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Antibacterial activity of some plants and formulations and determination of Minimum Inhibitory Concentration (MIC) by microtitre plate dilution method

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Traditional Medicine Formulation (TMF-01) [mixture of *Cyperus rotundus* Linn. (မြက်မုန်ညင်း), *Alpinia galangal* Walld (ပဒေကောကြီး**)** and *Piper betle* Linn. (ကွမ်း)] and plants [*Alpinia galangal* Walld. (ပဒေကောကြီး) *Acorus calamus* Linn. **(vif;ae)**, and *Piper longum* Linn. (ပိတ်ချင်း)] were tested for their antibacterial activity on 20 different types of bacteria (*Escherichia coli*=6 types, *Proteus morganii, Shigella boydii, S. dysenteriae, S. flexneri, S. sonnei, S. derby, S. krefeld, Staphylococcus aureus*; *S. epidermidis*, *Vibrio cholerae* O1, *V. cholerae* O139, *V. cholerae* Inaba, *V. cholerae* Ogawa and *V. fluvialis*) from clinical sources. The bacteriostatic and bactericidal activities of extracts were tested by microtitre plate dilution method and the optical density was determined by microplate reader. It was found that water extract of TMF had antibacterial activity on 20 different types of bacteria with various inhibition zone sizes ranging from 14 to 26 mm. Similarly, water extract of ash and water extract of ash from water extract of *Alpinia galangal* Walld (ပဒေကောကြီး) possess antibacterial activity with the zone size of 7-18 mm. The asarone and methyl piperate compound obtained from *Acorus calamus* Linn. (လင်းနေ), and *Piper longum* Linn. (ပိတ်ချင်း) respectively also showed antibacterial activity on *E. coli, S. flexneri, S. aureus* and *P. aeruginosa.* The Minimum Inhibitory Concentration (MIC) of water extracts of TMF -01 was ranging from 0.16 to 0.32mg/ml. Similarly, the MIC of water extract of ash of *Alpinia galangal* Walld (ပဒေကောကြီး) was 0.16 mg/ml and water extract of ash from water extract of *Alpinia galangal* Walld (ပဒေကောကြီး) ranged from 0.078 to 0.625mg/ml. Moreover, the MIC of asarone from *Acorus calamus* Linn. (လင်းနေ) was 0.06-0.12mg/ml and that of methyl piperate from *Piper longum* Linn. (ပိတ်ချင်း) was 0.03mg/ml.

INTRODUCTION

Herbal medicines, which have formed the basis of health care throughout the world since the earliest days of mankind, are still widely used and are of considerable importance in international trade. Recognition of their clinical, pharmaceutical and economic values is not only widespread but popularity is also maintained for historical and cultural reasons [1,2]. Plants, *Alpina galangal* Walld. (ပဒေကောကြီး), *Acorus calamus* Linn. **(**လင်းနေ**)** and *Piper longum* Linn. (ပိတ်ချင်း) [3] and Traditional Medicine Formulation 01 are well-known traditionally and are used by traditional medicine practitioners . Thus, this study is to determine the antibacterial activity of different preparations of extracts and to determine the minimum inhibitory concentration by microtitreplate dilution method.

MATERIALS AND METHODS

*Traditional Medicine Formulation(TMF-01)***:** It contains *Cyperus rotundus* Linn. (မြက်မုန်ညင်း), A*lpinia galangal* Walld. (ပဒေကောကြီး), and *Piper betle* Linn. (ကွမ်း).

Plants tested:
Alpinia galangal Walld. (ပဒေကာကြီး), *Acorus calamus* Linn. (လင်းခေ) and *Piper longum* Linn. (ပိတ်ချင်း).

Bacteria tested:

Escherichia coli ETEC, *E. coli* ATCC, *E. coli* LT, *E. coli* STLT, *E. coli* YCH 149, *Proteus morganii, Shigella boydii, S. dysenteriae, S. flexneri, S. sonnei, S. derby, S. krefeld, Staphylococcus aureus, S. aureus* M20, *Vibrio cholerae* O1, *V. cholerae* O139, *V. cholerae* Inaba, *V. cholerae* Ogawa, and *V. fluvialis* which were control strains and were isolated from clinical sources at Bacteriology Research Division, DMR (LM).

Extraction methods used for screening

Preparation of water extract

Sample powder either of the plants or formulations was weighed to 100g and was mixed thoroughly with 500 ml of distilled water by stirring overnight. The solution was spinned, filtered and then allowed to evaporate on a waterbath and was dessicated.

