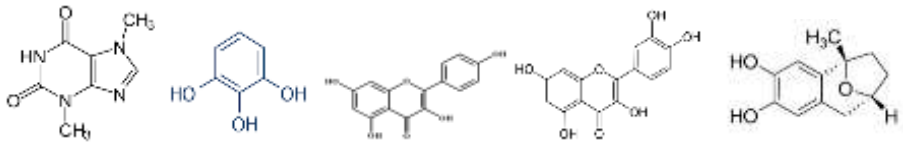
BIOLOGICAL PROPERTIES AND CHEMICAL INVESTIGATION OF MYANMAR FERMENTED TEA LEAF (*CAMELLIA SINENSIS* L.)

San San Aye[1], Ei Ei San[2], Myat Kyaw Thu[3], Ni Ni Than[4]

Abstract

Tea leaves possess many useful biological activities and are consumed in the daily life of the Myanmar people which have been the reason for the selection of this plant for the present paper. Bioassay offers special advantages to know about the biological activity of plant extracts and provide information to isolate active compounds which is a preliminary key step for drug discovery system. Bioassays such as antimicrobial activity, antiproliferative and anti-inflammatory activities were determined. The main aim of the present research was to evaluate the biological properties of *Camellia sinensis* L. leaves. The antimicrobial activity of polar and non-polar extracts of the fermented tea leaves was screened by using agar well diffusion method. Among the tested extracts, flavonoid extract of fermented tea leaves possessed higher antimicrobial activity (30 mm- 35 mm) than the other extracts. *In vitro* antiproliferative activity of ethanol and watery extracts of the fermented tea leaves was investigated against human lung cancer and human cervix cancer by MTT assay. The IC_{50} values of ethanol and watery extracts for human lung cancer were observed 95.34 µg/mL and 123.44 µg/mL, respectively. In the case of human cervix cancer, the IC_{50} values for ethanol and watery extracts were found to be 116.26 µg/mL and 124.39 µg/mL, respectively. No anti-inflammatory activity was found in both water and 70 % EtOH extracts by % NO inhibition assay and there was also no toxicity effect up to >100 µg/mL concentration. The fermented tea leaves were extracted with 70 % ethanol and followed by column chromatographic separation technique. Five compounds were isolated from the ethanol extract. The structures of isolated pure compounds were elucidated by ^{1}H NMR, ^{13}C NMR and ESI-MS spectroscopic methods. In conclusion, 70 % ethanol extracts of the fermented tea leaves possess significantly anticancer activity and antimicrobial activity. Therefore, fermented tea leaves (*C. sinensis* L.) may be used for preventing cancer and used as antimicrobial agent.



caffeine pyrogallol kaempferol quercetin bruguierol B

Keywords: spectroscopic methods, quercetin, kaempferol, caffeine, pyrogallol, bruguierol B

Introduction

Camellia sinensis L. (Tea Leaf)

Plants have a significant role in maintaining human health and improving the quality of human life. The world health organization (WHO,1999) estimated that 80 % of the people rely on traditional medicine. The medicinal plant (Tea leaf), *C. sinensis* L., belonging to the family, Theaceae, whose leaves and leaf buds are used to produce tea. There are three types of commercially available tea as snack or beverage in Myanmar: fermented tea, black tea, and green tea. Myanmar fermented tea leaf is a common signature and national ancient food that is eaten by all people in the country. Apart from the drinking form of tea, fermented pickled tea, the so- called *lapphet*, is another form of tea leaf. *Lapphet* is of Myanmar origin and not derived from other cultures. It is an essential dish for traditional ceremonies in Myanmar. Because of *Lapphet* is consumed in the daily life of the Myanmar people, *Lapphet* products can be easily found

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everywhere in Myanmar markets around the country. Most families have a habit, and use *Lapphet* as daily snacks and as a treat for their guests. Tea leaf contains a very large number of polyphenols, which is the most specific feature of tea. *Lapphet* is very unique in the culture of the Myanmar people and habitually consumed as a food in Myanmar. The department of Chemistry the University of Yangon has made much progress in the research on tea leaf "Myat Kyaw Thu (2003) studied on "The Mode of Formation of Myanmar Fermented Tea" and "Myat Kyaw Thu (1993) studied on "Caffeine Contents of Tea Leaves in Various Climates and Ages". In this study, the caffeine contents of various tea samples for tea consumers as beverage were determined. Although many studies relating to the medicinal properties of fermented tea leaves have been made, antiproliferative activity (human cervix cancer and human lung cancer) of fermented tea leave is still lacking. Therefore, it is needed to investigate antimicrobial, anticancer activities and chemical constituents present in Myanmar fermented tea leaves.

Materials and Methods

Plant materials

The fermented tea leaves (*C. sinensis* L.) were procured from local market, Yangon Region. Fermented tea leaves were left in the open air till they were completely dried. The dried sample was ground in a grinding machine. The drug powders were then stored in an air-tight container.

