ANTIMICROBIAL ACTIVITY AND PHYTOCHEMICAL CONSTITUENTS OF *EMBLICA OFFICINALIS* GAERTN. (ZI-BYU) AND *TERMINALIA CHEBULA* RETZ. (PHAN-KHA)

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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 medicinal plants (Zi-Byu and Phan-Kha) 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. Polar and non-polar extracts of the selected fruits were tested on six microorganisms by agar well diffusion technique. Phytochemical examination for types of organic constituents present in Zi-Byu and Phan-Kha fruits were carried out. The polar extracts of Zi-Byu and Phan-Kha showed antimicrobial activities (17 mm - 40 mm) against all the six microorganisms tested. Gallic acid (0.9 %) and β-sitosterol (0.36 %) were isolated from Zi-Byu and Phan-Kha, respectively. The isolated gallic acid was identified by UV, FT IR, ^{1}H NMR, ^{13}C NMR and mass spectrometry. β-sitosterol isolated from Phan-Kha was confirmed by authentic β-sitosterol and also compared with melting point determination. Moreover, isolated gallic acid showed antimicrobial activity (12 mm - 15 mm) against *Staphylococcus aureus*, *Candida albicans* and *Mycobacterium* species.

Keywords*:* antimicrobial activities, gallic acid, β-sitosterol, phytochemical

Introduction

Emblica officinalis Gaertn. (Zi-Byu)

The medicinal plant, *Emblica officinalis* Gaertn. (Zi-Byu), belonging to the family, Euphorbiaceae, is commonly known as 'Amla' or 'amlaki' in Bengali and 'Indian gooseberry' in English. This species is medium sized deciduous tree with 8-18 meters height (Rai **et al**., 2012). Amla is rich in fiber, carbohydrate, iron and is reported as the richest source of vitamin C (Singh **et al**., 2011). Supplements of fresh amla fruit is very favorable to individuals suffering from anemia. The juice of fresh amla fruit is given as diuretic, anti-bilious remedy and as a tonic. It is also helpful in over thirst, dyspepsia, burning sensation and other complaints of digestive system (Kumar *et al.,* 2012).

Terminalia chebula Retz. (Phan-Kha)

Terminalia chebula Retz. of the family Combretceae is an important tree of pharmaceutical and trade value. It is distributed in the forests of Northern India, Eastern India and the Southern Peninsula. The fruit of the plant is rich in tannin and commonly known as myrobalan or chebulic myrobalan. In Indian pharmacopoeia, fruit of *T. chebula* is extensively used as adjuvants to other medicines in almost all diseases e.g jaundice, splenopathy, hiccough, cephalagia, epilepsy, leprosy and as astringent, anti-inflammatory, digestive, cardiotonic (Phetkate *et al.,* 2012). Effect of tannin from *T. chebula* on the infectivity of potato virus X was also reported.

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The present research work deals with antimicrobial screening of polar and non-polar extracts and the column chromatographic isolation of some organic constituents of *E. officinalis* and *T. chebula* fruits.

Materials and Methods

Plant materials

The fruits of Zi-Byu and Phan-Kha were collected from local market in Yangon Region. These samples were ground to get a fine powder by grinding machine. The drug powders were then stored in an air-tight container.

Preparation of crude extracts

100 g of each dried powdered sample were 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, acetone and 80 % EtOH extracts of each dried powdered sample were prepared according to the above procedure. In the preparation of watery extract; 100 g of each dried powdered sample were soaked in 500 mL of distilled water into the conical flask. These flasks were boiled on water bath for 6 h 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

Antimicrobial activity of the prepared 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 well was 0.1 mL. The petridish was then incubated at 37 ºC for 48 h, and the diameters of clear inhibition zones around the wells, if appeared, were measured (Finegold and Martin, 1982).

