IDENTIFICATION AND EXTRACTION OF ANTIBACTERIAL METABOLITES OF SELECTED ENDOPHYTIC FUNGUS SL-37 AGAINST *ESCHERICHIA COLI*

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Abstract

The present work was focused on identification of selected endophytic fungus SL-37 from *Prunus persica* (L.) BATSCH., Met-mon leaves, (Mogok Township, Mandalay Region in 2017) and extraction of antibacterial metabolites. According to the result of macroscopic and microscopic characters, SL-37 was identified as *Aspergillus* sp. According to the paper chromatography values, *Aspergillus* sp was produced antibacterial metabolites by using ethyl acetate solvents. The equal ratio (1:1 v/v) ethyl acetate extract was showed higher inhibitory effect (23.91 mm) than (1:1 v/v) n-butanol extract (20.15 mm) against *E coli*. Crude ethyl acetate (5g) was obtained from 20 Liter of fermented broth SL-37. Then the crude extract of SL-37 was adjusted to pH 4, 5, 6, 7, 8, 9 and 10 and pH 6.0 was showed the maximum inhibitory antibacterial activity (23.18 mm) than other pH against *E coli*. These results suggested that the selected endophytic fungus may be utilized for screening the antibacterial metabolites.

Keywords: Endophytic fungi**,** identification, extraction

Introduction

Endophytic fungi inhabit a biotype that is not well studied (Nithya and Muthumary, 2011). This means the opportunity to find new and targeting natural products from interesting endophytic microorganisms, among the myriad of plants in different niches and ecosystems, is great. Endophytes are the chemical synthesizers inside plants (Owen and Hundley, 2004). Endophytic organism especially fungi have enormous potential to produce large range of bioactive secondary metabolites in order to protect their host plant against pathogens (Strobe, 2003). Thus, the search for novel secondary metabolites should concentrate on organisms that inhabit novel biotypes.

These microorganisms are recognized as potential sources of novel natural products for exploitation in medicine, agriculture, and industry. These microorganisms are recognized as potential sources of novel natural products for exploitation in medicine, agriculture, and industry.

The morphological characterization of the fungal isolates were observed and described based on the method of Photita *et al,* 2004. Further identification of fungal isolates was based on the standard taxonomic key included colony diameter, texture, colour, morphology of hyphae and conidia Hyde *et al,* 2000. Traditionally, clinical microbiology laboratories have relied heavily on morphology-based identification methods to differentiate *Aspergillus* species. However many species, especially members of the section *Fumigati* have overlapping morphological characteristics, which has allowed several genetically distinct species to be misidentified (Balajee *et al,* 2005, 2007). This has led to the clustering of species with overlapping morphologies into "species complexes", so that laboratories may report more accurately morphology-based identifications.

Paper Chromatography is a technique that is used to separate and to identify components of a mixture. Paper chromatography is one of the most important and simple chromatographic methods. Paper chromatography has proved to be very successful in the analysis of chemical

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compound and lipid sample in particular. In this chromatography, it uses paper as the stationary phase and a liquid solvent as the mobile phase the sample mixture is placed on a piece of paper, the edge of the paper is carefully immersed in a solvent, after that the solvent moves up the paper due to capillary action. Components of the mixture are carried along with the solvent up the paper to varying degrees, in other words the components of the mixture rise up at different degrees and thus are separated from one another depending on the compound's preference to be adsorbed onto the paper versus being carried along with the solvent. In order to obtain the extent of movement of a component in a paper chromatography, we can calculate retention factor "R_f value" for each separated component in the developed chromatogram. The R_f value is a number that is defined as the ratio of the distance traveled by the solute to the distance traveled by the solvent.

In this present study, *Aspergillus* was isolated from *Prunus persica* (L.) BATSCH, Metmon plant and extraction of antibacterial compounds were studied. Especially, the large number of study have carried out on the antimicrobial compounds produced from endophytic plants. In Myanmar, *Aspergillus* and its antibacterial compounds from *Prunus persica* (L.) BATSCH., (Metmon) leaves has not been carried out yet. Therefore, identification and extraction of antibacterial metabolites produced by *Aspergillus* against *E coli* was mainly studied in this research. In this study, the aim and objectives of present research were to identify the selected endophytic fungus and extraction of antibacterial compounds from this fungus.