Chemicals

Column chromatography was run on Kiesel gel 60 (Merck) and TLC on Alufolien Kiesel gel 60 GF_{254} (Merck). Other chemicals were procured from the BDH and E. Merck.

Instruments

JEOL, 500 MHz NMR spectrometer, Agilent Technologies 6420 Triple Quad LC/MS spectrometer, autoclave, incubator

Preparation of crude extracts

Dried powdered sample (100 g) was percolated in 500 mL of petroleum ether (PE, 60-80 °C) for one week and filtered. This procedure was repeated for three times. Then the filtrate was concentrated by a vacuum rotatory evaporator to get respective PE extract. Similarly, ethyl acetate and 70 % EtOH extracts of dried powdered sample were prepared according to the above procedure. After removing each solvent by rotary evaporator, crude extract was dried and kept in desiccator. In the preparation of watery extract; 100 g of dried powdered sample was soaked in 500 mL of distilled water into the conical flask. These flasks were boiled on water bath for 6 hours and filtered. This process was carried out for three times. The combined filtrates were to dryness over a water bath at 100 °C to get the corresponding watery extract.

Antimicrobial screening by agar well diffusion method

Antimicrobial activity of the crude extracts was determined by agar well diffusion method. Four small holes of 10 mm diameter each were cut out in the inoculated agar to place samples to be tested. The volume of each sample placed in each hole was 0.1 cm^3. The Petri dish was then incubated at 37 ºC for 48 hours, and the diameters of clear inhibition zones around the holes, if appeared, were measured (Finegold,1982).

Determination of antiproliferative activity by MTT assay

The anticancer or antiproliferative activity of ethanol and watery extracts of ***C. sinensis*** L. leaves samples were determined against two cancer cell lines such as Hela (cervix cancer) and A 549 (lung cancer) by MTT assay (Fatma *et al*., 2015). The sample solution with cell and medium was added with 100 µL MTT reagent. And then the 96 well plates were incubated in an incubator for 3 hours. After the incubation, (100 µL) of DMSO was added in the 96 well plates. After 15 minutes, the absorbance of each solution was measured at 570 nm by using UV-visible spectrophotometer. The percent cell viability activity was calculated by the following equation.

$$\% \text{ Cell viability} = [(\text{Abs (test sample)} - \text{Abs (blank)})/(\text{Abs (control)} - \text{Abs (blank)})] \times 100$$

Where,

$\text{Abs}_{(\text{test sample})}$ = absorbance of test sample solution
$\text{Abs}_{(\text{control})}$ = absorbance of DMSO solution
$\text{Abs}_{(\text{blank})}$ = absorbance of MTT reagent

IC_{50} (50 % inhibitory concentration) values were calculated by linear regressive excel program. The standard deviation was also calculated by the following equation.

$$\text{Standard Deviation (SD)} = \sqrt{\frac{(\bar{X}-X_1)^2+(\bar{X}-X_2)^2+\cdots\cdots(\bar{X}-X_n)^2}{(n-1)}}$$

Where,

$\bar{X}$ = average % inhibition
$x_1, x_2, \ldots, x_n$ = % cell inhibition of test sample solution
n = number of times

Determination of anti-inflammatory and cell viability activity

Anti-inflammatory activity of the ethanol and watery extracts was evaluated by % NO inhibition assay according to the method of (Jin *et al*., 2010) with some modifications. The 100 µL of cells (1×10^4/well) were seeded in the 96-well plates and incubated for 24 h at 37 °C in a humidified atmospheric containing 5 % CO_2. The cells were then treated with 50 µL each of LPS (100 ng/mL) and different doses of samples for 24 h. NO production was monitored by measuring the accumulation of nitrite in the culture supernatant using Griess reagent (Schmidt *et al*., 1996). In brief, 100 µL each of the supernatant from 96-wells was mixed with equal volume of Griess reagent (0.5 % sulfanilamide and 0.05 % naphthylenediamide dihydrochloride in 2.5 % H_3PO_4) in the new 96 well plates and allowed to stand for 15 minutes at room temperature. The absorbance at 540 nm was measured using microplate reader. On the other hand, the effect of the samples on the cell proliferation was evaluated by MTT assay. The remaining medium from the original plate was discarded and 100 µL each of 10 % MTT solution (5 mg/mL) in the medium was added. After 3 h incubation, the medium was discarded and 100 µL each of DMSO was added to dissolve the formazan crystals and the absorbance at 570 nm was recorded by micro plate reader. The percentage of NO inhibition and that of cell viability was calculated as follows:

NO inhibition (%) = $[\text{Abs}_{(\text{control})}-\text{Abs}_{(\text{sample})}/\text{Abs}_{(\text{control})}] \times 100$, where $\text{Abs}_{(\text{control})}$ and $\text{Abs}_{(\text{blank})}$ are the absorbance of the control group treated by LPS alone and the absorbance of the samples

Cell viability (%) = $100 \times [\text{Abs}_{(\text{test sample})} - \text{Abs}_{(\text{blank})}/\text{Abs}_{(\text{control})}-\text{Abs}_{(\text{blank})}]$

Isolation of compounds from *C. sinensis* L. leaves

Dried powered sample of fermented tea leaves (*C. sinensis*) was percolated in 70 % ethanol with occasional shaking for one week and filtered. This procedure was repeated three times. The combined filtrate was concentrated under vacuum evaporator to obtain ethanol crude extract. The ethanol extract was concentrated to dryness and the residue was used for column chromatographic separation. The ethanol extract (15 g) was chromatographed on a silica gel column using $CHCl_3$: EtOAc (9:1, 4:1) solvent mixture. Finally, five pure compounds were obtained.

