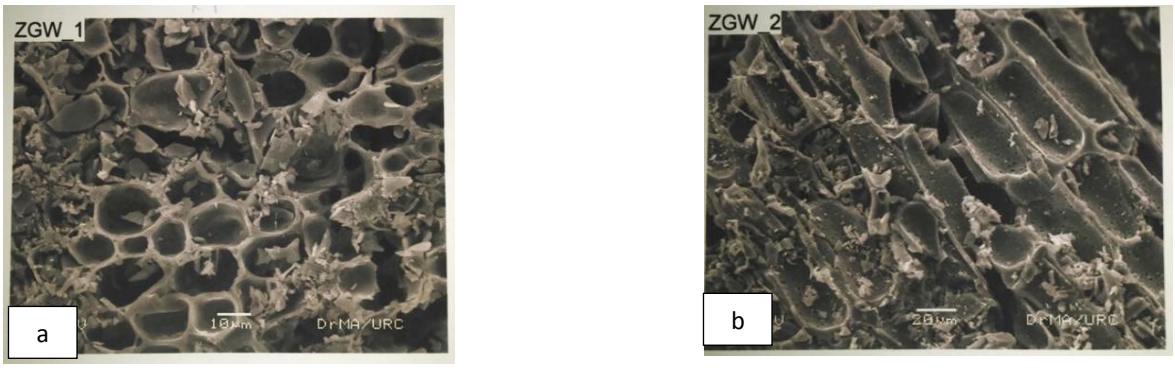

Figure 12 SEM Micrograph of White Charcoal from: (a) Kyathaungwa (b) Kyakhatwa prepared by Forced Air type Updraft kiln Using Sagger

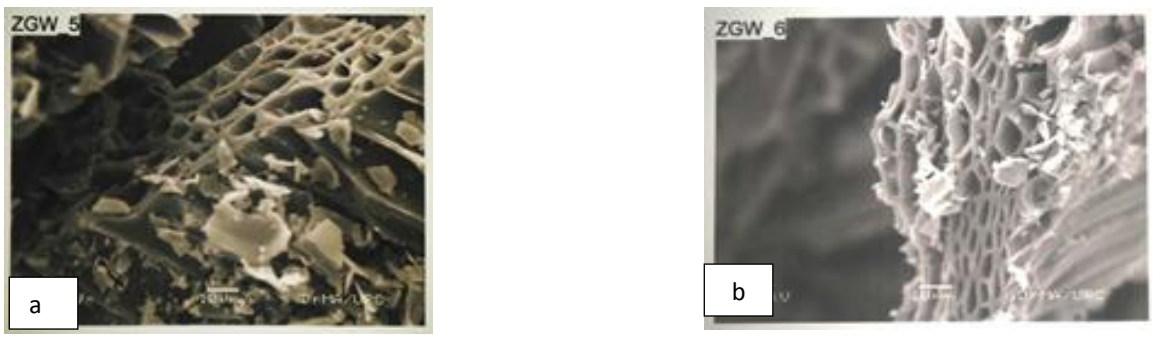

Figure 13 SEM Micrograph of White Charcoals from: (a) Kyathaungwa (b) Kyakhatwa Prepared by Forced Air Type Updraft Kiln Without Using Sagger

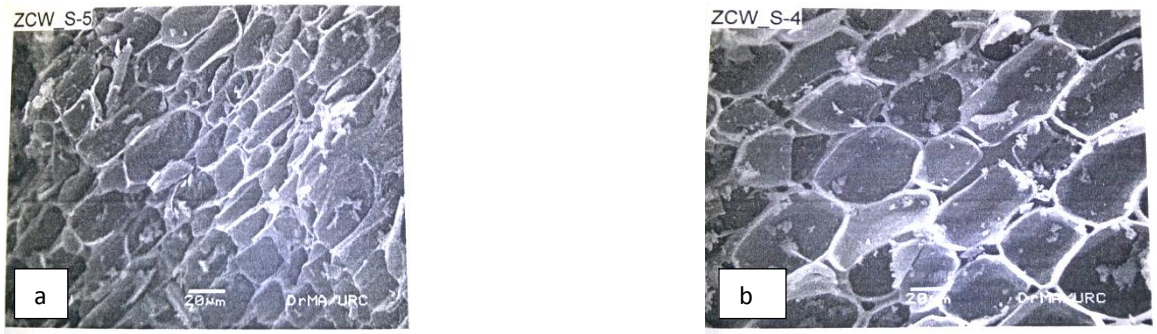

Figure 14 SEM Micrograph of White Charcoals from: (a) Kyathaungwa (b) Kyakhatwa Prepared by Makeshift Furnace

As shown in Table (1), the moisture contents of two types of bamboo (Kyakhatwa (*Bambusa arundinacea*) and Kyathaungwa *(Bambusa polymorpha)*) were 7.21%w/w and 8.21%w/w respectively. The moisture content of bamboo directly affects the time of carbonization and fuel consumption. If the moisture content is too high, the drying time of bamboo pyrolysis will take longer and the carbonizing cycle will continue with higher fuel consumption. The lower moisture content of bamboo makes the bamboo pyrolysis process faster. However, the strength of charcoal is reduced if the moisture content of bamboo is too low (Jian,2004). The ash contents of two types of bamboo were 2 %w/w and 4 %w/w respectively. The volatile matter contents of both types of bamboo was nearly the same as that of wood and other biomass (approximately 80 %w/w). These values were 82.40 %w/w and 79.21 %w/w respectively. The fixed carbon contents of raw bamboos were 8.7 %w/w and 8.58 %w/w respectively.