Water extract of ash

Sample powder was pre-ashed on a sand-bath until all the combustible materials were burnt out. The basin containing pre-ash samples was then placed inside a furnace (electric muffle furnace) and heated gradually by raising the temperature until 450˚C. The process of heating, cooling and weighing was repeated until constant weight of ash was obtained. Ash samples were then stirred in 200 ml of distilled water overnight. The solution was then centrifuged, filtered and evaporated and dessicated.

Water extract of ash from water extract

The above water extract was pre-ashed on the sand bath until all the combustibles were burnt and the procedure was as in the preparation of water extract of ash.

Preparation of asarone and methyl piperate

From ethyl acetate extract of *A. calamus* and *P. longum*, asarone (0.712%) and methyl piperate (0.084%) were obtained from Yangon University, reported by Ei Ei Khine [4] respectively and identified by phytochemical test, melting point thin layer chromatography (TLC), ultra violet (UV), Fourier transform infraRed spectroscopy (FT-IR) and mass spectrometric (MS) methods [4].

Screening for antibacterial activity by agar disc diffusion technique

It was done as described in Mar Mar Nyein *et al.,* (1991) [5].

Determination of minimum inhibitory concentration (MIC) by microplate dilution method

The bacteriostatic and bactericidal activities of extracts were tested by microtitre plate dilution method and the optical density was determined by microplate reader.

First, an inoculum of pure culture of respective organisms was seeded in 5 ml of trypticase soy broth (TSB) and incubated at 37˚C for 3-4 hours to obtain a turbidity of 0.05 by MacFarland nephelometer which corresponded to a bacterial suspension of 10^6 organisms per ml. If necessary, the broth cultures were diluted with sterile normal saline to meet the criteria. Prior to the experiment, 50 l of TSB was introduced into all wells of 96-well microtitre plate (Falcon 3072). The extracts to be tested were weighed, calculated and allowed to dissolve in a minimum amount of solvent and the amount required was made up by adding TSB to obtain the required concentration. Generally, approximately 2 mg/ml and 20 µg/ml of crude and pure compound respectively were utilized for the experiment. The prepared extract to be tested was introduced into all the wells of the first row of the plate (1A-H). With the aid of a 8-channel micropipetter (Titre Tek) 50 µl of the mixture were transferred to the wells of the second row of the microtitre plate (2A-H). The solution was mixed thoroughly by the pipetter and then transferred to the third row (3A-H) and the

same procedure was carried out up to the tenth row (10 A-H) and the remaining 50 µl was discarded. The 11th row contained solvent mixed with TSB and the 12th row served as media control. Before transferring the contents of each well, the mixture was mixed thoroughly with the multichannel pipetter. After that 50 µl of diluted inoculum preparation of each bacteria to be tested (approximately 10^6 organisms/ml) were introduced to the respective wells (1-11 A-H) except 12A-H wells. Thus, eight types of bacteria could be tested in each plate. The plates were then incubated at 37˚C for 18 hours. Prior to the spectrophotometric recordings, the mixtures were allowed to mix thoroughly by gently rocking the plates mechanically on a shaking machine. Growth of microorganisms was determined by an automated microplate reader (BioRad) at a wave length of 450 nm. From each and every well, 0.02 µl of broth suspension was inoculated onto nutrient agar, incubated at 37˚C for 18 hours and the growth of the respective organisms was recorded. The concentration of the extract in the last well with no growth of bacteria on nutrient agar was the minimum inhibitory concentration of the tested extract.

RESULTS

Antibacterial activity and minimum inhibitory concentration (MIC)

The mean diameters (in millimeter) of inhibition zones recorded by agar disc diffusion assay are shown in Table 1. It shows that water extract of TMF has antibacterial activity on 20 different types of bacteria with the zone sizes ranging from 14-26 mm. Similarly, water extract of ash and water extract of ash from water extract of *Alpinia galangal* Walld. (ပဒဲကောကြီး) possess antibacterial activity with the zone size of 7- 18 mm. The aserone and methyl piperate compound obtained from *Acorus calamus* Linn. (လင်းနေ) and *Piper longum* Linn. (ဝိတ်ချင်း) respectively also showed antibacterial action on *E. coli, S. flexneri, S. aureus* and *P. aeruginosa.* The Minimum Inhibitory Concentrations (MIC) of the tested extracts are shown in Table 2. It shows that the MIC of TMF-01 ranged from 0.16 to 0.32 mg/ml concentration of the extracts. Similarly, the MIC of water extract of ash of of *Alpinia galangal* Walld. (ပဒဲကောကြီး) was 0.16 mg/ml and water extract of ash from water extract of *Alpinia galangal* Walld. (ပဒဲကောကြီး) ranged from 0.078- 0.625 mg/ml. Moreover, the asarone from *Acorus calamus* Linn. (လင်းနေ) showed the MIC of 0.06-0.12 mg/ml and methyl piperate from *Piper longum* Linn. (ဝိတ်ချင်း) was 0.03 mg/ml.