Materials and Methods

Identification of selected fungus SL-37

The selected fungus was cultured on eight differential media ie. Blakeslee's Malt Extract Agar (BMEA) medium, Czapek-Dox Agar (CZA) medium, Malt Extract Agar (MEA) medium, Glucose Ammonium Nitrate Agar (GAN) medium, Dichloran-Rose Bengal- Chloramphenicol Agar (DRBC) medium, Potato Dextrose Agar (PDA) medium, low carbon Agar (LCA) medium and Water Agar (WA) medium (after five days of incubation) were observed for macroscopic characteristics such as colony diameter, colony colour and microscopic characteristics including conidiophore, vesicles, phialides and conidia. For microscopic characteristics slides were stained with cotton blue and mounted in lactophenol cotton blue.

Microscopic examination with lactophenol cotton blue

The drop of LPCB was placed on a clean glass slide. With a bent dissection needle, a small portion of the colony was removed from the agar surface and it was placed in the drop LPCB with two dissection needles, apart the mycelial mass of the colony gently teased on slide, with a coverslip was covered, and under the light microscope was observed with low power (X40) magnification.

Paper Chromatography (Tomita, 1988)

The filter paper and four solvents (20% NH_4Cl n-Butanol saturated with water, n-Butanol-Acetic- Water (3:1:1) and ethyl acetate saturated with water) were used for preliminary characterization of compounds. The obtained fermentated broth sample (100 µL) was applied on the paper and allowed to dry. The papers were chromatographed in each solvent. Then, bioautography was done to check the antibacterial activity of each paper. Each paper was placed on assay agar plate. After one hour the paper was taken out, the plates were incubated for 24-36 hours. In this case, the inhibitory zone was measured yielding the R_f value for the corresponding bioactive compound.

$$R_f \text{ value} = \frac{\text{Distance travelled by compound}}{\text{Distance travelled by solvent}}$$

Extraction of antibacterial metabolite (Natarajan *et al*, 2010)

The fungus was cultivated on BMEA by inoculating selected endophyte culture in 500 mL conical flask containing 250 mL of the medium. The flask was incubated at 30˚C for 5 days with static. After incubation period, fermentation broth of the fungus was filtered with filter paper. The filtrate was extracted with equal ratio of ethyl acetate. Then the mixture was shaken in separating funnel. The organic layer was separated and collected.

The effect of pH on extraction with ethyl acetate (Vasconcelos *et al,* 2015)

The collected organic layer (ethyl acetate layer) was tested adjusting at pH 4, 5.0, 6.0, 7.0, 8.0, 9.0 and 8.0 with desired) 0.1 M NaOH or 0.1 M HCL. Then each adjusted pH sample was tested by using agar well diffusion assay.

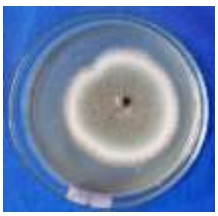
Results

Identification of selected fungus SL-37

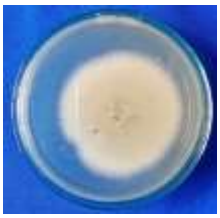
Table 1 Morphological character and colony size of selected fungus SL-37

Sr. No	Culture media	Surface colour	Reverse colour	Colony size (cm)
1	BMEA	Greenish white	Gray	6.5 - 6.4
2	CZA	Gray	Cream	4.7 - 4.7
3	MEA	Greenish white	Yellow	5.5 - 3.1
4	GAN	Pale gray	Cream	1.5 - 1.2
5	PDA	Greenish white	Yellow	7.4 - 7.0
6	LCA	Pale gray	Cream	4.6 - 4.0
7	WA	White	White	2.0 -1.6

A



Surface colour on BMEA



Reverse colour on BMEA

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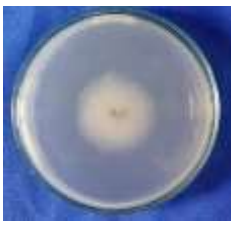
B