The acid soluble lignin in both types of bamboo were carried out to determine the hardness of bamboo that was associated with the lignin content. Wood contains about 20-30% lignin. The raw bamboo contains the lignin content of 25.24 %w/w and 25.60 %w/w respectively. Therefore, it might be proved that both types of bamboo are as hard as wood and suitable for charcoal production. Solvent extractives was used to know the amount of solvent-soluble, nonvolatile materials in raw bamboo. The alcohol-benzene extractives values of bamboo were 3.2%w/w and 4.29 %w/w respectively. The amount of solvent extractable matter is markedly influenced by seasoning or drying of bamboo (Jian,2004). 1% NaOH extractives was done to determine the 1%NaOH solubility of bamboo. 1% NaOH extractives values of bamboo were 20.24 %w/w and 24.92 %w/w respectively. The solubility has been related to the strength of bamboo (Jian,2004). Appropriate bamboo moisture content is therefore essential for pyrolysis, and Jian, 2004, reported that 15 percent ~20 percent bamboo moisture content is favorable for carbonization in an external heating pyrolyzing kettle.

Based on the results from Table (1), the strength, hardness and other physico-chemical properties of two types of bamboo were not different significantly. Therefore, both types of bamboo were suitable for the preparation of charcoal. Charcoal made by makeshift furnace from Kyathaungwa *(Bambusa polymorpha*) was found to have the highest fixed carbon content (86.24 %w/w) among the charcoals samples made by different methods and also higher than that of two commercial white charcoals. The fixed carbon content of white charcoal samples from Myanmar and Japan were 74.98 %w/w and 85.9 %w/w respectively. Due to the difference in heating methods and types of furnaces, bamboo white charcoals contained fixed carbon from 55 %w/w to 86.24 %.w/w. With the increasing carbonizing temperature, the corresponding

percentage of fixed carbon in bamboo white charcoal might increase. The charcoal prepared from Kyakhatwa using makeshift furnace had the lowest moisture content of 3.33 %w/w. The highest moisture content was found for the charcoal prepared from Kyathaungwa using forced air type updraft kiln with sagger. The moisture of charcoal determines the quality of the product.

The bamboo charcoal releases gaseous offspring such as CO, CO_2, H_2, CH_2 and other hydrocarbons labeled as volatile matter when the bamboo is carbonized under high temperature. The lowest volatile matter content (4.37 %w/w) was found for the charcoal made from Kyathaungwa using makeshift furnace. The charcoals produced from Kyakhatwa *(Bambusa arundinacea)* by forced air type updraft kiln using sagger and without sagger contain the high volatile matter contents (28 %w/w and 29 %w/w). The ash of bamboo charcoal is its inorganic constituent, which is a white or shallow red substance after bamboo charcoal has been burned completely at high temperature. Bamboo charcoal ash elements are complex, all inorganic components in bamboo, mostly Si, K, Mg, Na, Mn, etc. remain in ash. The ash content of white charcoal samples from Kyakhatwa *(Bambusa arundinacea)* and Kyathaungwa *(Bambusa polymorpha*) produced by force air type updraft kiln without sagger were found to have the same value (11 %w/w). This high value of ash content showed that both samples contain a large amount of silicon. From these results, it is possible to predict that the carbonization was not completed. The lowest ash content (3%w/w) was found for the charcoal sample prepared from Kyakhaungwa using forced air type updraft kiln with sagger. The lower contents of moisture, volatile matter and ash, and the higher content of fixed carbon revealed that the prepared charcoal samples are of good quality. In comparison of the physico-chemical properties especially fixed carbon content of prepared charcoal with commercial white charcoal from Japan and Myanmar, the fixed carbon contents of charcoal prepared from both types of bamboo were higher than that of commercial white charcoal. These values were 83.31 %w/w and 86.24 %w/w respectively.

The decolourization property of prepared charcoal and commercial charcoals were determined using potassium permanganate solution. The results were shown in Table (3) and Figures (7 to 10). According to the results, the colour absorbance value of potassium permanganate solution before decolourization was 1.832. After decolourization with prepared charcoal, the absorbance value of solution was reduced noticeably. Among them, the absorbance values of solution that was decolourized using Kyakhatwa prepared by both makeshift furnace and forced air type updraft kiln with and without using sagger were lowered than that of prepared charcoal (Kyathaungwa) and commercial charcoals. These values were 0.027,0.023 and 0.021 respectively. However, the white charcoal (Kyathaungwa) prepared by using forced air type updraft kiln without using sagger also showed lower absorbance values (0.027) than that of commercial charcoals. It means that both types of white bamboo charcoal prepared by forced air type updraft kiln without using sagger has higher decolourization property than that of commercial white charcoal.