Table 1. The mean diameters in millimeter of Inhibition zones by agar disc diffusion assay

Tested bacteria	TMF	Badegawgyee		Lin-ne	Peik-chin
	water extract	Water extract of ash	Water extract of ash from water extract	Asarone	Methyl piperate
Escherichia coli ETEC	24	18	18	NT	NT
E. coli ATCC	17	10	16	33	37
E. coli LT	23	10	10	NT	NT
E. coli STLT	20	8	10	NT	NT
E. coli YCH 149	24	8	14	27	16
Vibrio cholerae O1	16	8	18	22	32
V. cholerae O139	15	8	12	NT	NT
V. cholerae Inaba	18	8	12	NT	NT
V. cholerae Ogawa	20	12	18	NT	NT
V. fluvialis	18	12	18	NT	NT
Shigella boydii	20	10	14	NT	NT
S. dysenteriae	14	9	16	NT	NT
S. flexneri	18	14	21	24	30
S. sonnei	18	12	8	NT	NT
Salmonella derby	20	10	10	NT	NT
Salmonella krefeld	16	8	14	NT	NT
Staphy-lococcus aureus	26	8	12	28	31
S. aureus M20	24	7	16	NT	NT
Proteus morganii	15	10	10	NT	19
Pseudomonas aeruginosa	20	8	14	12	42

NT= Not tested due to minute amount of the compound

Table 2. Minimum Inhibitory Concentrations of TMF and some plant extracts in mg/ml

Tested bacteria	TMF	Badegawgyee		Lin-ne	Peikchin
	Water extract	Water extract of ash	Water extract of ash from water extract	Asarone	Methyl piperate
Escherichia coli ATCC	0.16	0.16	0.078	0.12	0.03
Escherichia coli STLT	NT	NT	NT	0.63	0.03
Escherichia coli LT	0.16	0.16	0.078	0.06	0.03
Proteus morganii	0.16	0.16	0.625	NT	0.03
Salmonella derby	0.16	0.16	0.625	NT	0.03
Salmonella krefeld	0.32	0.16	0.625	NT	0.03
Staphylo-coccus aureus ATCC	0.32	0.16	0.625	0.12	0.03
Staphylo-coccus aureus	0.32	0.16	0.625	0.06	0.03
Vibrio fluvialis	0.32	0.16	0.625	NT	NT

NT= Not tested

The minimum inhibitory concentration of *Alpinia galangal* Walld. (ပဒဲကောကြီး) extracts AW (water extract of ash) and WAW (water extract of ash from water extract) tested on *Escherichia coli* ETEC is shown in Fig. 1 .

DISCUSSION

In this study by agar disc diffusion technique, antibacterial activity was found in the water extract of ash of TMF-01. Water extract of ash and water extract of ash from water extract of *Alpinia galangal* Walld. (ပဒဲကောကြီး) also showed a significant inhibition zone. In determination of MIC, it showed that the MIC of water extract of ash from water extract of *Alpinia galangal* Walld (ပဒဲကောကြီး) was 0.078 mg/ml. Extraction method plays an important role in determination of antibacterial testing. Moreover, the MICs of asarone from *Acorus calamus* Linn. (လင်းနေ) was 0.06-0.12 mg/ml and methyl piperate from *Piper longum* Linn. (ပိတ်ချင်း) was 0.03 mg/ml and these plants were known to possess antibacterial activity [6]. These plants were used for various ailments by Myanmar traditional medicine practioners especially for gastrointestinal disorders [7, 8].