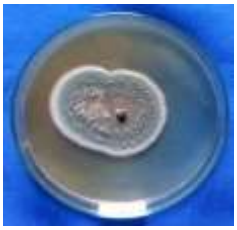
Surface colour on CZA



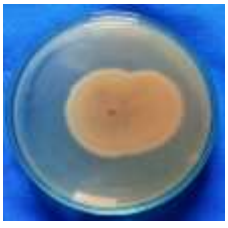
Reverse colour on CZA

X40

C



Surface colour on MEA



Reverse colour on MEA

X40

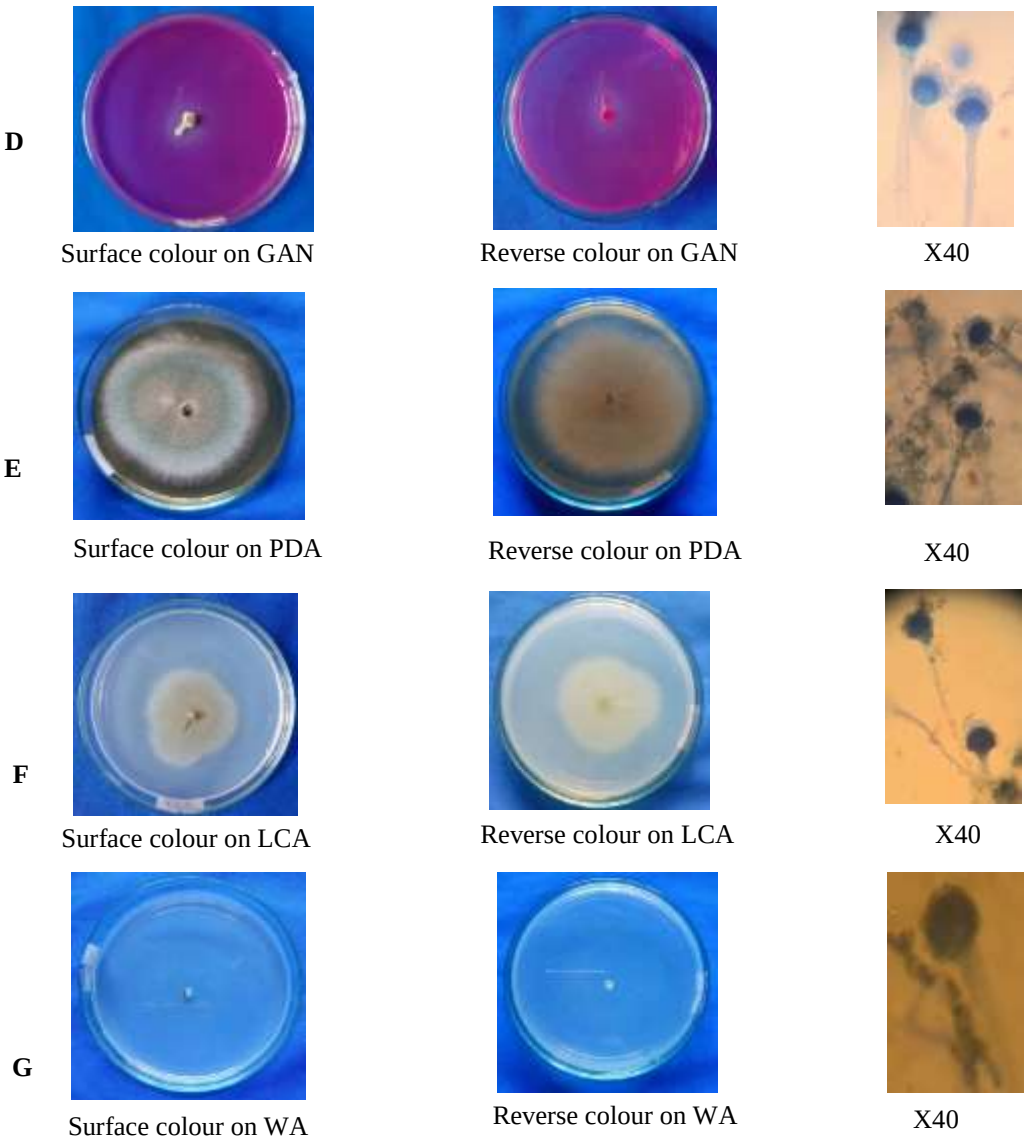


Figure 1 Morphological, colony size and microscopical character of isolated fungus SL-37 on (A) BMEA and (B) CZA media (C) MEA (D) GAN (E) PDA(F) LCA and (G) WA media