Adsorption capability is one of bamboo charcoal's important characteristics. Since bamboo charcoal forms a lot of pores after pyrolyzing at high temperatures, similar to wood charcoal, it has a broad specific surface adsorption ability (Jian, 2004). The pore structures of the prepared white charcoal samples and commercial white charcoal samples were examined by SEM. Figures (11 to 14) showed SEM micrographs of Kyakhatwa *(Bambusa arundinacea)* and Kyathaungwa *(Bambusa polymorpha*) charcoal prepared by makeshift furnace and forced air type updraft kiln, and of commercial white charcoal samples. It was obvious that all white

charcoal samples prepared from bamboo had a greater number of pores than commercial white charcoals. Furthermore, charcoal samples prepared from Kyakhatwa *(Bambusa arundinacea)* and Kyathaungwa *(Bambusa polymorpha)* by makeshift furnace contained the high number of pores which indicated relatively large surface areas for absorption (Figure 14).

Conclusion

According to the results, the physico-chemical properties (i.e in terms of fixed carbon content) of white charcoal made from Kyathaungwa *(Bambusa polymorpha*) by using Makeshift furnace were higher than that of commercial white charcoal. It can be concluded that Kyathaungwa *(Bambusa polymorpha*) could be able to use for white charcoal preparation and Makeshift furnace is a suitable furnace for carbonization of bamboo to produce white charcoal. It is predictable that white charcoal made from bamboo would give the higher absorption capacity for decolourization than the white charcoal made from other (like oak, mangrove, etc.,) because of its abundant porous structure detected by SEM.

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A STUDY ON THE PREPARATION OF MODIFIED STARCH FROM BROKEN RICE

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Abstract

This research was emphasized on the preparation of modified broken rice starch using both acid treatment method and cross-link method. Broken rice (Paw Hsan Hmwe) was collected from Bago Township, Bago Region. The most suitable parameters for the preparation of native starch were 1:8 (w/v) ratio of broken rice to water at 4 hr settling time. The optimum conditions for the preparation of modified broken rice starch by acid treatment were 1 mL of 10% HCl, 1mL of 1% NaOH at reaction temperature 65°C for 15 min of reaction time. In cross-link method, the optimum parameters were 5mL of 2.5% sodium tripolyphosphate,5mL of 1% NaOH, 5mL of 5 % HCl at 45°C for 10 min. The characteristics of modified starch such as ash, moisture, pH and gelatinization temperature, solubility, swelling power, amylose and amylopectin content were determined. The morphology properties, molecular components and structures of native and modified broken rice were determined with Scanning Electron Microscopy (SEM) and FT-IR Analysis.

Keywords: Native starches, acid treatment method, cross-link method

Introduction

Starch is a basis of food and plays a major role in industrial economy. The most abundant substance in nature is starch. Starch consists of semi crystalline carbohydrate synthesized in plant roots, seeds, rhizomes and tubers. It is a polymer of glucose and consists of two types of glucose polymers such as amylose and amylopectin. These polymers have different structures and properties. Starch can be converted into many diverse products such as paper, beverages, pharmaceuticals, plastics, textiles and confectioneries either through chemical or biological process depending on the physical and chemical characteristics of the starch (Tester et al. 2004; Nand et al. 2008). Native starches are pure forms of starch. They can be obtained from the source such as corn, wheat, potato, rice, cassava and tapioca. The native starches are modified physically, enzymatically, or chemically to enhance their performance in different applications. Modified starches are better than native starches due to their functional properties.(Kavlani N et al, 2012).

Rice (*Oryza sativa* L.) is an important cereal grain which feeds nearly half of the world's population. Broken rice is fragments of rice grains obtained during drying, during transport or by milling. It is nutritious like the unbroken rice. Broken rice is used to make starch which is used as laundry starch and in foods, cosmetics and textile manufacture. Due to economic reasons the rice starch is extracted preferably from broken rice which is most valuable for numerous industries like food and cosmetic industry. It is also favourable because of its unusual characteristics like small particle size, white color and neutral taste. The objectives of this research work are-to investigate the different chemical modification reactions of starch and to determine the characteristics of modified broken rice starches.

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Materials and Methods

Raw Materials

Broken rice (Paw Hsan Hmwe) was collected from Bago Township, Bago Region. The chemicals used were Analar grade hydrochloric acid (HCL), sodium hydroxide (NaOH), sodium tripolyphosphate ($Na_5P_3O_{10}$).

Methods

Preparation of Broken Rice Starch

Broken rice (100 g) was thoroughly washed with water to remove impurities such as dirt, girt and dust. Then, broken rice was ground in a grinder and the resulting broken rice paste was soaked in water in the ratio of 1:4(w/v %) for 60 min at room temperature by stirring with a constant rate of 150 rpm. The broken rice starch slurry was filtered through a nylon cloth to obtain the starch solution. The settling of starch solution was taken about 4 hr. The supernatant layer of water was decanted to remove out the starch. Water washing of native starch was carried out two times to obtain pure starch. Then, the starch was sundried and then ground in mortar and pestle to get fine powder. Effect of broken rice to water ratio (1:5), (1:6), (1:7), (1:8) and (1:9) (w/v) and effect of setting time (1, 2, 3, 4, 5) hr on the yield of broken rice starch were conducted according to the above procedure.

