COMPARATIVE STUDIES OF ANTIBACTERIAL ACTIVITY AND PHYTOCHEMICAL CONSTITUENTS OF SOME MYANMAR INDIGENOUS MEDICINAL PLANTS

San San Aye[1], Hnin Hnin Aye[2], Daw Hla Ngwe[3], Saw Hla Myint[4]

Abstract

Developing countries, where dysentery and diarrhea are endemic, depend strongly on traditional medicine as a source for inexpensive treatments because it is based on plants which are abundantly available in these countries. Consequently, Myanmar indigenous medicinal plants (*Curcuma longa* L., *Nigella sativa* L. and *Piper betel* L.) which are used for the treatment of dysentery and diarrhea in Myanmar were selected to study in order to find the scientific basis for such use. The selected plants were screened for antibacterial activity by using agar disc diffusion technique. Polar and non-polar extracts of the selected plants were tested on 33 species of standard and hospital bacterial strains. The Minimum Inhibitory Concentration (MIC) of the active extracts was also determined by agar plate dilution method. Three curcuminoids, namely curcumin (5.9 %), desmethoxy curcumin (0.018 %) and bisdesmethoxy curcumin (0.0136 %) were isolated from *C. longa* rhizomes. *N. sativa* seeds were fractionated by column chromatography to give thymoquinone (0.01 %), kaempferol (0.12 %) and quercetin (0.001 %). Eugenol (0.1 %) was isolated from essential oil of *P. betel*. The isolated compounds were identified by UV, FT IR, ^{1}H NMR, ^{13}C NMR and mass spectroscopic methods. The isolated compounds were also found to show bactericidal activity. Thus the extracts of the three plants have high potential for the production of combined formulation to treat infections caused by bacteria.

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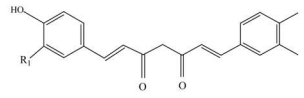
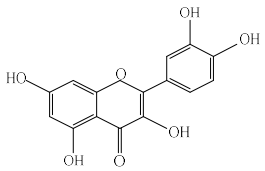
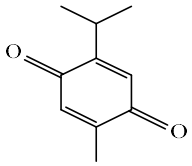


$R_1 = R_2 = OCH_3$: Curcumin
$R_1 = H, R_2 = OCH_3$: Desmethoxy curcumin
$R_1 = R_2 = H$: Bisdesmethoxy curcumin



Quercetin



Thymoquinone

Kaempferol

Eugenol

Keywords*:* bactericidal activity, curcuminoids, essential oil, spectroscopic method

INTRODUCTION

Curcuma longa L. (Nanwin)

The medicinal plant (tumeric), *Curcuma longa* L., belonging to the family, Zingiberaceae, is a perennial herb 2 to 3 feet high with a short stem and tufted leaves and is cultivated in India. It is used as a domestic remedy. It is used as a food additive for fish and meat (Burkill, 1996). Major constituents of rhizomes are pale yellow to orange-yellow volatile oil (6 %) composed of a number of monoterpenes and sesquiterpenes including zingiberence, curcumene, α- and β–turmerone (WHO, 1999). The colouring principles

(5 %) are curcuminoids. 50-60 % of which are a mixture of curcumin, desmethoxy curcumin and bisdesmethoxy curcumin. The antioxidant properties of curcuma powder are probably due to the phenolic character of curcumin (FAO/ WHO, 1978).

Nigella sativa L. (Samon-net)

Samon-net (black seed), *Nigella sativa* L., belonging to the family, Ranumculaceae, is an annual herb leaves. Flowers are pale blue on solitary

long stalks. Fruit composed of 5-12 more or less united follicles. It is found in upper Myanmar (Mya Bwin and Sein Gwan, 1973).

Black seed is native to the Mediterranean and is also known as the "Seed of blessing". Black seed has rich nutrient composition which comprises 15 amino acids including all essential acids, carbohydrates and proteins as well as essential fatty acids and minerals: calcium, iron, sodium and potassium. The chief constituents of the seeds are volatile oil and a fixed oil. The seed oil possesses hypotensive effect. *N. sativa* L. seeds have many medicinal properties such as bronchodilator, hypotensive, antibacterial, antifungal, analgesic, anti-inflammatory and are universally accepted as a panacea (Khan, 1999).