Results and Discussion

Antimicrobial activity

In the present work, the sample was tested on 6 strains of bacteria which include *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus pumilus, Candida albicans* and *Escherichia coli*. The measurable zone diameter, including the agar well diameter, shows the degree of antimicrobial activity. It was found that all the extracts of fermented tea leaves showed antimicrobial activity (12 mm- 35 mm) against microorganisms tested except of *Escherichia coli.* Among the tested extracts, flavonoid extract of fermented tea leaves (30 mm-35 mm) has more pronounced antimicrobial activity than the other extracts (Figure 1 & Table 2).

Antiproliferative activity

The antiproliferative activity of ethanol and watery extracts of fermented tea leaves against human lung cancer and human cervical cancer cell lines were evaluated by using MTT assay. The anticancer effect was expressed as IC_{50} values (50 % inhibitory concentration). The lower the IC_{50} value, the higher is the antiproliferative activity. In the case of human lung cancer cell line, the IC_{50} value of ethanol (95.34 µg/mL) was lower than the watery extract (123.44 µg/mL). The IC_{50} value of ethanol (116.26 µg/mL) was found to be lower than that of the watery extract (124.39 µg/mL) against human cervical cancer. Although the tested extracts were observed to show the antiproliferative activity against both cancer cell lines, the ethanol extract has more antiproliferative activity than the watery extract (Table 3 & 4).

Anti-inflammatory activity

Anti-inflammatory activity of ethanol and watery extracts of fermented tea leaves was determined by % NO inhibition assays. If the extract exhibits only anti-inflammatory effect, the IC_{50} values in % NO inhibition should be less than IC_{50} values in % cell viability. However, the IC_{50} values in % NO inhibition of the tested extracts was found to be higher than 100 µg/mL. Therefore, no anti-inflammatory activity was observed in fermented tea leaves up to 100 µg/mL concentration. Moreover, IC_{50} values of % cell viability were greater than 100 µg/mL. It indicates that no toxicity effect in fermented tea leaves up to 100 µg/mL concentration (Table 5).

Identification of isolated compounds

The isolated compounds were identified by ^{1}H NMR, ^{13}C NMR (Figures 2,3,4,5,6) HMBC and ESI-MS spectroscopic methods. The observed ^{13}C NMR data were compared with the reported data (Table 6).

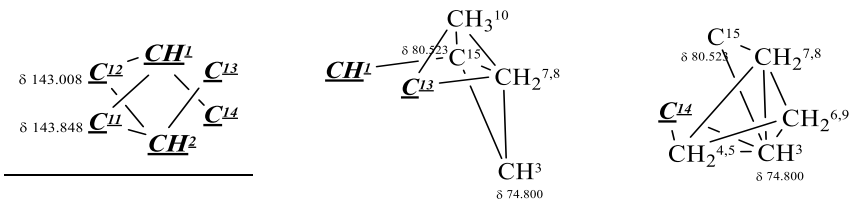
I (Caffeine): Caffeine is a typical natural alkaloid that can stimulate the nervous system and heart. It is white colourless needles with the melting point of 235-237 ˚C. ESI-MS m/z: 195 $[M+H]^+$, 217 $[M+Na]^+$; ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 7.486 (1H, S, H-8), 3.968 (3H, s, CH_3-7), 3.558 (3H, s, CH_3-3), 3.381 (3H, s, CH_3-1)

II (Pyrogallol): It is white, water soluble solid (m.p. 131-134 ˚C). ESI-MS m/z: 125 [M-H]; ^{1}H NMR (500 MHz, DMSO-D6), δ (ppm): 6.381 (1H, t, J = 8.0 Hz, H-5), 6.214 (2H, d, J = 8.0 Hz, H-4 and H-6), 8.556 (2H, brs)

III (Kaempferol): It is yellow crystalline solid (m.p. 276-278 ˚C). It is slightly soluble in water and well soluble in hot ethanol and diethyl ether. It reduces the risk of chronic diseases especially cancer. ESI-MS m/z: 285 $[M-H]^{+}$; ^{1}H NMR (500 MHz, mthanol-D4) δ (ppm): 8.065 (2H, dd, H-2' and H-6'), 6.883 (2H, dd, H-3' and H-5'), 6.366 (1H, d, H-8), 6.157 (1H, d, H-6)