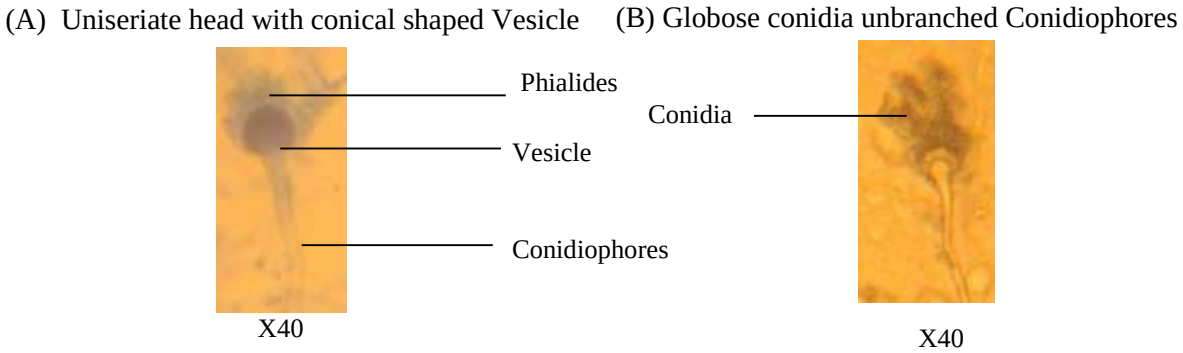


Figure 2 Microscopical character of SL-37

Description of microscopic characters of selected fungus SL-37

Isolated from *Prunus persica* (L.) Batsch. Mogok Townshop, Mandalay Region on seven medium.

Culture medium – BMEA, CZA, MEA, GAN, PDA, LCA and WA

1. Hyphae - aseptate
2. Conidium
   (a) Shape - simple, globose
   (b) Septum - amerospore
   (c) Production - drop
   (d) Colour - hyaline
   (e) Surface - texture
   (f) Size - 29.45 µm
3. Conidiophore - with aseptate, with simple and unbranch
4. Conidiophore development - elongate along with conidium production
5. Conidium development
6. Conidiogenous cell

   Conidium locus - multi loci

According to the distinct characters, selected fungus SL-37 may be identified as the genus *Aspergillus.*

Scientific classification

Kingdom : Fungi
Division : Ascomycota
Class : Eurotiomycetes
Order : Eurotiales
Family Trichocomaceae
Genus : *Aspergillus sp.*

Identification key of selected fungus SL-37

1. Isolated was predominantly uniseriate ……..….....….....……..... 2
1. They had uniserate conidia heads ……..….....….....……..... 2
   2. Conidial heads on MEA, columnar…….....….....……..... 3
   2. Conidia globose to subglobose ……..….....….....…….… 3
3. The colony color was greenish white, reverse gray on BMEA …….....…..... 4
3. The colonies were gray on CZA, reverse cream …….....….....….....4
   4. On DRBC, colonies were greenish white, reverse color gray ….....….....…...5
   4. On GAN and LCA, colony color were pale gray and reverse pale yellow ……..……..……..…….....….....……..…….. 5

5. Colonies on MEA, the colonies were gray and reverse yellow…….... 6

5. Colony diameter after incubation for 7 days, 6.5cm on BMEA, 4.7cm on CZA, 5.5cm on MEA, 1.5cm on GAN, 7.4cm on PDA, 4.6cm on LCA and 2.0cm on WA …….…... 6

    6. Conidiophore stipes are short and hyaline ……..…..…….. 7

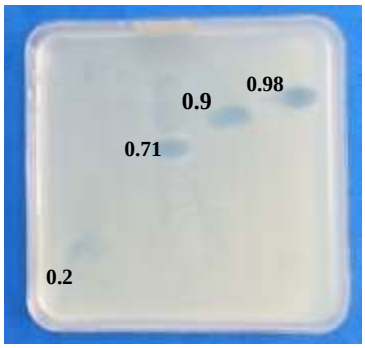
    6. Conidiophores have conical-shaped terminal vesicles ……..….7

7. Vesicles 163 µm, diameter and clavate shape ……*Aspergillus sp.*

7. Vesicles have a single row of phialides on the upper two thirds of the vesicles ……...……..……..…….. *Aspergillus sp.*

Paper chromatography

In this study, four kinds of solvents (20% NH_4CL, n-Butanol, ethyl acetate -acetic-acid-water (3:1:1), n-Butanol saturated with waterand ethyl acetate saturated with water were used. According to the R_f value, 0.98, ethyl acetate was more extractable the antibacterial metabolites than other solvent, follow by n-Butanol solvent (0.9), Ethyl acetic- Water 3:1:1 (0.71) and the lower R_f value, but 20% NH4CL was not showed and R_f value.



