PHYTOCHEMICAL INVESTIGATION AND ANTIMICROBIAL ACTIVITY FROM FRUIT EXTRACT OF *HAPLOPHRAGMA ADENOPHYLLUM* (WALL.) DOP. (PHET-THAM)

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Abstract

The present work was conducted to investigate the phytochemical constituents and antimicrobial activity of fruits of *Haplophragma adenophyllum* (Wall.) Dop. (Phet-tham) on different microbial strains. From the phytochemical investigation, fruits of Phet-tham showed positive for alkaloids, α-amino acids, carbohydrates, flavonoids, glycosides, phenolic compounds, reducing sugars, saponins, steroids, tannins, terpenoids and showed negative results for starch and cyanogenic glycoside. Elemental analysis by EDXRF method revealed that fruits of Phet-tham contained K, Ca and Cl as major elements. By silica gel column chromatographic separation, three terpenoid compounds including lupeol (0.033 %, m.pt.=213-214 °C) were isolated from pet-ether extract of fruits of *H. adenophyllum*. Antimicrobial activities of pet-ether, ethyl acetate, 95 % ethanol, methanol and watery extracts from the fruits of *Phet-tham* were investigated against six species of microorganisms such as *Bacillus subtilis*, *Bacillus pumilus*, *Staphylococcus aureus*, *Pseudomonas areuginosa*, *Escherichia coli* and *Candida albicans* by agar well diffusion method at Pharmaceutical Research Department (PRD), Yangon. Among all tested crude extracts EtOAc extract exhibited the highest antimicrobial activity with the inhibition zone diameter in the range of 13~33 mm against all microorganisms tested.

Keywords: antimicrobial activity, *Haplophragma adenophyllum* (Wall.) Dop., lupeol,

Introduction

Haplophragma adenophyllum (Wall.) Dop. (Phet-tham), a member of the Bignoniaceae family, is a deciduous tree grown in tropical and subtropical climates of Southeast Asia and Africa (Jassbi *et al.*, 2004). These trees are commonly known in English as Karen wood. This tree is grown as an ornamental plant in parks and gardens due to the unique magical shape of its pods that distinguishes it amongst other trees of horticultural significance. *Haplophragma adenophyllum* (Wall.) Dop. has been used for various ailments treatment which includes cancer, gastrointestinal disorders, cholera, rheumatoid arthritis, hepatic disorders, leucorrhea and diabetes (Rahmatullah *et al*., 2010). In Myanmar, the plant is distributed in various regions and used as remedy for various medicinal purposes. Hnin Wutt Yee Win reported that the bark of *H. adenophyllum* possesed antimicrobial activity (Hnin Wutt Yee Win, 2013). In this research work, the fruits of *H. adenophyllum* (Phet-tham) were chosen for determination of some phytochemical constituents and evaluation of antimicrobial activity.

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Botanical Aspect of *Haplophragma adenophyllum* (Wall.) Dop.

Scientific classification

Family : Bignoniaceae
Botanical name : *Haplophragma adenophyllum* (Wall.) Dop.
Myanmar name : Phet-tham
English name : Karen wood
Synonyms : *Fernandoa adenophyllum*
Part used : Fruits

Figure 1 *Haplophragma adenophyllum* (Phet-tham) Fruits

Uses of *Haplophragma adenophyllum* (Wall.) Dop. (Phet-tham)

The fruits of *H. adenophyllum* (Phet-tham) is a traditional medicinal tree used for the prevention and treatment of various diseases. In Thai traditional medicine, the leaves are used for external treatment of skin diseases. As an ingredient in message oils, it is supposed to ease muscular tension sparingly cultivated as an ornamental tree. Folk medicinal uses of *H. adenophyllum* roots are used in piles, constipation and also prescribed as drink in viper bite. *H. adenophyllum* leaves and seeds have been used since centuries in traditional medicinal for the skin, urinary tract infections, antidiarrheal and anti-diabetic agents. It is used for various ailments treatment which includes cancer, gastrointestinal disorders, cholera, rheumatoid arthritis, hepatic disorders, leucorrhea and diabetes (Rahmatullah *et al*., 2010). In Myanmar, the boiled fruits of *H. adenophyllum* are used to eat with fish sauce as diet. In Myanmar, no scientific study was carried out to assess antimicrobial activities of the fruits extracts of *H. adenophyllum*. Therefore, present study was conducted to determine the bioactivities of various crude extract of fruits of *H. adenophyllum*.