Preparation of Acid Treated Modified Broken Rice Starch

(5) g of native broken rice sample was mixed thoroughly with 1mL of 10% (w/v) hydrochloric acid in a 250 mL beaker and stirred for 15 min at 30°C. The slurry was neutralized with 1 mL of 1 % of sodium hydroxide. The slurry was washed with water and settled for one day. After that, it was separated by decantation of water. The starch was sun-dried for 3 hr and then ground in motor and pestle to obtain fine powder. Effect of volume of 10% hydrochloric acid (0.5, 1, 1.5,2 ,2.5)ml, effect of concentration of 1 mL of hydrochloric acid(5, 10, 15, 20 , 25)(v/v %) , effect of reaction time(5, 10, 15, 20 ,25) min and effect of reaction temperature (35, 45, 55, 65 , 75) °C on the yield of modified broken rice starch were determined according to the above procedure.

Preparation of Cross-Linked Modified Broken Rice Starch

(5) g of native broken rice sample was mixed thoroughly with 10mL of water for 10min. 5mL of 2.5% sodium tripolyphosphate and 5mL of 1% sodium hydroxide were added into the starch slurry and stirred with small scale batch type mixer for 30min at 30˚C. The pH of the obtained slurry was adjusted to 6-7 using 5mL of 5% hydrochloric acid. The slurry was washed two times with water and settled for one day. Then, water was decanted to separate out the starch. The starch was sun-dried for 90min and ground in motor and pestle to obtain fine powder. The yield percent of modified broken rice starch were determined by varying the effect of volume of 2.5% sodium tripolyphosphate (1, 3, 5, 7 and 9) mL, effect of concentration of 5mL of sodium tripolyphosphate (0.5, 1, 1.5, 2, 2.5 and 3)% (w/v) ,effect of reaction temperature (35, 45, 55, 65 and 75) ˚C and effect of reaction time (5, 10, 15, 20 25) using according to the above procedure.

Determination of the Physicochemical Properties of Native and Modified Broken Rice Starch

The p**hysicochemical properties** of native and modified broken rice starch such as, ash, gelatinization temperature, moisture, pH, solubility, swelling power, amylose and amylopectin content were determined.

Results and Discussion

Figure (1) shows the results of the effect of ratio of broken rice to water on the yield percent of broken rice starch. Although the ratio of broken rice to water was increased from 1:8 (w/v) to 1:9 (w/v), the yield percent of broken rice starch does not apparently increased. So, 1:8 (w/v) ratio of broken rice to water was chosen as the most suitable ratio from the economic point of view. The yield percent of modified broken rice starch were determined by varying the effect of settling time in the range of 1,2,3,4 and 5 hr. Among them, 4 hr settling time was selected as the most suitable condition from the economic point of view. The results are shown Figure (2).

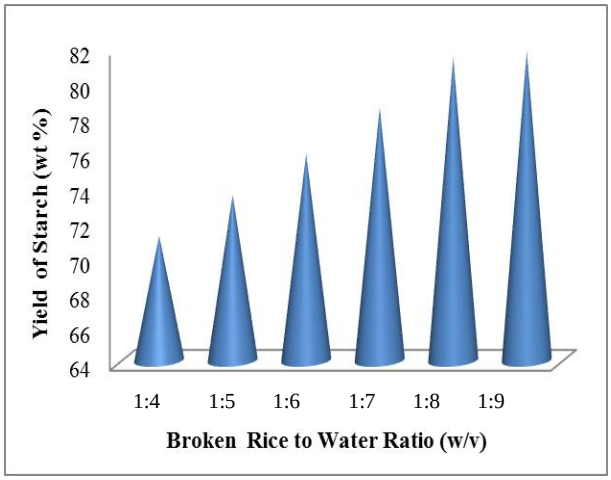


Figure 1 Effect of Broken Rice to Water Ratio on the Yield of Broken Rice Starch

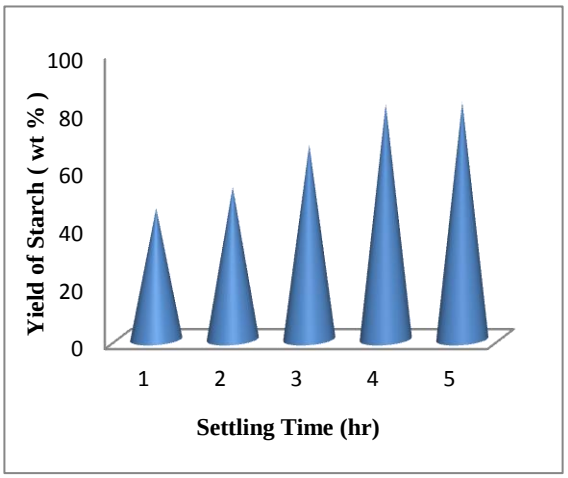


Figure 2 Effect of Settling Time on the Yield of Broken Rice Starch

The results of Table (1) show the effect of volume of 10 % hydrochloric acid on the yield of acid treated modified broken rice starch The volume of 10 % hydrochloric acid beyond 1 mL caused slightly rough texture and the yield percent of modified broken rice starch was decreased. When higher concentration of hydrochloric acid was used in the treatment of starch, non-starchy materials were broken down from slurry. Therefore, 1 mL of 10% hydrochloric acid was chosen as the most suitable condition.