Isolation of compound from *E. officinalis* (Zi-Byu)

The dried fruit powder (50 g) was soaked overnight in 200 mL of PE at room temperature (Harborne, 1984). The mixture was filtered and the filtrate (PE extract) kept for later use; the residue was extracted with 500 mL of 70 % EtOH by maceration for one week and the EtOH extract was evaporated to dryness in rotary evaporator. The residue was dissolved in 2 M 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 in rotary evaporator and the residue ("flavonoid" extract) was used for column separation. Thus, ethyl acetate extract (0.6 g) was chromatographed on a silica gel column using toluene: ethyl acetate (7:3) solvent mixture as eluent. Finally, three main fractions were obtained. Fraction No.3 yielded compound 1 as pure crystals (0.9 % gallic acid) upon evaporation at room temperature. The isolated compound 1 was identified by spectroscopic method (^{1}H, ^{13}C and MS). The NMR spectra (^{1}H, ^{13}C) of isolated compound was recorded in Pyridine with TMS as internal standard at 400 MHz for ^{1}H, and at 100 MHz for ^{13}C NMR. Moreover, mass spectrum (ESI-FT-ICR) of isolated compound was recorded by a Bruker Apex III Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer.

Isolation of compound from *T. chebula* (Phan-Kha)

The dried fruit powder (50 g) was extracted with petroleum ether (60-80 ºC) for about one week by percolation method followed by filtration. PE extract of Phan-Kha was fractionated by column chromatography using PE: ethyl acetate (9:1, 5:1) as eluent. Finally, colorless crystals of β-sitosterol (0.36 %) were isolated. β-sitosterol is one of several phytosterols with chemical structure similar to that of cholesterol. It is a white, waxy with a characteristic odour. The isolated compound was subjected to Co-TLC analysis with a standard β-sitosterol sample. The chromatogram was developed in the chosen solvent system for the compound isolated. After the plate has dried, the R_f value of isolated sample was measured. Localization of spot was made by viewing directly under UV 254 nm and 365 nm light. And then, it was treated with 5 % H_2SO_4, anisaldehyde-H_2SO_4 and ceric sulphate followed by heating, respectively.

Results and Discussion

Antimicrobial activity

In the present work, the samples were tested on six strains of microorganisms which include *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus pumilus, Candida albicans* and *Mycobacterium* species. The measurable zone diameter, including the agar well diameter, shows the degree of antimicrobial activity. It was found that the polar extracts of *E. officinalis* and *T. chebula* showed antimicrobial activities against all the six microorganisms tested (Figure 1 and Table 1 and Figure 2 and Table 2). It was also observed that, the PE extracts of *E. officinalis* and *T. chebula* did not show any antimicrobial activity. The gallic acid isolated from Zi-Byu indicated the antimicrobial activity against *Staphylococcus aureus, Candida albicans* and *Mycobacterium* species (12 mm-15 mm). According to the experimental results, antimicrobial activity of ethyl acetate crude extracts (40 mm) of Zi-Byu fruit was found to be more potent against *Bacillus subtilis* than 80 % EtOH crude extracts (26 mm) of Phan-Kha fruits. The larger the inhibition zone diameter, the greater is the antimicrobial activity.

Identification of isolated compounds

The two compounds namely gallic acid and β-sitosterol were isolated from Zi-Byu fruits and Phan-Kha fruits, respectively.

1 (Gallic acid): White crystalline compound (0.9 %), mp. 230 ºC (225-230 ºC) (Merck Index, 2001); UV(nm): 228, 262, 284; FT IR ν_{max}^{kBr} cm^{-1}: 3418 ($^{\upsilon}$O-H), 1701 ($^{\upsilon}$C=O), 1618, 1451, 1246, 1027; ^{1}H NMR (400 MHz, Pyridine-d5) δ (ppm): 8.11(s) (Figure 3); ^{I3}C NMR (100 MHz, pyridide-d5) δ (ppm): 169.50, 147.48, 110.45 (Figure 4); ESI - FT-ICR mass spectrum m/z (%): M-H, 169 (Figure 5).