Piper betel L. (Kun)

Kun (Betel leaf), *Piper betel* L., belonging to the family, Piperaceae, is shout glabrous climber with leaves of 5-20 cm long broadly ovate, slightly cordate and unequal at the base, shortly accumulate with yellowish or bright green shining on both size. The pungent taste possessed by all organs of the plant is one of the vital characteristics of the genus. This taste owes to the presence of alkaloid piperine and the aromatic odour is due to that of volatile oil (Chopra *et al.*, 1956).

Betel is widely cultivated in South East Asia countries especially in Myanmar for its leaves serve both as indigenous and modern medicines. It is one of the well-known medicinal plants and is widely cultivated for commercial purpose in Myanmar.

Betel leaf oil consists mainly of phenol and terpenes, that relative proportions varying with the original portion of the leaves. The highest the proportion of phenols in the oil, the better the quality. The predominant phenolic constituent in the betel leaf oil is eugenol which is used extensively as a raw material in the synthetic preparation of vanillin and as an antiseptic in pharmaceutical and dental preparation (Guenther, 1960).

Materials and Methods

Plant Materials

The rhizomes of *Curcuma longa* L. and *Nigella sativa* L. were procured from Sandi indigenous medicine shop. The leaves of *Piper betel* L. were collected from Yangon Region market. The rhizomes and seeds were ground to get a fine powder. The drug powders were then stored in an air-tight container. Betel leaves were left in the open air till they were completely dried. The dried sample was ground in a grinding machine.

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.

Microbial Strains

The bacterial strains used were obtained from the Department of Medical Research (Lower Myanmar), Yangon.

Instruments

Shinmadzu UV-240, UV-visible spectrophotometer, Perkin-Elmer spectrum GX FT IR spectrophotometer, Bruker 400 MHz NMR spectrometer, autoclave, incubator.

Antibacterial Screening of Crude Extracts

For the examination of *in vitro* antibacterial activity of crude extracts, agar disc diffusion method was used. Discs obtained by filter paper (Toyo No.26, Japan) punched to 8 mm diameter, were used to impregnate the extracts to obtain approximately 20 μg/disc, and prior to adherence on the culture plates. The discs were allowed to dry at 42 ℃ incubator (Finegold and Martin, 1982).

The bacterial suspension from trypticase soy broth was streaked evenly onto the surface of the trypticase soy agar plates with sterile cotton swab. After the inoculums had dried (5 mins), the dried discs were placed on the agar with flamed forceps and gently pressed down to ensure proper contact. A disc impregnated with solvent only was placed alongside the test discs for control and comparing purposes.

The plates were incubated immediately or within 30 mins after inoculation. After overnight incubation at 37 ℃, the zones of inhibition diameter including 8 mm discs were measured.

Determination of mMinimum Inhibitory Concentration (MIC) of the Active Extracts by Agar Plate Dilution Method

The minimum inhibitory concentration (MIC) of the active extracts were determined by plate dilution method (Cruickshank *et. al.,* 1975; Finegold and Martin, 1982).

The active extracts were dissolved with their respective solvents (e.g. ethyl acetate extract with ethyl acetate) and diluted with trypticase soy agar to obtain the following concentrations: 2 mg/mL, 1 mg /mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, 0.0625 mg/mL, 0.03125 mg/mL, 0.015625 mg/mL and 0.0078125 mg/mL.

The bacterial suspension (0.02 mL) was applied as streak/ droplet onto the surface of the prepared agar plates. Then the plates were incubated at 37 ℃ overnight. After overnight incubation, the lowest concentration showing no growth of the organisms was taken as the minimum inhibitory concentration (MIC), expressed in mg/mL. The experiments were repeated three times at exactly the same parameters, and the mean results were taken.

Isolation of Compounds from *C. longa*

The ethyl acetate extract (0.6 g) was chromatographed on a silica gel column using dichloromethane - methanol (95:5) solvent mixture as eluent, Finally, 3 main fractions were obtained after combining the similar fractions.

Isolation of Compounds from *N. sativa*

From the soxhlet extraction ***N.*** *sativa* seed powder (100 g) with PE (60-80 ºC), 40 g of an oil, i.e. 40 % of the drug, were obtained. Thus, oil yielded upon steam distillation was 0.04 g of volatile oil, i.e. 0.04 % of the drug. The volatile oil in turn yielded 0.0632 g of yellow oil of thymoquinone, i.e. 0.01 % based on drug (0.6 g) by column chromatography on a silica gel column with PE-toluene (2:3) solvent mixture as eluent.