Phytochemical testing showed that water extract of TMF 01 contains α-amino acids, glycosides and saponins. Water extract of *Alpinia galangal* Walld. (ပဒဲကောကြီး) contains alkaloids, α-amino acids, glycosides, phenolic compounds, saponins,steroids, terpenoids, tannins and reducing sugar. Sodium (9.74%), magnesium (1.52%), potassium (1.01%), calcium (1.23%) were found as major elements in TMF-01. In *Alpinia galangal* Walld. (ပဒဲကောကြီး), sodium (0.07%), magnesium (0.34%), potassium (2.24%), and calcium (0.08%) were found. Iron was found as a minor element; chromium, zinc, and arsenic were found as trace elements in both TMF and *Alpinia galangal* Walld. (ပဒဲကောကြီး) [9].

According to their study, these plants possess not only the antibacterial activity but also salts and nutrients that show antisecretory activity in gastrointestinal infections.

The microplate dilution method also elaborates the specificity, sensitivity and the least amount required for media, reagents and glassware. It also saves time and working space in conducting the experiments.

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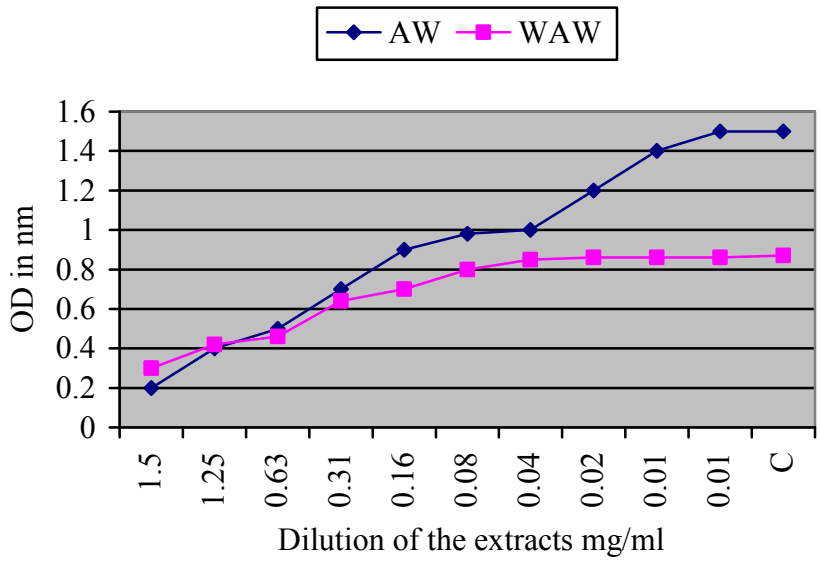

Fig.1. Minimum Inhibitory Concentration of Badegawgyee extracts AW (water extract of ash) and WAW (water extract of ash from water extract) tested on *Escherichia coli* ETEC

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REFERENCES

1. Albert, F. H. Economic Botany. A Text book of Useful Plants and Plant Products second edition, 1976.

2. ASEAN Symposium. Progress in Chemistry of Medicinal Plants in Asia. *Proceedings of the sixth Asean Symposium on Medicinal Plants and species.*, Bandaung, 1989.Ashin Nagathein Pon pya say ah beadan (Myanmar version). Mingalar Press, Yangon, 1978.

4. Ei Ei Khine. Isolation, identification and screening of antibacterial activities of some active principles of *Acorus calamus* Linn. (Lin-ne) and *Piper longum* Linn (Peikchin) for thetreatment of dysentery and diarrhea. *PhD Dissertation Chemistry*, 2005.

5. Mar Mar Nyein, Chit Maung & Mya Bwin *In vitro* testing of various indigenous plant extracts on human pathogenic bacteria. *Myanmar Health Sciences Research Journal* 1991; 3(2): 89-99.

6. Mar Mar Nyein, Nwe Yee Win, Win Myint, Aye Aye Thein, Mi Mi Htwe, Win Win Maw & Aye Than. Plants possessing antibacterial activity. *Myanmar Health Sciences Research Journal* 1999; 11 (1-3): 26-32.

7. San San Aye, Mar Mar Nyein, Than Soe, Saw Hla Myint & Maung Maung Htay . Antibacterial screening and chemical investigation of Myanmar Traditional Medicine Fomulation (TMF-06) used to treat dysentery. *Journal of Myanmar Academic Arts and Science* 2002, 1(3): 79-99.

8. Mar Mar Nyein and Mi Mi Htwe. Activity of Traditional Medicine Formulations (TMF-06 and TMF-23) on some pathogenic bacteria. *Myanmar Health Sciences Research Journal* 2004; 16 (1): 18-22.

9. Ba Han. Chemical and antibacterial studies of some Traditional Medicine Formulations. *PhD Dissertation, Chemistry*, 2003.