2 (β-sitosterol): The isolated compound was UV inactive. It was identified by some colour tests on TLC, 5 % H_2SO_4, ceric sulphate solutions and anisaldehyde H_2SO_4 were employed as spraying agents followed by heating. It was also identified by comparison study (Co-TLC) with standard β-sitosterol (Figure 6). The same R_f values (0.27) were observed for the isolated compound and standard β-sitosterol with PE: EtOAc (5:1). The coloration of isolated compound was found to be identical with that of standard β-sitosterol with each spraying reagent. It gave violet, brown and bluish-purple colors when it was treated with 5 % H_2SO_4, anisaldehyde-H_2SO_4 and ceric sulphate followed by heating, respectively.

β-sitosterol: Colorless needle (0.36 % yield, mp. 138-140 ºC), FT IR ν_{max}^{kBr} cm^{-1}: 3400 ($^{\upsilon}$O-H), 2939-2877 ($^{\upsilon}$C-H), 1643 ($^{\upsilon}$C=C), 1049 ($^{\upsilon}$C-O).

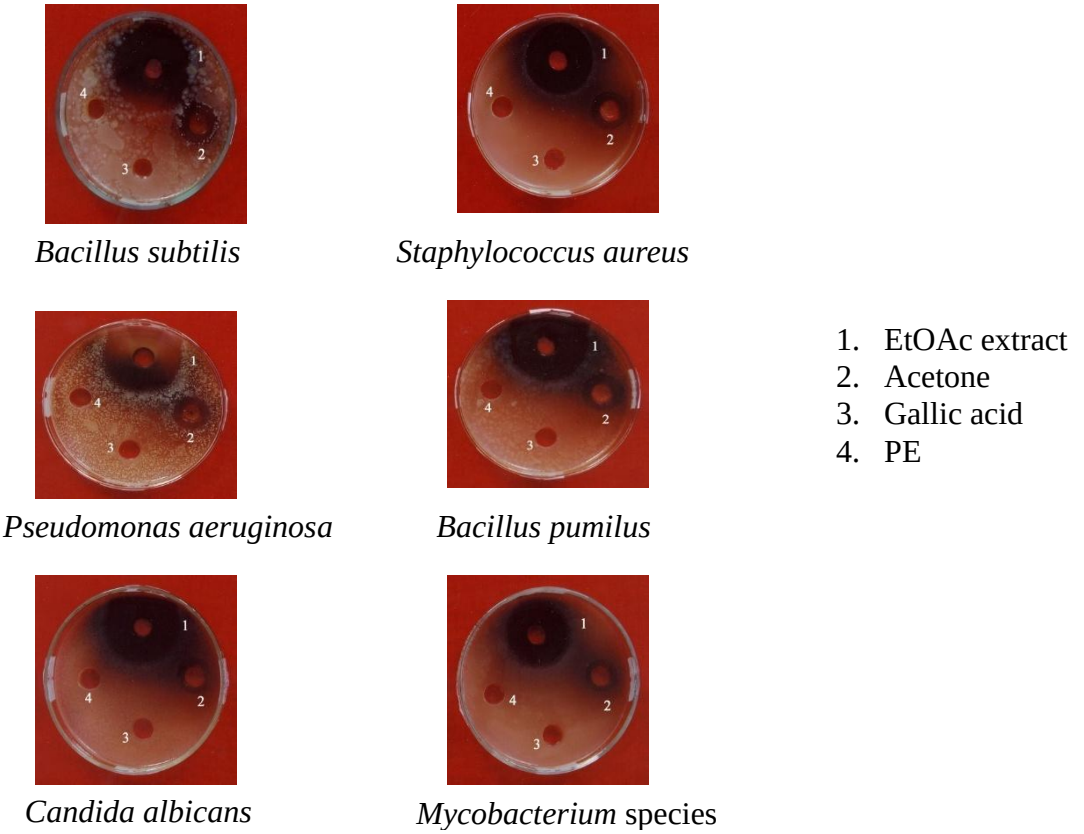


Figure 1 Agar well diffusion tests of *E.officinalis* (Zi-Byu) fruit extract on six microorganisms