1. 20%NH4Cl
2. ethylacetate,n-Butanol-acetic-water (3:1:1)
3. n-Butanol saturated with water,
4. Ethyl acetate saturated with water

Figure 3 Paper chromatography bioautography assay

Comparison of antibacterial activity of metabolite in SL-37 extracted with different volume of EtOAc and n- BuOH against *E. coli*

Using ethyl acetate extract (1:1) resulted in higher inhibition zone 23.91mm, followed by 21.90mm and 20.73mm in ethyl acetate extract (2:1) and (3:1) respectively as well as inhibitory zone 20.15mm was found in n-butanol extract (1:1), 17.57mm in n-butanol extract (2:1) and 16.66mm in n-butanol extract (3:1). Therefore, ethyl acetate extract (1:1) of SL-37 displayed higher inhibition zone than n-butanol extract (1:1). There was no antibacterial activity at all of lower layer. These results were shown in table 2 and figure 4.

Table 2 Comparasion of antibacterial activity of metabolite in SL-37 extracted with different volume of EtOAc and n- BuOH against *E. coli*

Different ratio of solvent	Inhibition diameter zone (mm)	
	EtOAc extract	n- BuOH extract
1:1	23.91mm	20.15mm
2:1	21.90mm	17.57mm
3:1	20.73mm	16.66mm

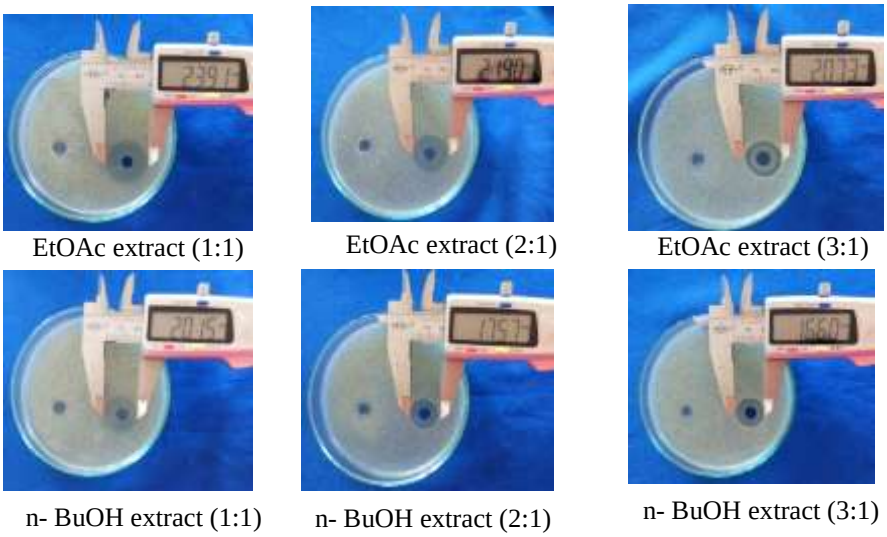


Figure 4 Comparison of antibacterial activity of SL-37 extracted with different volume of EtOAc and n- BuOH against *E. coli*

The antibacterial activity on extracted pH of SL-37

The collected organic layer (ethyl acetate layer) was tested adjustind at pH 4, 5, 6, 7, 8, 9, and 10. The minimum inhibitory zone was found at pH-5 (20.56mm) while maximum inhibitory zone occured in pH 6 in (23.18mm), followed by pH-4 and 5 (19.57mm, 20.56mm) and pH 7 and 8 (20.39mm and 19.68mm) respectively. The negative results were found as pH 9 and 10 (Table 3 and Figure 5).

Table 3 The antibacterial activity on extracted pH of SL-37

pH range	Inhibition zone (mm)
4	19.57
5	20.56
6	23.18
7	20.39
8	19.68
9	17.47
10	-