The dried seed powder (100 g) was soaked in 500 mL of 70 % ethanol and heated for 10 min on a water bath and kept at room temperature overnight (Harborne, 1984). The mixture was filtered and the filtrate was concentrated to a volume of 100 mL. The solution was extracted twice with 200 mL of PE (60 – 80 ºC). The aqueous layer was evaporated to dryness. The residue was dissolved in 2M hydrochloric acid solution and hydrolyzed for 45 min on a boiling water bath. The mixture was cooled and filtered. The filtrate was extracted with ethyl acetate. The ethyl acetate layer was concentrated to dryness and the residue was used for column chromatographic separation. The ethyl acetate (0.6 g) was chromatographed on a silica gel column using Toluene-EtOAc (70:30) solvent mixture. Finally, quercetin and kaempferol were obtained.

Extraction of Essential Oil from *P. betel*

Extraction of essential oil from *P. betel* was carried out by steam distillation method. The dried powder (100 g) and distilled water (500 mL) were placed in 1 L round-bottomed flask. The flask was fitted for steam distillation and heated. The steam was passed into the flask. The condensed oil and water were collected in a flask and the oil was extracted with PE in a separating funnel. The PE extract was dried over anhydrous sodium sulphate. After filtration, the filtrate was evaporated to get the essential oil which was weighed and kept in air tight bottle for further analysis (Pauli, 2001).

Isolation of Eugenol from *P. betel*

The essential oil of *P. betel* was prepared by steam distillation method. Eugenol was isolated from essential oil of *P. betel* by column chromatographic method using toluene and ethyl acetate (9:1).

Results and Discussion

Antibacterial Activity

It was observed that ethyl acetate and 95 % ethanol extracts of *C. longa* and essential oils of *N. sativa* and *P. betel* showed antibacterial activity on the tested bacteria strains. Therefore, in this screening test, *C. longa*, *N. sativa* and *P. betel* found to possess bactericidal activity (Table 1).

The bactericidal activity of extracts was tested by test tube serial dilution method by determining the turbidity and growth on nutrient agar. All the tested extracts showed the bactericidal activity which coincide with the MIC values. The minimum inhibitory concentrations of EtOAc extract of *C.longa* ranged from 1 to 〉2 mg/mL. The lowest MIC of 1 mg/mL was obtained with *E.coli* (ID-6), *E.coli* (ID-12), *Proteus morganii* (ID-1), *Salmonella typhi* (ID-3), *Salmonella typhi* (ID-24), *Staphylococcus aureus* (ID-15), *Staphylococcus aureus* (ID-26) and the remaining bacteria had MIC of 〉2 mg/mL.

The MIC's of EtOAc extract of *N.sativa* were in the range of 0.01562 to 〉2 mg/mL. The lowest MIC of 0.01562 mg/mL was obtained from *Shigella boydii* (ID 47). *Staphylococcus aureus* (ID-26) was 0.03125 mg/mL and *E.coli* (ID-35), *Plesiomonas shigelloides* (ID-23), *Salmonella typhi* (ID-3), *Salmonella typhi* (ID-19) and *Shigella boydii* (ID-53), the MIC of 0.0625 mg/mL was observed. The remaining bacteria showed MIC value of 2 mg/mL. The MIC'S of essential oil of *P.betel* were from 〉1 to 〉2 mg/mL. The lowest MIC of 〉1 mg/mL was obtained with *E.coli* (ID-35) and *Shigella boydii* (ID-53). In case of *Plesiomonas shigelloides* (ID-23), *Salmonella typhi* (ID-3), *Salmonella typhi* (ID-19), *Shigella dysenteriae* (ID-25), *Shigella boydii* (ID-22), *Staphylococcus aureus* (ID-15) and *Staphylococcus aureus,* (ID-26), the MIC value was 2 mg/mL and the rest showed 〉2 mg/mL. The minimum inhibitory concentrations (MIC) of antibacterial active extracts of *C. longa*, *N. sativa* and *P. betel* are shown in Table 2.

The isolated compounds namely curcumin from *C. longa*, kaempferol, quercetin and thymoquinone from *N. sativa* and eugenol from *P. betel* were found to show bactericidal activity against *Salmonella typhi, Plesiomonas*

shigelloides, Escherichia coli, Staphylococcus aureus, Shigella boydii, Bacillus subtilis and Salmonella paratyphi (Table 3 and Figure 1).