Table 1 Results of Antimicrobial Activity of Zi-Byu Fruit

No	Organisms	Inhibition zone diameter (mm)			
		PE	**Gallic acid**	**Acetone**	**EtOAc**
1.	*Bacillus subtilis*	–	–	20	40
2.	*Staphylococcus aureus*	–	15	20	35
3.	*Pseudomonas aeruginosa*	–	–	17	40
4.	*Bacillus pumilus*	–	–	20	38
5.	*Candida albicans*	–	12	19	38
6.	*Mycobacterium species*	–	12	18	33

* Agar well -10 mm, No activity (–)

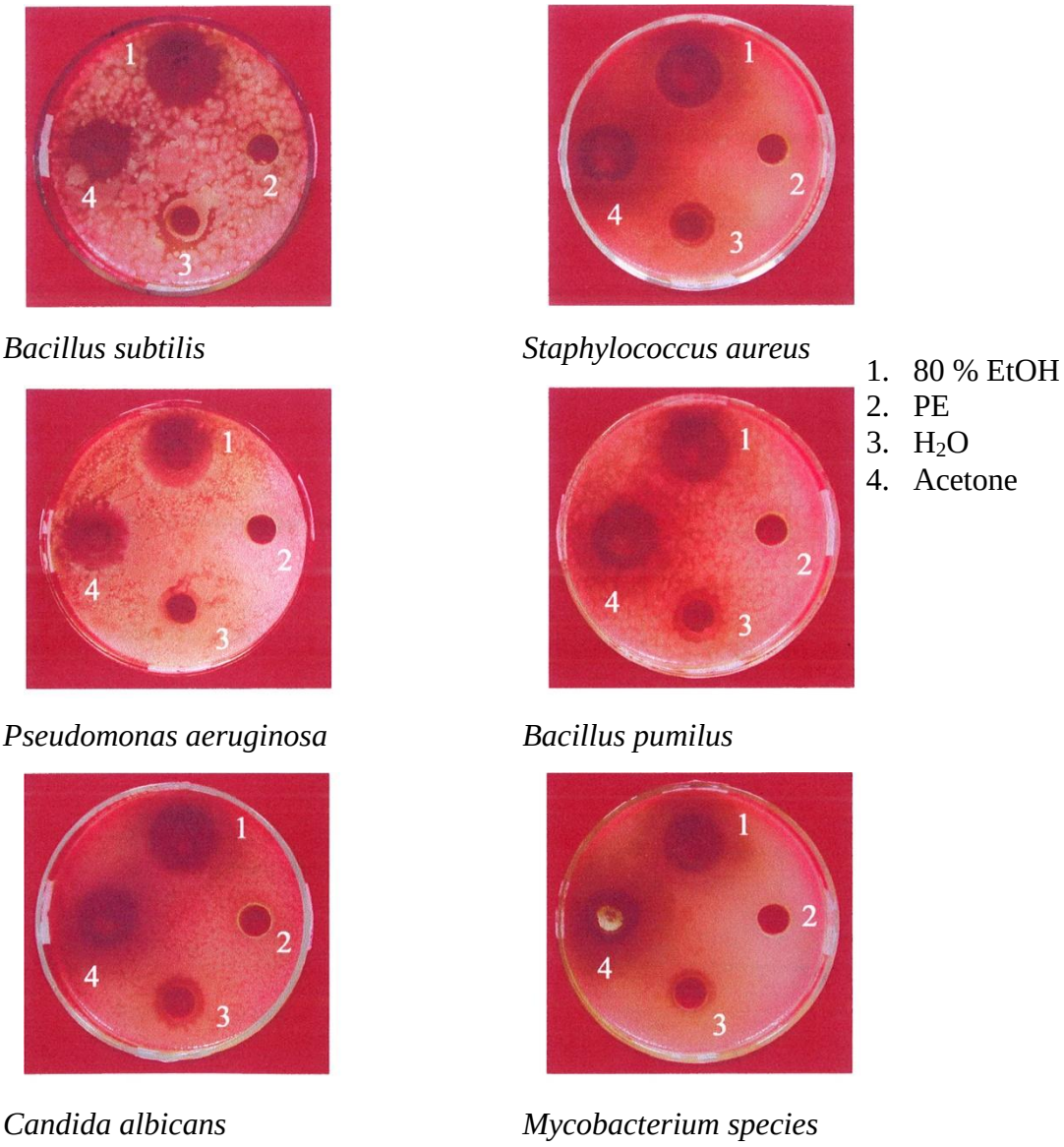


Figure 2 Agar well diffusion tests of *T. chebula* (Phan-Kha) fruit extracts on six microorganisms

Table 2 Results of Antimicrobial Activity of Phan-Kha Fruit