The effect of concentration of 1 mL of hydrochloric acid on the yield of modified broken rice starch is shown in Table (2).The lower the acid concentration, the higher the yield percent of rice broken starch was obtained. Moreover, lower concentration of hydrochloric acid gave a smooth texture of modified broken rice starch and it was more economic. It can also reduce the side effect of the application of high concentration of hydrochloric acid. Therefore 10% of 1 mL hydrochloric acid was selected as the most suitable condition.

Table 1 Effect of Volume of 10% Hydrochloric Acid on the Yield of Acid Treated Modified Broken Rice Starch

Weight of Native Starch Sample - 5 g
Volume of 1%(w/v) NaOH - 1 mL
Reaction Time - 15 min
Reaction Temperature - Room Temp

Sr. No.	Volume of 10% HCl (mL)	Yield (w/w%)	Colour	Texture
1	0.5	84.0	White	Smooth
2	1*	84.8	White	Smooth
3	1.5	84.1	White	Slightly Rough
4	2	83.2	Odd-White	Slightly Rough
5	2.5	83.0	Odd-White	Slightly Rough

*Most suitable condition

Table 2 Effect of Concentration of 1mL Hydrochloric Acid on the Yield of Acid Treated Modified Broken Rice Starch

Weight of Native Starch Sample - 5 g
Volume of HCl - 1 mL
Volume of 1(w/v)% NaOH - 1 mL
Reaction Time - 15 min
Reaction Temperature - Room Temperature

Sr. No.	Concentration of 1mL HCl (v/v%)	Yield (w/w%)	Colour	Texture
1	5	83.8	White	Smooth
2	10*	84.8	White	Smooth
3	15	83.4	White	Slightly Rough
4	20	83.2	Odd-White	Slightly Rough
5	25	83.1	Odd-White	Slightly Rough

*Most suitable condition

Table (3) shows the effect of reaction time on the yield of acid treated modified broken rice starch. The highest yield percent (87.6%) of modified rice broken starch was obtained on the reaction time of 15 min. The yield percent of modified broken rice starch was also investigated by varying the effect of reaction temperature in the range of (35, 45, 55, 65 and 75 °C). The results are shown in Table (4). Among them, 65°C reaction temperature gave the highest yield percent of modified broken rice starch (92.8%) than others. Beyond this temperature, it can be seen that the texture was slightly rough and the yield percent of modified broken rice starch was decreased.

Table 3 Effect of Reaction Time on the Yield of Acid Treated Modified Broken Rice Starch

Weight of Native Starch sample - 5 g
Volume of 10 (v/v) %HCl - 1 mL
Volume of 1(w/v)% NaOH - 1 mL
Reaction Temperature - Room Temperature

Sr. No.	Reaction Time(min)	Yield (w/w%)	Colour	Texture
1	5	86.0	White	Smooth
2	10	86.6	White	Smooth
3	15*	87.6	White	Smooth
4	20	85.6	White	Smooth
5	25	84.8	White	Slightly Rough

*Most suitable condition

Table 4 Effect of Reaction Temperature on the Yield of Acid Treated Modified Broken Rice Starch

Weight of Native Starch Sample - 5 g
Volume of 10% (v/v) HCl - 1 mL
Volume of 1% (w/v) NaOH - 1 mL
Reaction Time - 15 min

Sr. No.	Reaction Temperature (°C)	Yield (w/w%)	Colour	Texture
1	35	87.6	White	Smooth
2	45	89.8	White	Smooth
3	55	91.2	White	Smooth
4	65*	92.8	White	Smooth
5	75	92.9	White	Slightly Rough

*Most suitable condition

By varying the volume of 2.5(w/v)% sodium tripolyphosphate such as 1,3,5,7 and 9 mL, the effect of volume of 2.5 (w/v)% of sodium tripolyphosphate on the yield of modified rice broken starch was studied. It was found that 5mL of 2.5% sodium tripolyphosphate gave 90.0 wt% of modified rice broken starch, which is the highest yield among them. The results are shown in Table (5). Highest concentration of sodium tripolyphosphate gave white and smooth texture of modified rice broken starch. 2.5 % and 3 % of sodium tripolyphosphate gave the 94.6 % and 93.8 % of modified rice broken starch respectively. Between them, 2.5 % of sodium tripolyphosphate was selected as the most suitable condition due to its higher yield percent .The results of the effect of concentration of sodium tripolyphosphate on the yield percent of cross-linked modified broken rice starch are shown in Table (6).

The yield percent of modified rice broken starch was investigated by varying the effect of reaction temperature on in the range of (35, 45, 55, 65 and 75)˚C. Among them, 45˚C of reaction temperature gave the highest yield percent of modified rice broken starch 99.6% than the others.

Beyond this temperature modified rice broken starch absorbed water to form gel. The results are shown in Table (7).The longer the reaction time, the lower the yield % of modified rice broken starch and also the colour of modified rice broken starch was found to be the odd white colour. So, reaction time 10 min was selected as the most suitable condition. The results are shown in Table (8).