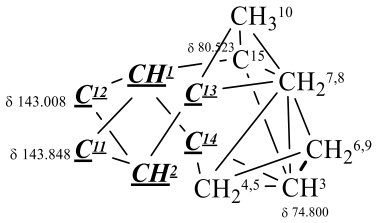
IV (Quercetin): It is a yellow crystalline powder (m.p. 316-317 ˚C) and is a potent antioxidant flavonoid. It is soluble in ethanol and DMSO. ESI-MS m/z: 301 $[M-H]^{+}$; ^{1}H NMR (500 MHz, methanol-D4), δ (ppm): 7.711 (1H, d, J= Hz, H-2'), 7.618 (1H, dd, H-3'), 6.867 (1H, d, H-5'), 6.382 (1H, d, H-8), 6.155 (1H, d, H-6)

V (Bruguierol B): Bruguierol has the molecular mass of 206 from ESI-MS (m/z 205 for [M-H] peak in the negative ion mode). Four quaternary sp^2 carbons and two methine sp^2 carbons showing two one-proton singlet suggests a 1,2,4,5-tetrasubstituted aromatic ring. Twelve numbers each of protons and carbons were observed in ^{1}H and ^{13}C NMR (C12H12) (Table 1), adding up to mass of 156. This gives the mass balance of 206-156= 50, which suggests presence of 2 OH and an O, and the molecular formula is $C_{12}H_{14}O_3$. The DBE is 6, 4 for benzene ring and 2 for further 2 rings, since no more sp^2 carbons are present. The two OH groups must be logically placed ortho to each other at the two deshielded sp^2 carbons on one side of the benzene ring, while the opposite side is the common bond shared between the aromatic ring and a nonaromatic ring. Another two deshielded sp^3 carbons suggest an oxygen bridge in the nonaromatic ring dividing it into two rings, resulting in a total of DBE 6. The methyl group which appears as a three-proton singlet should be attached to the quaternary bridgehead carbon. The two bridgehead carbons must be chiral centers, which results in the observed three methylene carbons bearing diastereotopic protons. This requires that two of these three methylene groups need to be on either side of one of the bridgeheads. The molecular connectivity/correlation diagrams (MCD) (Soong et al., 2020) were constructed based on the HMBC correlation data (Table 1). In drawing the MCD, Chem Draw Professional 15 software was used, bearing in mind the requirements considered above. Partial molecular connectivity/correlation diagrams (MCD) were constructed. Thus, MCDs for aromatic portion a, for methyl and quaternary sp^3 oxygenated carbon containing portion b and oxygenated sp^3 methine carbon containing portion c were constructed. (In the molecular connectivity/correlation diagram (MCD), the lines represent two bond (2JCH), three bond (3JCH) or four bond (4JCH) correlations. Bold, italic, underlined font is used to represent sp^2 hybridization and normal font is used to represent sp^3 hybridization.

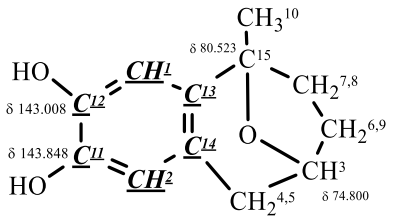


a **b** **c**

From these partial MCDs, the combined MCD d was obtained. The corresponding molecular structure of d was drawn and the two OH and an O were inserted in logical positions to give the deduced structure e of the isolated compound, which is that of bruguierol

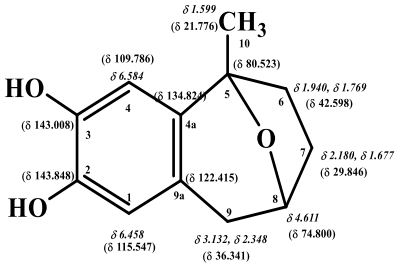


d

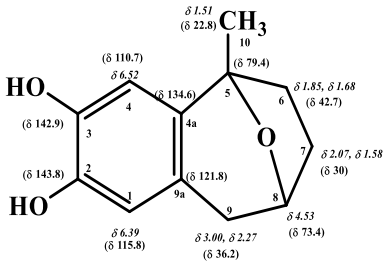


e (bruguierol B)

B. The assignment of the peaks is of the isolated compound and bruguierol B is also compared below using the same numbering system for the position of atoms.