Identification of isolated compounds

1 (Curcumin) : yellow needles, mp. 183°. MS m/z ; 368 (M^+), 350, 340, 232, 137 (Figure 2). UV λ_{max}^{EtOH} nm 260, 425 ; UV $\lambda_{max}^{EtOH+NaOH}$ nm : 467, FT IR υ_{max}^{kBr} cm^{-1} : 3400 (υ_{O-H}), 2925, 2850 (υ_{C-H} of OCH_3), 1627 ($\upsilon_{C=0}$), 1602, 1510, 1460, 1427, 1280, 1150, 960. ^1H NMR δ (ppm) : 7.61 (2H, d, J = 16 Hz), 7.13 (2H, dd, J = 8.5 & 1.5 Hz), 7.05 (2H, d, J = 1.5 Hz), 6.94 (2H, d, J = 8.5 Hz), 6.5 (2H, d, J = 16.0 Hz), 5.90 (2H, s), 5.80 (1H, s), 3.95 (6H, s)

2 (Desmethoxy curcumin) : mp . 181 - 182°. MS m/z : 338 (M^+), 320, 202, 191, 147, 137 (Figure 3) : UV λ_{max}^{EtOH} nm : 250, 419 ; UV $\lambda_{max}^{EtOH+NaOH}$ nm : 462, FT IR υ_{max}^{kBr} cm^{-1} : 3400 (υ_{O-H}), 2925, 2850 (υ_{C-H} of OCH_3), 1627 ($\upsilon_{C=0}$), 1602, 1510, 1460, 1427, 1280, 1150, 960

3 (Bisdesmethoxy curcumin) : mp . 232 - 234°. MS m/z : 308 (M^+), 290, 202, 161, 147, 107 (Figure 4). UV λ_{max}^{EtOH} nm : 245, 415 ; UV $\lambda_{max}^{EtOH+NaO}$ nm : 443, FT IR υ_{max}^{kBr} cm^{-1} : 3400 (υ_{O-H}), 2925, 1627 ($\upsilon_{C=0}$) 1602, 1510, 1460, 1427, 1280, 1150, 960.

4 (Thymoquinone) : bright yellow crystalline compound mp. 49 - 50°. MS m/z : 164 (M^+), 149, 136, 121, 108, 93. UV λ_{max}^{MeOH} nm : 252, 292, FT IR υ_{max}^{kBr} cm^{-1} : 2922, 1458, 1373, 1636 ($\upsilon_{C=0}$). ^1H NMR (ppm) : 6.5 (1H, s), 6.43 (1H, s), 3.01 (1H, septet, J = 6.6 Hz), 2.02 (3H, s), 1.45 (6H, d, J = 6.6 Hz) (Figure 5).

5 (Kaempferol): Yellow crystalline compound, mp. 275º, MS m/z : 286 (m^+), 285, 258, 257, 241, 229, 213, 184, 153, 134 and 121 (Figure 6). UV λ_{max}^{MeOH} nm : 266, 366, FT IR υ_{max}^{kBr} cm^{-1} : 3422 (υ_{O-H}), 1630 ($\upsilon_{C=O}$), ^1H NMR δ (ppm) : 6.18 (1H, d), 6.38 (1H, d), 6.89 (2H, d), 8.07(2H, d).

6 (Quercetin): Yellow crystalline compound, UV λ_{max}^{MeOH} : 257, 375 (Figure 7), FT IR υ_{max}^{kBr} cm^{-1} : 3421 (υ_{O-H}) , 1647 ($\upsilon_{C=O}$).

7 (Eugenol): UV λ_{max}^{PE} 282 nm (Figure 8),FT IR υ_{max}^{kBr} cm^{-1}:3514((υ_{O-H}),1637 ($\upsilon_{C=O}$), 1268, 1234 (υ_{C-O-C}), 1634 ((υ_{C-O-H}).

Table 1. Antibacterial Activity of *C. longa* L., *N. sativa* L. and *P. betel* L.

No.	Tested bacteria	Inhibition zone diameter (mm)			
		***C. longa* L.**		***N. sativa* L.**	***P. betel* L.**
		EtOAc	**EtOH**	**Essential Oil**	**Essential Oil**
1.	*E. coli*	18	13	28	15
2.	*Salmonella paratyphi*	14	12	28	12
3.	*Shigella dysenteriae*	16	11	16	14
4.	*Vibrio parahaemolytics*	22	20	28	10
5.	*Shigella boydii*	16	18	18	16
6.	*Staphylococcus aureus*	14	16	28	20
7.	*Bacillus subtilis*	12	10	14	18
8.	*Salmonella typhi*	14	12	15	12
9.	*Plesiomonas shigelloides*	15	16	28	14
10.	*Shigella sonnei*	14	12	28	20

Table 2. Minimum Inhibitory Concentration of Active Extracts of *C. longa* L., *N. sativa* L. and *P. betel* L.