Table 5 Effect of Volume 2.5% (w/v) of Sodium Tripolyphosphate on the Yield of Cross-Linked Modified Broken Rice Starch

Weight of Native Starch Sample - 5g
Water - 10 mL
Volume of 1% (w/v) NaOH - 5 mL
Volume of 5% (v/v) HCl - 5 mL
Reaction Temperature - 38˚C
Reaction Time - 10 min

Sr. No.	Volume of 2.5% (w/v) of Sodium Tripolyphosphate (mL)	Yield (wt %)	Colour	Texture
1	1	86.8	White	Smooth
2	3	89.2	White	Smooth
3	*5	94.6	White	Smooth
4	7	86.8	White	Slightly Rough
5	9	84.8	Odd White	Slightly Rough

*Most suitable condition

Table 6 Effect of Concentration of 5mL of Sodium Tripolyphosphate on the Yield of Cross-Linked Modified Broken Rice Starch

Weight of Native Starch Sample - 5g
Water - 10 mL
Volume of 1% (w/v) NaOH - 5 mL
Volume of 5% (v/v)HCl - 5 mL
Reaction Temperature - 38˚C
Reaction Time - 10 min

Sr. No.	Concentration of 5mL of Sodium Tripolyphosphate (%)	Yield (wt %)	Colour	Texture
1	0.5	84.2	White	Smooth
2	1	90.6	White	Smooth
3	1.5	91.0	White	Smooth
4	2	91.2	White	Smooth
5	*2.5	94.6	White	Smooth
6	3	93.8	White	Slightly Rough

*Most suitable condition

Table 7 Effect of Reaction Temperature on the Yield of Cross-Linked Modified Broken Rice Starch

Weight of Native Starch Sample - **5g**
Water - 10 mL
Volume of 1(w/v)% NaOH - 5 mL
Volume of 5(v/v)% HCl - 5 mL
Volume of 2.5% STPP - 5mL
Reaction Time - 10 min

Sr. No.	Reaction Temperature (˚C)	Yield (wt %)	Colour	Texture
1	35	96.6	White	Smooth
2	*45	99.6	White	Smooth
3	55	95.2	White	Slightly Rough
4	65	94.8	White	Slightly Rough
5	75	90.0	White	Slightly Rough

*Most suitable condition

Table 8 Effect of Reaction Time on the Yield of Cross-Linked Modified Broken Rice Starch

Weight of Native Starch Sample - 5g
Water - 10 mL
Volume of 1(w/v)% NaOH - 5 mL
Volume of 5(v/v)% HCl - 5 mL
Volume of 2.5(w/v)% $Na_5P_3O_{10}$ - 5mL
Reaction Temperature - 45°C

Sr. No.	Reaction Time (hr)	Yield (wt %)	Colour	Texture
1	5	96.2	White	Slightly Rough
2	*10	96.6	White	Smooth
3	15	92.6	White	Smooth
4	20	92.4	White	Smooth
5	25	92.2	White	Smooth

*Most suitable condition

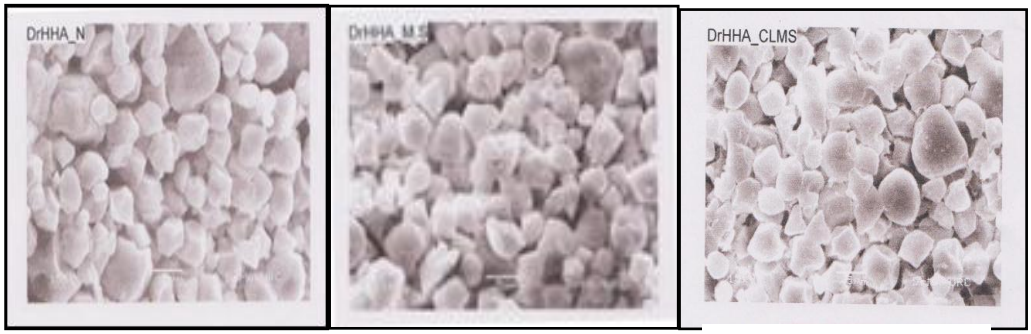
The physicochemical properties of native and modified broken rice starch such as amylose and amylopectin content, ash, gelatinization temperature, moisture, pH, solubility and swelling power, were determined and the results are shown in Table (9). Amylose was the major factor influencing the physico-chemical properties of starch. Starch swelling was property of amylopectin content whereas higher amylose content limited it. During modification, the amylose content was increased due to hydrolysis of amylopectin. Among the two methods of modification of starch, cross –link modification generated significant changes in starch behavior, gelatinization capacity, solubility and paste properties than acid treatment method.

Table 9 Characteristics of Native and Modified Broken Rice Starches

Characteristics	NS		AMS		CMS	
	E.V	***LV**	**E.V**	***LV**	**E.V**	***LV**
Ash (wt %)	1.57	-	1.53	-	1.52	-
Gel.Temp. (^{o}C)	60	-	68	-	65	-
Moisture (wt %)	8.66	-	8.36	-	8.41	-
Solubility	4.0	-	4.8	-	6.0	-
Swelling Power	1.12	-	1.00	-	0.89	-
pH	6.4	-	6.32	-	6.46	-
Amylose Content (wt%)*	24	15-35	32	38.62±0.6	40	48.41±0.6
Amylopectin Content (wt%)	76	65-85	68	61.39±0.6	60	51.59±0.6

NS- Native Starch
AMS- Acid Treated Modified Starch
CMS- Cross-Link Modified Starch
E-V- Experimental Value
*L-V – Literature Value
Source: www.scielo.br
www.onlinelibary.wiley.com

The native broken rice starch granules are polygonal shape with well-defined edges and smooth surface. The acid treated and cross link modified broken rice starch granules are oval, spherical and irregularly shaped with slightly rough surface and pores. The SEM photomicrograph of native and modified starches of broken rice is shown in Figure (3). SEM images of the granules of native and modified starches showed difference in their appearance.