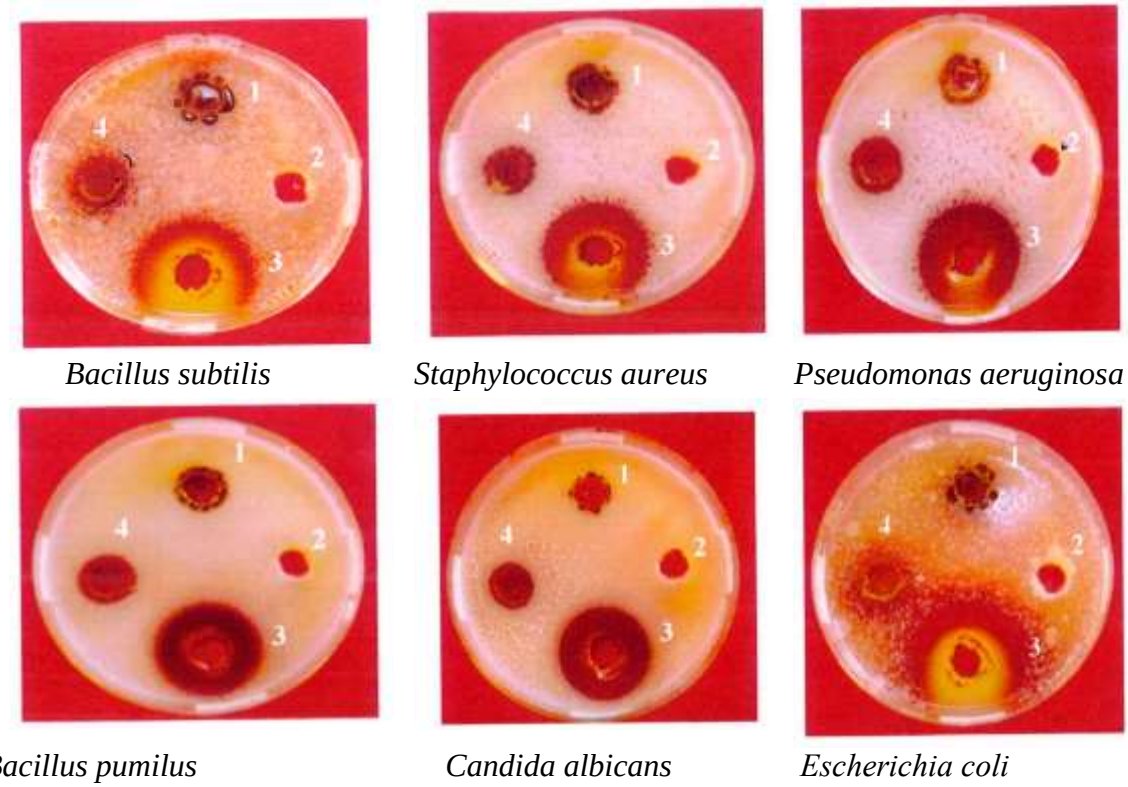
Isolated compound



Han *et al.*, 2005

Table 1 NMR Data for the Isolated Compound V (CD_3OD, 500 MHz)

ID	δ [ppm]		# of H	Type	Multiplicity	Connectivity Correlation
	^1H	^{13}C				HMBC (δ [ppm])
1	6.584	109.786	1	CH	s	80.523 ,122.415, 143.008, 143.848
2	6.458	115.547	1	CH	s	134.824, 143.008, 143.848
3	4.611	74.8	1	CH	s br	42.598, 80.523, 122.415
4	3.132	36.341	1	CH_2	dd	29.846, 74.8, 122.415
5	2.348	36.341	1	CH_2	d	29.846, 122.415
6	2.18	29.846	1	CH_2	q	36.341
7	1.94	42.598	1	CH_2	t	74.8
8	1.769	42.598	1	CH_2	m	36.341, 80.523, 134.824
9	1.677	29.846	1	CH_2	m	36.341
10	1.599	21.776	3	CH_3	s	42.598, 80.523, 134.824
4° Cs: 143.848, 143.008, 134.824, 122.415, 80.523 ID: 11,12,13,14,15						



Bacillus subtilis *Staphylococcus aureus* *Pseudomonas aeruginosa*

Bacillus pumilus *Candida albicans* *Escherichia coli*

1= PE, 2= solvent control, 3= Flavonoid extract, 4= 70 % EtOH

Figure 1 Antimicrobial screening of fermented tea leaves on six microorganisms

Table 2 Antimicrobial Screening of Fermented Tea Leaves

	Tested Organisms	Inhibition zone diameter (mm)		
		PE	**70 % EtOH**	**Flavonoid extract**
1.	*Bacillus subtilis*	13	15	30
2.	*Staphylococcus aureus*	15	18	30
3.	*Pseudomonas aeruginosa*	17	20	30
4.	*Bacillus pumilus*	16	17	30
5.	*Candida albicans*	12	17	35
6.	*Escherichia coli*	-	-	-

*Agar well diameter – 10 mm, No activity (-)

Table 3 Antiproliferative Activities of Crude Extracts of the Fermented Tea Leaves against Human Lung Cancer Cell

Extracts	**Human lung cancer cell**		**IC_{50} (µg/mL)**
	20 µg/mL	**200 µg/mL**	
Ethanol	113.90 ± 14.71	13.05± 0.57	95.34
Watery	145.11 ± 22.13	18.59± 0.14	123.44

Table 4 Antiproliferative Activities of Crude Extracts of the Fermented Tea Leaves against Human Cervix Cancer Cell