Extract (mg/mL)	Tested Bacteria*															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
C. longa (EtOAc)	1	>2	1	>2	1	>2	1	>2	1	>2	>2	>2	>2	1	1	>2
N. sativa (EtOAc)	>2	>2	>2	0.06 25	>2	0.06 25	0.06 25	0.06 25	>2	>2	0.5	0.01 562	0.06 25	0.5	0.03 125	>2
P. betel (*E-coli*)	>2	>2	>2	>1	>2	2	2	2	>2	>2	2	2	>1	2	2	>2

*

1. *Escherichia coli* (ID-6)
2. *Escherichia coli* EPEC (ID-7)
3. *Escherichia coli* EHEC (ID-12)
4. *Escherichia coli 0126* (ID-35)
5. *Proteus morganii*
6. *Plesiomonas shigelloides* (ID-23)
7. *Salmonella typhi* (ID-3)
8. *Salmonella typhi*(ID-3)
9. *Salmonella typhi*(ID-19)
10. *Shigella sonnei* (ID-14)
11. *Shigella dysenteriae* (ID-25)
12. *Shigella boydii* (ID-47)
13. *Shigella boydii* (ID-53)
14. *Staphylococcus aureus* (ID-15)
15. *Staphylococcus aureus* (ID-26)
16. *Vibrio fluvialis* (ID-70)

Table 3. Antibacterial Activity of Isolated Compounds from *C. longa* L., *N. sativa* L. and *P. betel* L.

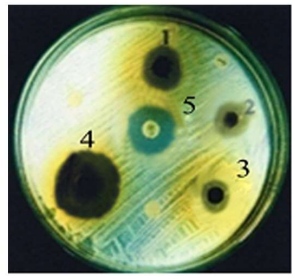
Disc diameter = 8 mm

Isolated Compound	**Inhibition zone diameter (mm)**						
	Tested Bacteria*						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Curcumin	13	NT	13	17	16	-	12
Thymoquinone	15	16	16	25	20	>25	>25
Quercetin	12	12	NT	12	14	10	12
Kaempferol	10	-	-	10	12	16	13
Eugenol	25	17	19	20	24	25	25

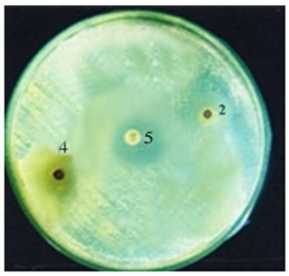
*** Bacteria**

1. *Salmonella typhi*
2. *Salmonella paratyphi*
3. *Plesiomonas shigelloides*
4. *Escherichia coli*
5. *Shigella boydii*
6. *Bacillus subtilis*
7. *Staphylococcus aureus*