Materials and Methods

Collection of *H. adenophyllum* Fruits Sample

The fruits of *H. adenophyllum* (Wall.), Dop., (Phet-tham) belonging to the family Bignoniaceae were collected from Laputta Township, Ayeyarwady Region, Myanmar, during January to February, 2018. The collected fresh fruit sample was washed and peeled. The fruit plups were dried at room temperature for two weeks and dried fruits were ground into powder and then it was stored in air tight container.

Qualitative Screening of the Phytochemicals

In order to classify the types of organic constituents present in fruits samples, preliminary phytochemical tests on samples were carried out according to the series of test tube methods (Trease and Evans, 1980; Marini-Bettole *et al.,* 1981; Shriner *et al.,* 1980; Robinson, 1983; and M-Tin Wa, 1972;).

Qualitative Elemental Analysis of the Fruits of Phet-tham by Energy Dispersive X-ray Fluorescence (EDXRF) Spectrometry

For this measurement, pellets of the powdered sample were first made. X-ray spectrometer permits simultaneous analysis of light element to heavy element. Energy dispersive X-ray fluorescence spectrometer (Shimadzu EDX - 700) can analyze the elements from Na to U under vaccum condition. X-ray fluorescence uses X-rays to excite and unknown sample. The individual elements in the sample are detected by using semiconductor detector [Si-Li] that permits multi-elements, simultaneous analysis. In this way, the elements present in fruits of Phat-tham were measured by EDXRF method using EDX-700 instrument at the Universities' Research Center (URC), Yangon.

Separation and Isolation of some Organic Constituents from the Fruits of Phet-tham
Preparation of crude extracts by successive solvent extraction method

Dried powered sample of fruits of Phet-tham (ca. 200 g) was percolated in 700 mL of 95 % EtOH with occasional shaking for one week and filtered. This procedure was repeated three times. The combined filtrate was concentrated under vacuum evaporator to obtain 95 % EtOH crude extract. The defatted marc was removed. 95 % EtOH crude extract was stirred with 500 mL PE (60-80 °C) and filtered. This procedure was repeated three times. The 95 % EtOH crude extract and PE crude extract were obtained. The 95 % EtOH crude extract was partitioned with EtOAc. After removal of the 95 % EtOH layer, EtOAc soluble extract was obtained. In the same procedure with different solvent, way, PE extract and 95 % EtOH extract from the fruits of Phet-tham were also obtained. These crude extracts were kept for separation of organic constituents and for screening of antimicrobial activities.

Isolation of some organic constituents from pet-ether extract of the fruits of Phet-tham

A glass chromatographic column (50 × 3 cm) with a tap attached was clamped so that it was perfectly vertical. The column was packed by the wet method, using PE:EtOAc (40:1 v/v). The column was plugged by pushing a small piece of cotton wool through the solvent with a glass rod. Care was taken so that no air bubbles were trapped in the cotton wool. Silica gel (ca. 50 g) was measured and placed in a beaker and made into slurry by mixing with pet-ether and the suspension was thoroughly stirred. A portion of the slurry was poured into the column and at the same time the tap was opened so that the solvent flowed at a slow but constant rate. As the column material slowly settled to the bottom, the column was lightly tapped with a rubber tubing around the outside wall so as to achieve an air bubble free, uniform packing. Column materials sticking to the upper walls of the column were washed down with the solvent. When the level of solvent had fallen to a few millimeters above the top of the silica gel column, the tap was closed. PE crude extract (3 g) was dissolved in PE and mixed with a little amount of silica gel. The mixture was allowed to evaporate with continuous agitation so that a free flowing dry silica gel on which the sample was uniformly adsorbed. By careful pouring of the adsorbed gel down the small funnel and adjusting the position of the lower end of the tube, a uniformed layer of adsorbed gel was placed on the top of the column. The top of the layer was wet with solvent. Some adsorbed gel sticking on the inner wall was washed down with the solvent. A piece of cotton wool was placed between the solvent and the column gel. The column was then completely filled with the solvent system and fraction was started; flow rate was adjusted to