Extracts	Human cervix cancer cell		IC_{50} (µg/mL)
	20 µg/mL	200 µg/mL	
Ethanol	95.00 ± 17.18	10.85 ± 1.06	116.26
Watery	99.85 ± 11.31	13.84 ± 0.28	124.39

Table 5 Anti-inflammatory and Cell Viability Activities of the Crude Extracts of the Fermented Tea Leaves

Extracts	% NO inhibition		IC_{50} (µg/mL)	% Cell viability		IC_{50} (µg/mL)
	10 µg/mL	100 µg/mL		10 µg/mL	100 µg/mL	
Ethanol	10.33± 0.21	38.53 ± 0.26	>100	81.81 ± 14.35	100.03± 11.17	>100
Watery	9.28 ±0.12	29.07 ± 0.46	>100	77.29 ± 2.97	104.38 ± 2.76	>100
*L-NMMA	18.49 ± 0.1	50.35 ± 0.1	98.25	100.32 ± 12.41	92.01 ± 1.02	>100

*L-*N*MMA(L-*N*-monomethyl-L-arginine) = positive control

Table 6 ^{13}C NMR Data for Isolated Compounds I to V from Fermented Tea Leaves

Position Carbon	I (δ,ppm)		II (δ,ppm)		III (δ,ppm)		IV (δ,ppm)		V (δ,ppm)	
	Obs. (A)	Lit[a] (A)	Obs. (B)	Lit[b] (C)	Obs. (D)	Lit[c] (D)	Obs. (D)	Lit[c] (D)	Obs. (D)	Lit[d] (B)
1	28.0	27.8	146.7	146.2	-	-	-	-	115.5	115.8
2	151.8	151.6	133.5	133.2	146.6	146.8	147.4	147.7	143.8	143.8
3	29.8	29.7	146.7	146.2	135.7	136.6	135.9	135.6	143.0	142.9
4	148.7	148.6	107.6	109.3	176.0	176.6	175.9	176.4	109.7	110.7
4a	-	-	-	-	-	-	-	-	134.8	134.6
5	107.6	107.5	119.0	121.4	161.1	162.3	161.1	161.2	80.5	79.4
6	155.5	155.3	107.6	109.3	97.9	99.2	97.8	98.7	42.5	42.7
7	33.6	33.5			164.2	164.9	164.2	164.4	29.8	30.0
8	141.4	141.5			93.10	94.4	93.0	93.8	74.8	73.4
9					156.9	157.7	156.8	156.1	36.3	36.2
9a					-	-	-	-	122.4	121.8
10					103.1	104.1	103.1	103.0	21.7	22.8
1'					122.3	123.3	120.3	121.9		
2'					129.2	125.9	114.6	115.0		
3'					114.9	116.3	144.8	145.0		
4'					159.2	160.1	146.6	145.8		
5'					114.9	116.3	114.8	115.6		
6'					129.2	125.9	122.7	124.5		
7'										
8'										

a (Sitkowski *et al*, 1995), b (Chemical book.com), c (Natural Product Science, 2017),
d (Han *et al*., 2005)
A (in $CDCl_3$), B (in DMSO-D6), C (in D_2O), D (in Methanol- D4)