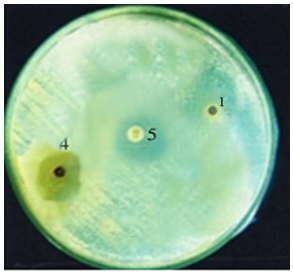
NT – Not Tested, - = No activity



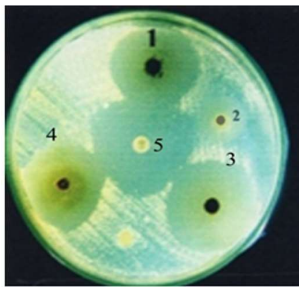
Salmonella typhi



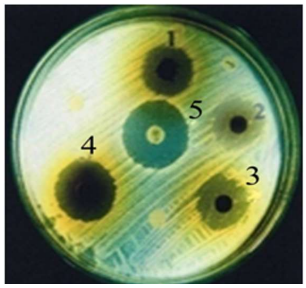
Salmonella paratyphi



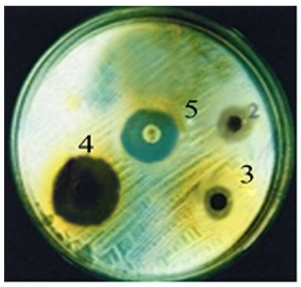
Plesiomonas shigelloides



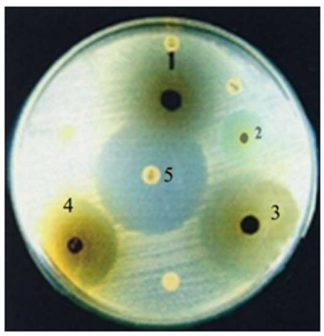
Escherichia coli



Shigella boydii



Bacillus subtilis



Staphylococcus aureus

1 = Curcumin
2 = Quercetin
3 = Kaempferol
4 = Eugenol
5 = Thymoquinone

Figure 1. Antibacterial activity of isolated compounds

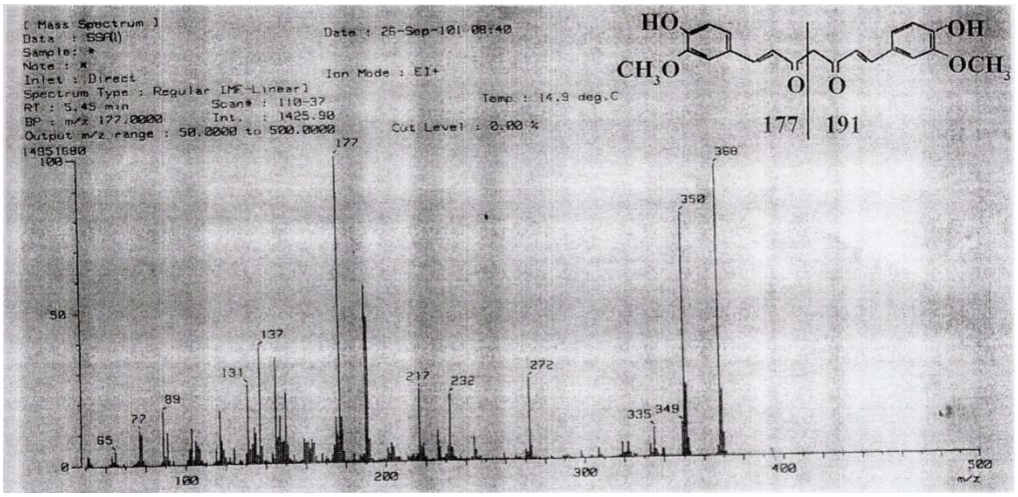


Figure 2. EI mass spectrum of isolated curcumin from *C. longa* L.

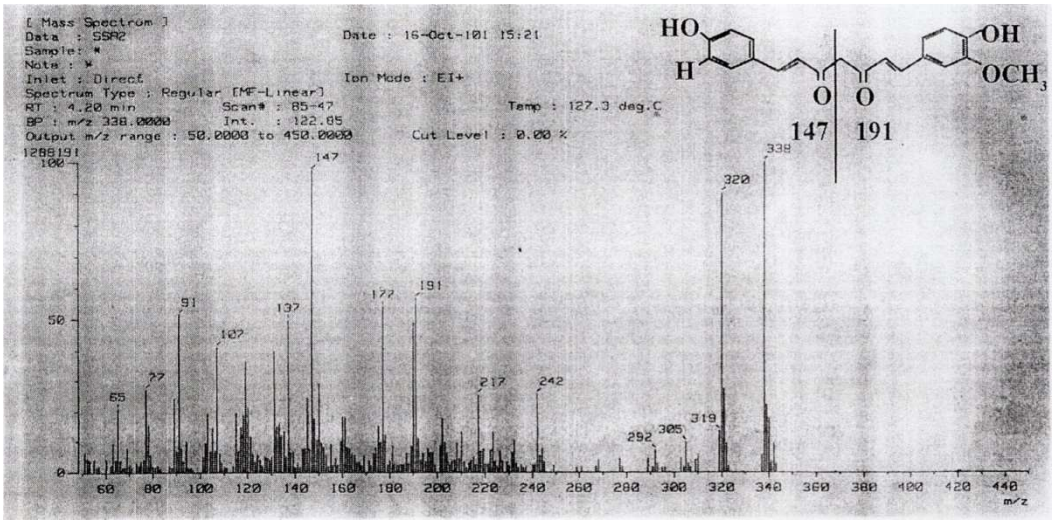


Figure 3. EI mass spectrum of isolated desmethoxy curcumin from *C. longa* L.