Native Broken Rice Starch | Acid Treated Modified Broken Rice Starch | Cross-Link Modified Broke Starch

Figure 3 SEM Photomicrograph of Native and Modified Broken Rice Starches

FT-IR spectroscopy was used to verify the structural changes of native and modified broken rice starch. Figures (3 and 4) show the FT-IR spectra of native and modified broken rice starches. In native and modified broken rice starch, the IR spectrum of starch samples was described by seven main modes, with maximum absorbance peaks near 3500, 2900, 1600, 1400, 1000, 800, and 500 cm^{-1}. The peaks at 3664 cm^{-1} and 2929 cm^{-1} in native starch and 3630 cm^{-1} and 2933 cm^{-1} in modified starches could be attributed to O-H and C-H bond stretching , while the peaks in native starch at 1672 cm^{-1} and 1373 cm^{-1} and the bonding modes of H-C-H ,C-H and O-H attributed to the peaks in modified starches at 1645 cm^{-1} and 1413 cm^{-1}. C-O-H stretching in both native and modified starches attributed to the peaks at 1300~1000 cm^{-1} .The bands at 763±10 cm^{-1} were attributed to

D-glucopyranosyl ring stretching. The 1645~1600 cm^{-1} bands were assigned to H_2O bending vibrations.

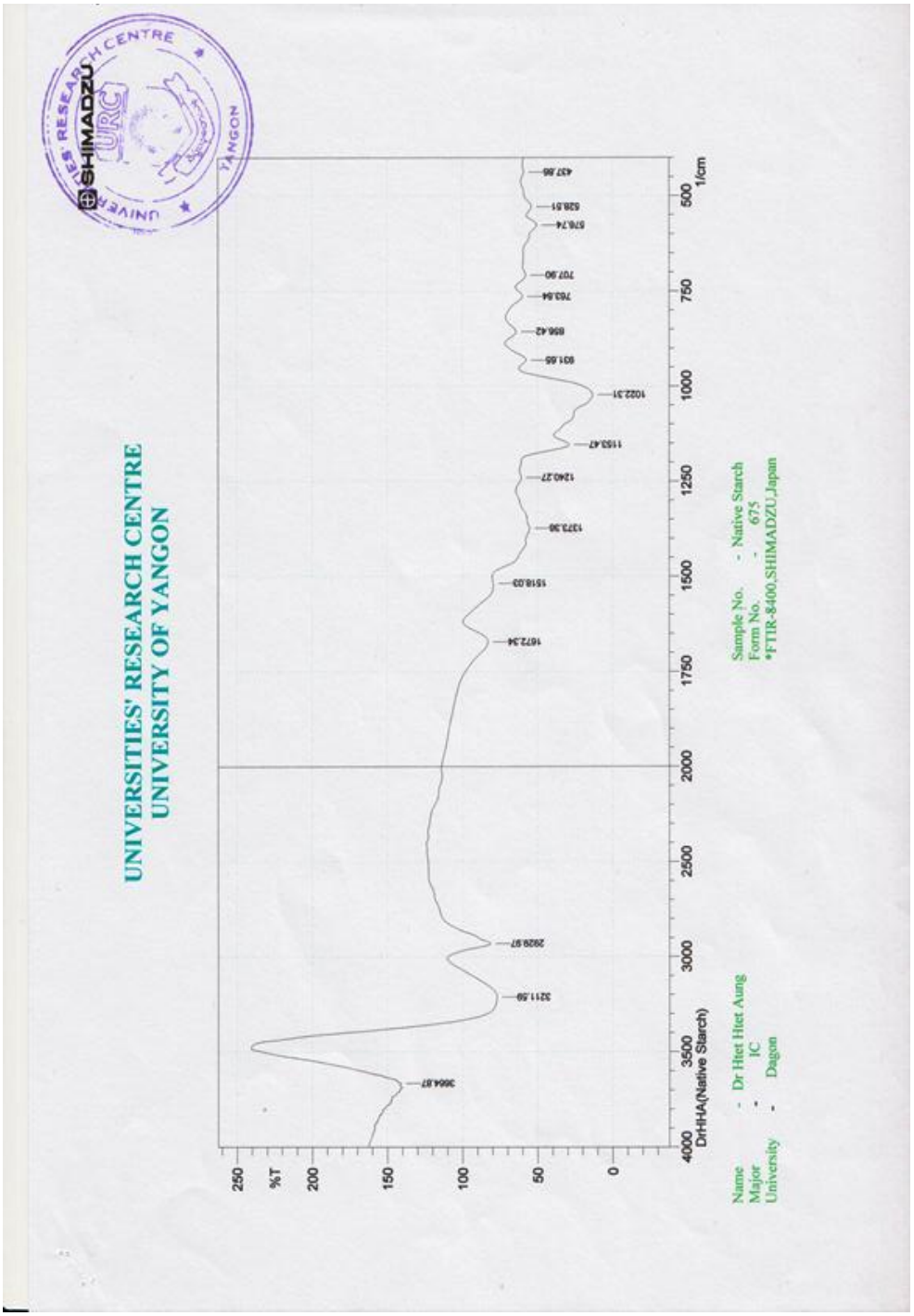


Figure 4 FT-IR Spectrum of Native Broken Rice Starch