No	Organisms	Inhibition zone diameter (mm)			
		PE	**80 % EtOH**	**Acetone**	**H_2O**
1	*Bacillus subtilis*	–	26	22	20
2	*Staphylococcus aureus*	–	23	20	15
3	*Pseudomonas aeruginosa*	–	22	19	15
4	*Bacillus pumilus*	–	24	25	18
5	*Candida albicans*	–	24	21	17
6	*Mycobacterium species*	–	20	19	17

* Agar well-10 mm, (–) No activity

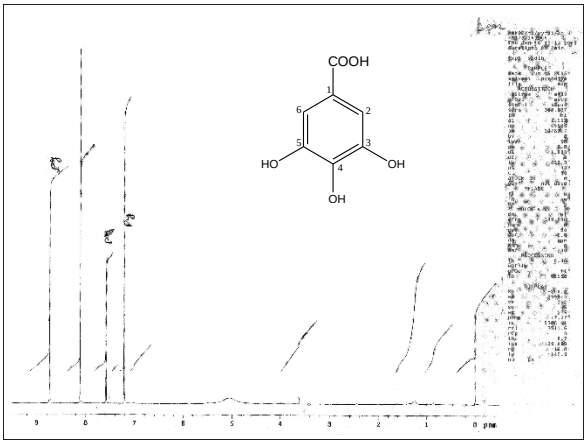


Figure 3 ^{1}H NMR spectrum of isolated gallic acid from *E. officinalis* (Zi-Byu)

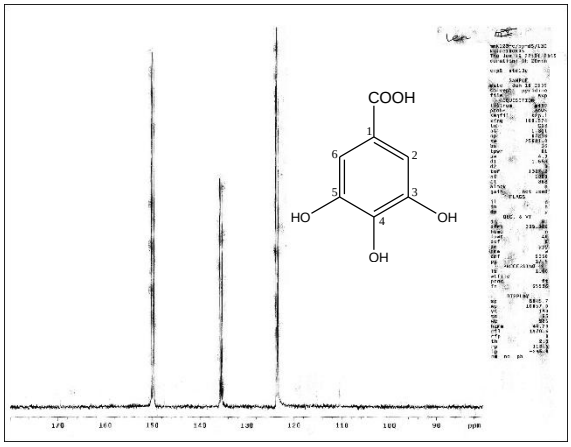


Figure 4 ^{13}C NMR spectrum of isolated gallic acid from *E. officinalis* (Zi-Byu)