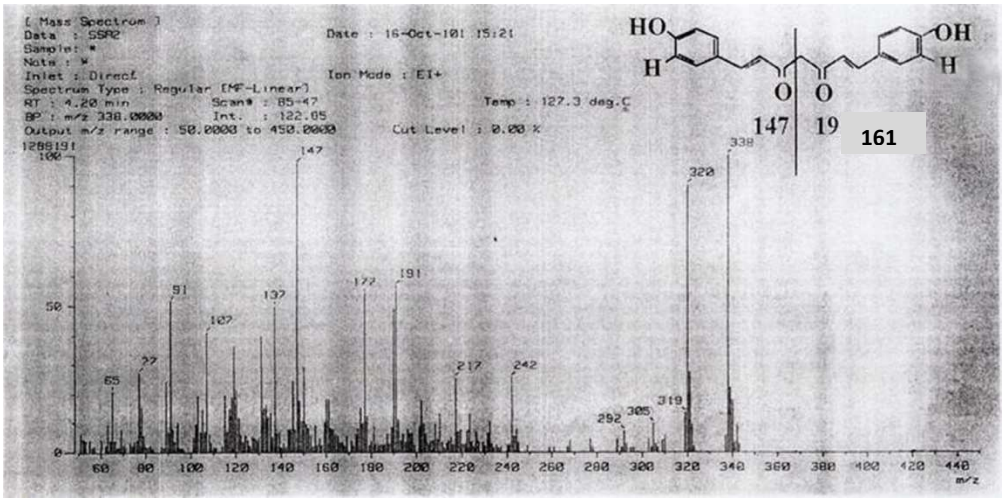


Figure 4. EI mass spectrum of isolated bisdesmethoxy curcumin from *C. longa* L.

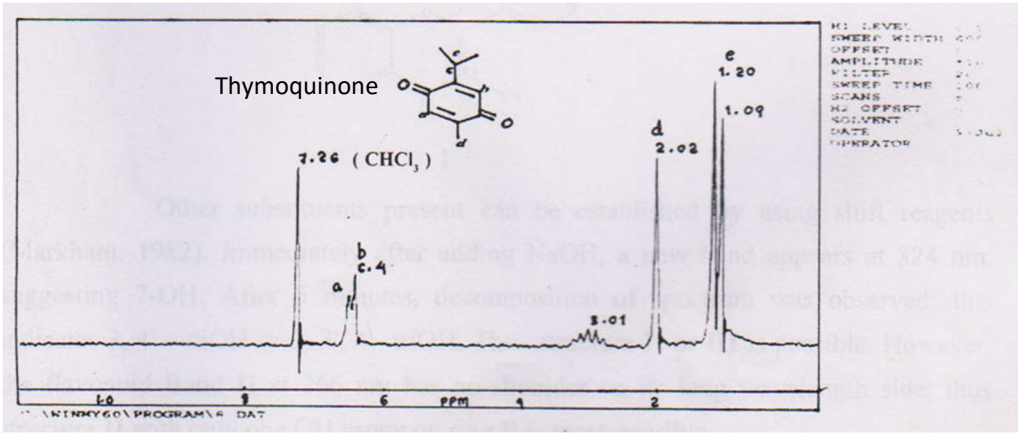


Figure 5. ^{1}H NMR spectrum of isolated thymoquinone from *Nigella sativa* L.

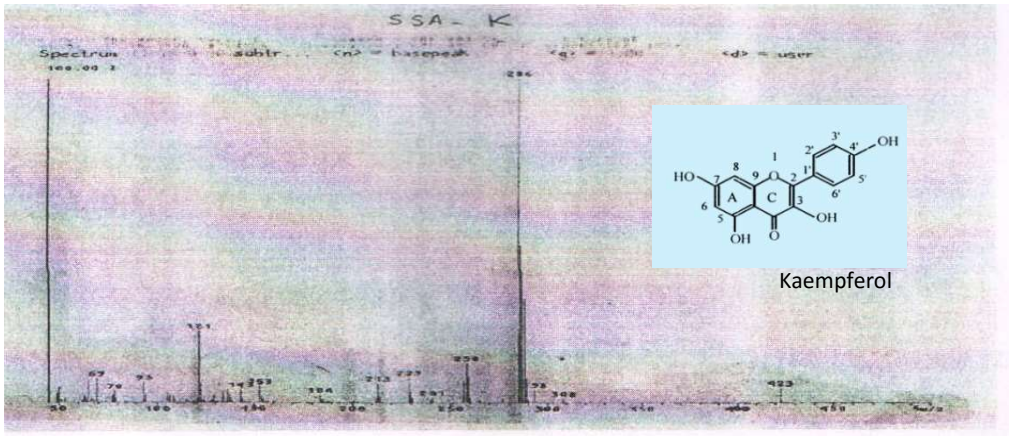


Figure 6. EIMS spectrum of isolated kaempferol from *Nigella sativa* L.