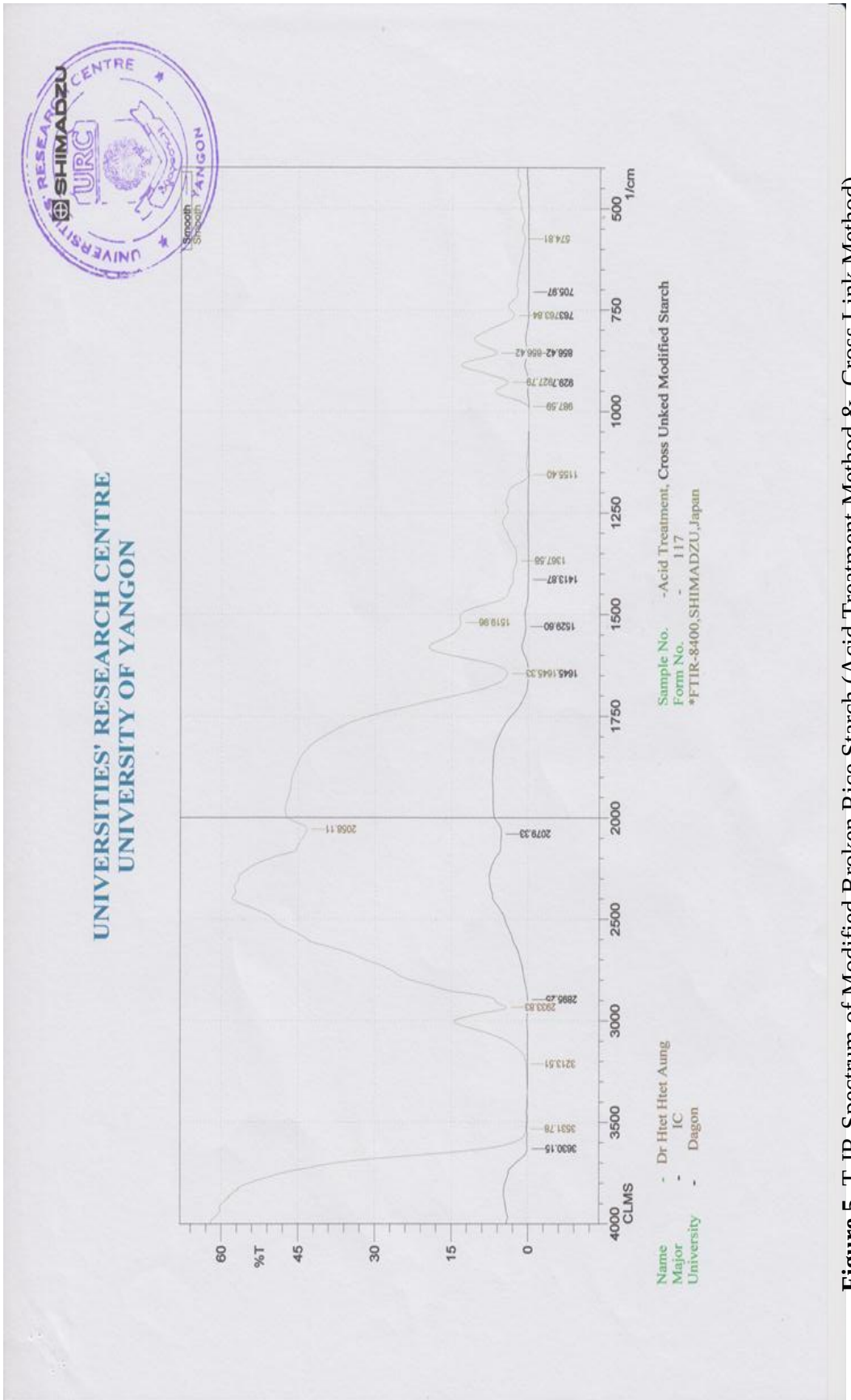


Figure 5 T-IR Spectrum of Modified Broken Rice Starch (Acid Treatment Method & Cross Link Method)

Conclusion

Native starch was prepared from broken rice and then it was modified using both acid treatment method and cross linked method. The most suitable conditions of the preparation of native starch were found to be 1:8 of the ratio of raw to water and 4 hr of settling time. The most favorable conditions for the preparation of modified starch by acid treatment were 1 mL of 10% HCl, 1mL of 1% NaOH at reaction temperature 65^{o}C for 15 min of reaction time and 5ml of 2.5% $Na_5P_3O_{10}$, 5mL of 1% NaOH, 5mL of 5 % HCl at 45^{o}C reaction temperature for 10 min of reaction time for cross-link method. The characteristics of native and modified starch were investigated. The morphology properties, molecular components and structures of native and modified broken rice were determined with Scanning Electron Microscopy (SEM) and FT-IR Analysis. . According to the results of the characteristics and yield % of modified starch using two different methods, the quality of cross-linked modified starch is better than the acid treated modified starch.

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