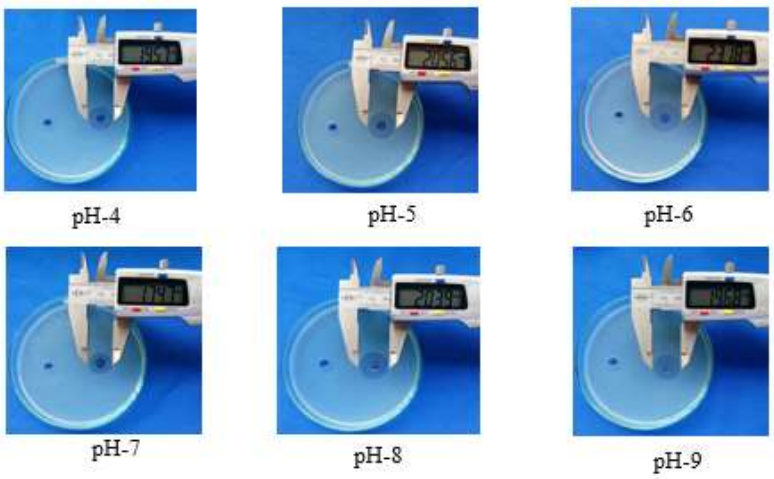


Figure 5 Antibacterial activity on extracted pH of SL-37

Discussion and Conclusion

Endophytes have been intensively studied in several unexplored environments around the world. Endophytes were distributed in each and every plant species and were investigated for endophytic microbial components (Carroll, 2004). Endophytes are chemical synthesizers inside plants (Owen, 2004).

In the identification of selected fungus SL-37, macro and micro characteristics have been studied on seven differential media. Colony diameter after incubation for 7 days, 7.4cm on PDA, 6.5cm on BMEA, 5.5cm on MEA, 4.7cm on CZA, 2.0cm on WA,4.6cm on LCA, and 1.5cm on GAN. It had short columnar and uniseriate conidia head; vesicle 163-239 µm diameter and conical shaped; majority had phialides covering half to three quarter of the vesicles; the conidiophore stipe measured 350 µm long and 45 µm wide, globose and smooth. Diagnostic features; of conidiophore stipe are short, smooth walled and have conical-shaped terminal vesicles which support a single row of phialides on the upper two third. The genus has been classified in to section based seriation either uniseriate, the shape of conidia head; globose, radiate, columnar or clavate.

According to the results, the selected fungus SL-37 was identified as genus *Aspergillus sp*. These results were agreement with Ando (2016), Larone and Davise (1995) and Barnette (1969).

Paper chromatography was performed by using four kinds of different solvent were applied to observe the optimum extraction ability of secondary metabolites. According to the R_f value, ethyl acetate was the excellent solvent for SL-37. Furtado *et al,* 2005 recorded antimicrobial activity of metabolite extracted from *Aspergillus fumigatus* within R_f values of 0.33 to 0.91. The antibacterial activity of SL-37 extracted with different ratio of ethyl acetate -and n-butanol (1:1, 2:1, 3:1) were used. The equal ratio of ethyl acetate extract was showed the highest activity of inhibition zone (23.91 mm).

Jain and Pundri 2011 reported that fermentation broth and ethyl acetate solvent (1:1) was applied and the mixture antimicrobial metabolite was obtained by using this ratio. Similarly, Anuhya *et al,* 2017 described that the extraction of the secondary metabolite was effectively done with ethyl acetate and broth culture in ratio (1:1). Garcia *et al,* 2012. reported that the ethyl acetate solvent system was most efficient method to extract endophytic fungi principle compound. In the extraction of antibacterial compounds, endophytic fungus SL-37 (20 liters) were fermented on suitable synthetic fermentation medium and extracted with equal ratio of EtOAc (1:1) to yield 5 g. Then the resultant extract of ethyl acetate solvent was adjusted to 4, 5, 6, 7, 8, 9 and 10. The maximum antibacterial activity was observed at pH 6.0 (23.18 mm). Vasconcelos *et al,* 2015 described that ethyl acetate extract (pH 7) showed antimicrobial activity of with zones of 20 mm and 22 mm.

The identification and extraction of antibacterial metabolites required for further research plan, the purification and identification of isolated compounds and minimum inhibitory concentration (MIC).

Acknowledgment

I would like to express my gratitude to Dr Zaw Win, Rector, Sagaing University, for his various guidance, suggestion and permissions to do the research. I am also grateful to thanks Dr Thazin, Pro-rectors, Sagaing University, for her suggestion and advices. I wish to express most sincere gratitude to Dr May Kyaut Khaing, Professor and Head, Department of Botany, Sagaing University, for their guidance, invaluable suggestions. Many thanks to Dr Zar Zar Yin, Associate Professor, Department of Botany, Bago University, for her advice, encouragement, understanding and cooperation of this research.

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