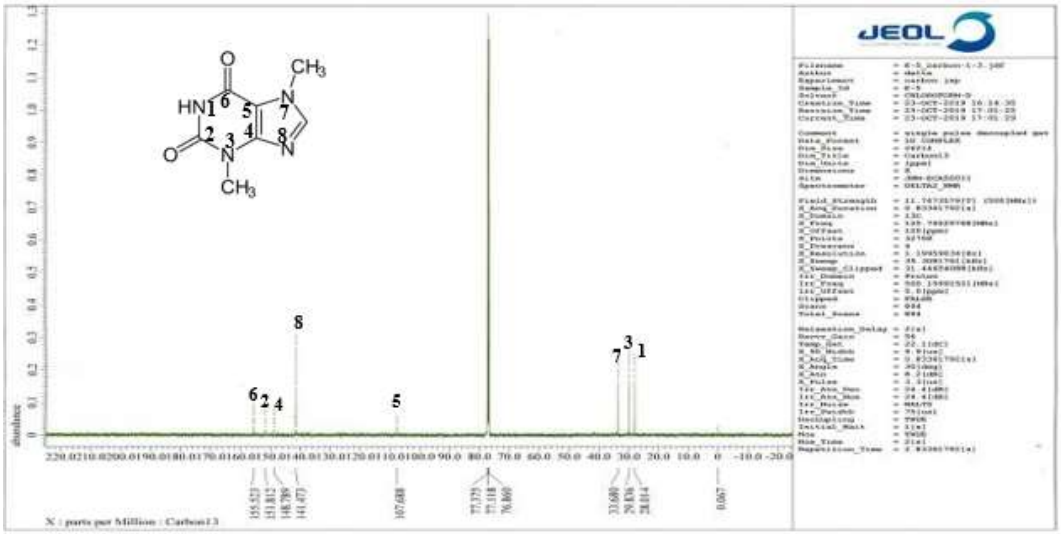


Figure 2 ^{13}C NMR spectrum of caffeine from fermented tea leaves

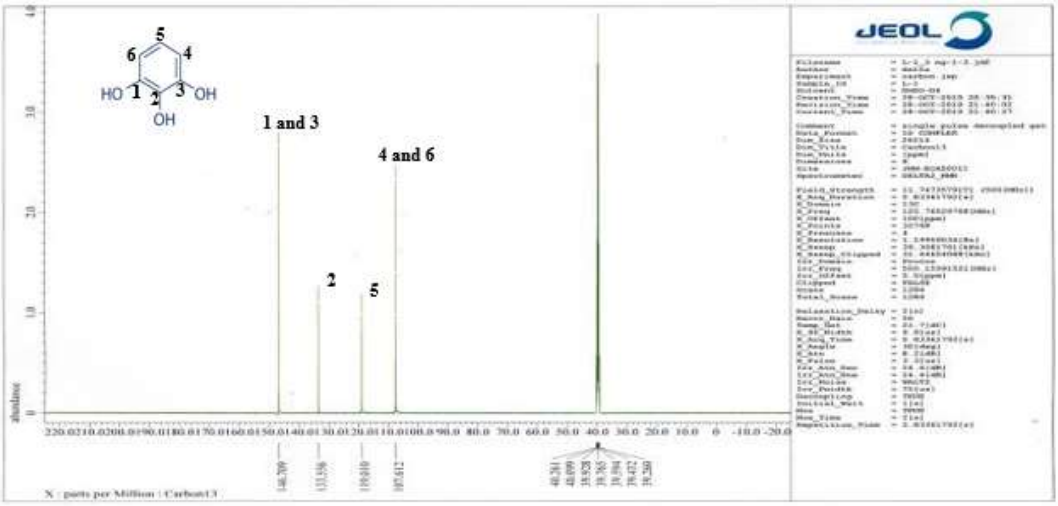


Figure 3 ^{13}C NMR spectrum of pyrogallol from fermented tea leaves

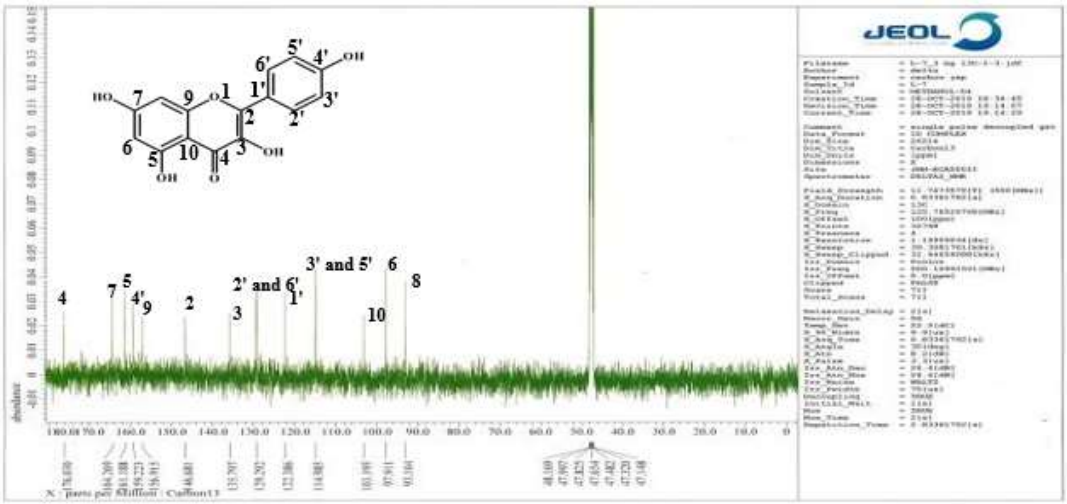


Figure 4 ^{13}C NMR spectrum of kaempferol from fermented tea leaves

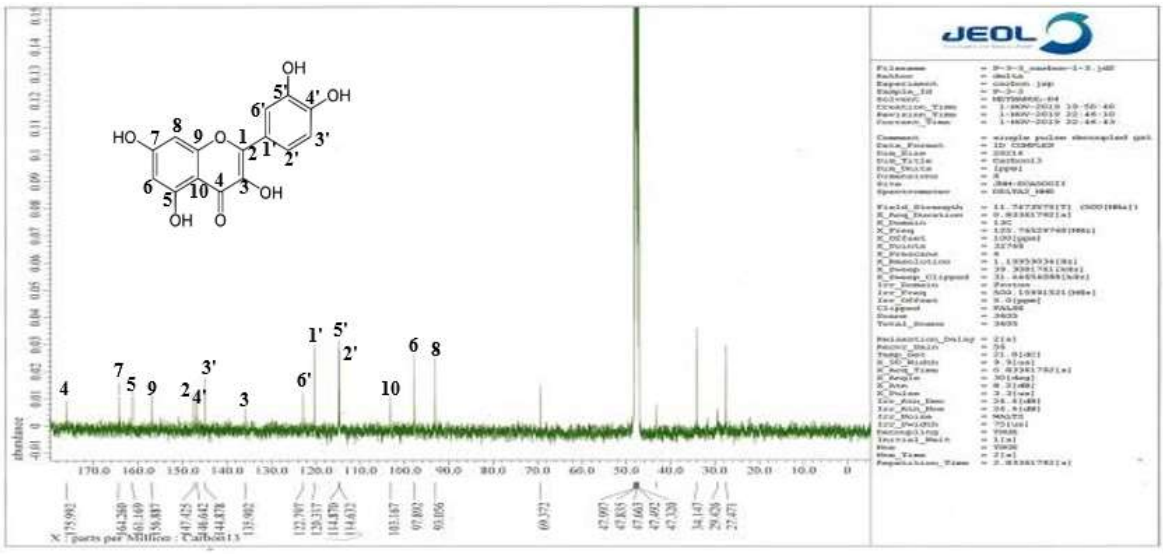


Figure 5 ^{13}C NMR spectrum of quercetin from fermented tea leaves

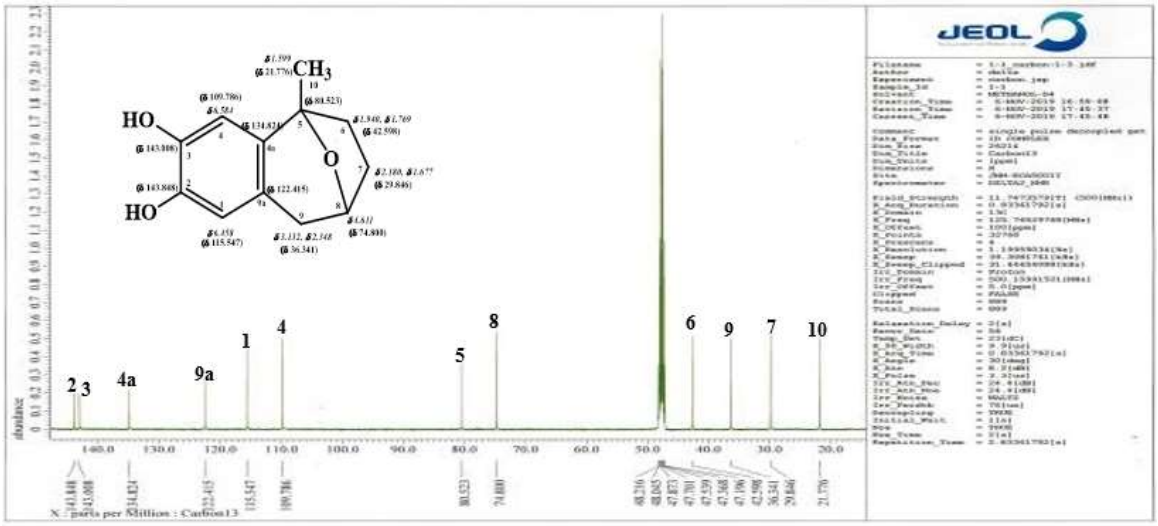


Figure 6 ^{13}C NMR spectrum of kaempferol from fermented tea leaves

Conclusion

From the present research work on "Biological properties and chemical investigation of Myanmar fermented tea leaves (*Camella sinensis* L.)", the following conclusions can be drawn.

The antimicrobial activity of the three crude extracts was screened by *in vitro* method using agar well diffusion technique on six microorganisms. Among the tested extracts, flavonoids extract (35 mm) has more pronounced antimicrobial activity than the other extracts (PE and 70 % EtOH). The ethanol and watery extracts (IC_{50} < 200 µg/mL) had antiproliferative activity against two cancer cell lines (Human lung cancer and Human cervix cancer). Although the tested extracts did not show anti-inflammatory activity by % NO inhibition assay, no toxicity effect was observed in the tested extracts up to 100 µg/mL concentration. Moreover, ethanol extract of fermented tea leaves was separated by column chromatographic method. Five compounds were isolated from fermented tea leaves and identified by ESI-MS and NMR data. The fermented tea leaves, one of the major traditional snacks, can be considered as good sources of natural antioxidant for medicinal uses due to the presence of the isolated flavonoids (quercetin and kaempferol), polyphenol

(pyrogallol) and alkaloid (caffeine). They are strong antioxidants and help to prevent oxidative damage of our cells. Moreover, the isolated bruguierol B has antibacterial activity against mycobacteria and resistant strains. Due to the presence of these active isolated compounds containing fermented tea leaves *Lapphet* definitely provides health benefits such as antimicrobial and anti-cancer to Myanmar people.

Acknowledgements

The authors would like to express their profound gratitude to the Department of Higher Education (Yangon Office), Ministry of Education, Yangon, Myanmar, for provision of opportunity to do this research and Myanmar Academy of Arts and Science for allowing to present this paper.

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