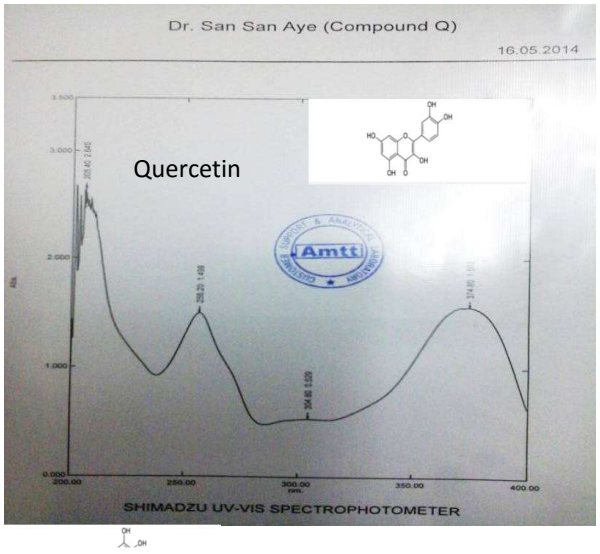


Figure 7. UV spectrum of isolated quercetin from seeds of *Nigella sativa* L. L. (Kun)

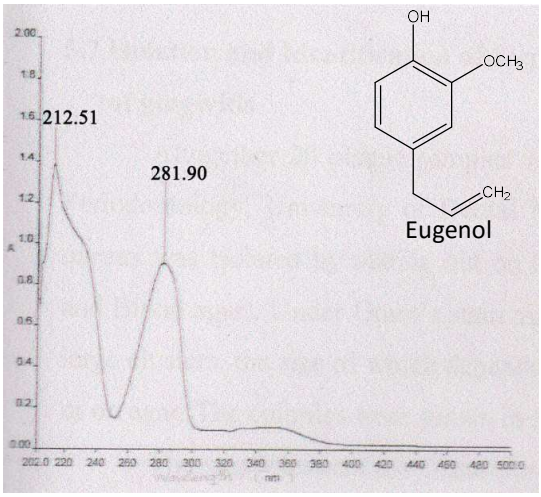


Figure 8. UV spectrum of isolated eugenol from leaves of *Piper betel* L. (Kun)

Conclusion

From the present research work on "Comparative studies of antibacterial activity and phytochemical constituents of some Myanmar indigenous medicinal plants", the following conclusions can be drawn.

Crude extracts have been prepared from *C. longa*, *N. sativa* and *P. betel* by using non-polar and polar solvents. The antibacterial activity of the crude extracts was screened by *in vitro* method using agar disc diffusion techniques on 33 bacteria which include *S. aureus, E. coli, Shigella, Salmonella* and *Vibrio*. Ethyl acetate and ethanol extracts of the *C. longa*, essential oil of *N. sativa* and *P. betel* showed antibacterial activity against all tested organisms.

The minimum Inhibitory Concentration (MIC) of the active extract 1mg/ mL for *C. longa*, 0.0156 mg/ mL for *N. sativa* and 2mg/ mL for *P. betel* were also determined by using serial dilution technique.

Ethyl acetate extract of *C. longa* was separated by column chromatography and curcumin (5.9 %), desmethoxycurcumin (0.018 %) and bisdesmethoxy curcumin (0.0136 %) were obtained in pure form as crystal. Kaempferol (0.12 %) and quercetin

(0.001 %) were isolated from flavonoid extract of *N. sativa* by column chromatographic method.

Essential oil of *N. sativa* was fractionated by column chromatography to yield thymoquinone (0.01 %). Eugenol (0.1 %) was also isolated from essential oil of *P. betel.* The isolated compounds were identified by UV, FT IR, ^{1}H NMR and mass spectroscopic methods.

The antibacterial activity of isolated compounds was tested by agar disc diffusion technique. From the experimental results, all the isolated compounds showed antibacterial activity against all tested organisms (12 mm-25 mm). Among these thymoquinone was found to be the most active compound (> 25 mm).

C. longa (Nanwin), *N. sativa* (Samon-net) and *P.betel* (Kun) may be used for the treatment of dysentery and diarrhea since they have bactericidal action against *Escherichia coli* responsible for diarrhea and *Shigella boydii* responsible for dysentery. These three plants were used in traditional medicine formulation (TMF-06) to treat the diseases caused by bacteria. From the experimental results that *N. sativa* (Samon-net seed) is the most effective plant to treat dysentery and diarrhea. Therefore, these three plants have a good potential for the production of combined formulation to treat diseases caused by bacteria.

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