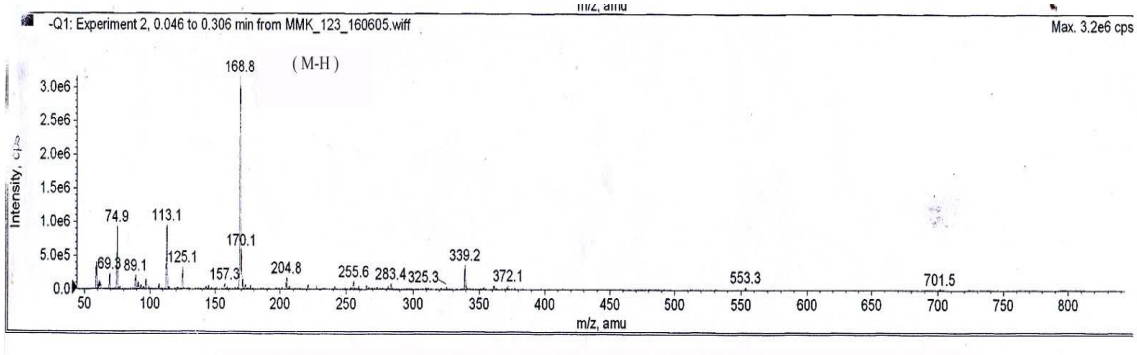
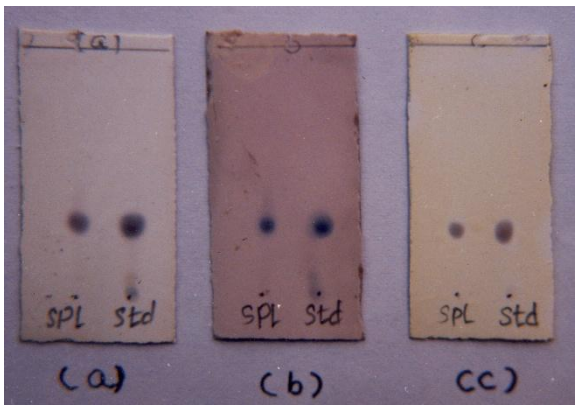


Figure 5 ESI-FT-ICR mass spectrum of isolated gallic acid from *E. officinalis* (Zi-Byu)



SPL = Sample
Std = standard β-sitosterol
Spraying agents
(a) = 5 % H_2SO_4
(b) = Anisaldehyde H_2SO_4
(c) = $CeSO_4$
Stationary phase: Silica gel
Mobile phase : PE: EtOAc (5:1)

Figure 6 Thin layer chromatograms of isolated β–sitosterol from *T. chebula* (Phan-kha)

Conclusion

From the present research work on "Comparative studies of antimicrobial activity and phytochemical constituents of *E. officinalis* (Zi-Byu) and *T. chebula* (Phan-Kha)", the following conclusions can be drawn.

Crude extracts have been prepared from Zi-Byu and Phan-kha by using non-polar and polar solvents. The antimicrobial activity of the crude extracts was screened by *in vitro* method

using agar well diffusion techniques on six microorganisms which include *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus pumilus, Candida albicans* and *Micobacterium* species. Polar extracts of Zi-Byu and Phan-Kha showed antimicrobial activity against all tested microorganisms (17 mm - 40 mm). However, non-polar extracts of both fruits did not show antimicrobial activity. In the case of *Bacillus subtilis* and *Pseudomonas aeruginosa*, ethyl acetate extract of Zi-Byu fruit has more antimicrobial activity than acetone extract. 80 % ethanol extract of Phan-Kha has higher antimicrobial activity than acetone extract against *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans* and *Mycobacterium species.* From the experimental results, Zi-Byu (40 mm) has more pronounced antimicrobial activity than Phan-Kha fruit (26 mm). Colorless crystal of gallic acid (0.9 %) was obtained from ethyl acetate extract of Zi-Byu by column chromatographic method. It was confirmed by UV, FT IR, ^{1}H NMR, ^{13}C NMR and mass spectrometry. Pet-ether extract of Phan-Kha was separated by column chromatographic method to give β-sitosterol (0.36 %). It was confirmed by standard β-sitosterol and melting point determination. Moreover, isolated gallic acid from Zi-Byu also showed antimicrobial activity (12 mm-15 mm). Gallic acid is a well-known natural antioxidant. Therefore, gallic acid containing Zi-Byu fruit may be used as natural antioxidant and antimicrobial agents.

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