about one drop per five seconds. Gradient elution was performed successively with increasing polarity (PE: EtOAc, 40:1, 20:1, 10:1, 5:1, 2:1, 1:1 and 1:2). Successive fractions obtained were combined on the basic of their behavior on TLC. Finally eleven main fractions (F_I to F_{XI}) were collected. Fraction F_I, F_{III}, F_V, F_{VI}, F_{VIII}, F_{IX}, F_X and F_{XI} were found as mixtures. Fraction F_{II} was evaporated and washed with PE and PE: EtOAc (20:1 v/v) and then recrystallized from PE, yield (49.6 mg, 0.033 %) of compound I as colorless needle shaped crystal. Fraction F_{IV} provided the solid material. The solid materials were washed with PE and PE:EtOAc (20:1 v/v) and then purified by recrystallization from PE and EtOAc, to give (110.7 mg, 0.074 %) of compound II as colorless needle shaped crystal. Fraction F_{VII} provided the solid material. The solid materials were washed with PE and PE: EtOAc (10:1 v/v) and then purified by recrystallization from MeOH, to give (6.9 mg, 0.004 %) of compound III as colorless crystal.

Screening of Antimicrobial Activity of Different Crude Extracts

Preparation of crude extracts by direct extraction method

Each dried powdered sample (50 g) was extracted with 150 mL of PE (60-80 °C) for 6 h by using soxhlet extractor. The filtrate was concentrated by removal of the solvent under reduced pressure to give the respective pet-ether crude extract. Preparation of ethyl acetate extract, 95 % ethanol, methanol, and watery extracts were also prepared by similar manner mentioned in above procedure. Each extract was dried at normal pressure on a water bath and stored under refrigerator for screening some bioactivities.

The antimicrobial activities of different crude extracts such as PE, EtOAc, 95% EtOH, MeOH and H_2O extracts from fruits of Phet-tham were determined against six species of microorganisms such as *Bacillus pumilus* (N.C.I.B - 8982), *Bacillus subtilis* (N.C.T.C - 8236), *Candida albicans, Escherichia coli* (N.C.I.B - 8134), *Pseudomonas aeruginosa* (6749) and *Staphylococcus aureus* (N.C.P.C - 6371) by employing agar well diffusion method at the Pharmaceutical Research Department, Ministry of Industry, Yangon, Myanmar.

Results and Discussion

From preliminary phytochemical analysis of the fruits of Phet-tham the results showed that the fruits of Phet-tham contain alkaloids, α-amino acids, carbohydrates, flavonoids, glycosides, phenolic compounds, reducing sugars, saponins, steroids, tannins and terpenoids. But starch and cyanogenic glycosides were found to be absent in the fruits of Phet-tham.

EDXRF elemental analysis revealed that the fruits of Phet-tham contained K, Ca and Cl as major elements. Moreover, S, Br and Mn as minor elements, Cu, Rb, Zn and Br as trace elements were also present in Phet-tham fruits.

By silica gel column chromatographic separation, compound I (lupeol, colourless needle shaped crystals, 0.033 %, m.pt. = 213-214 ^{0}C), compound II (a terpenoid compound, colourless needle shaped crystals, 0.074 %, m.pt. = 121-122 ^{0}C) and compound III (a terpeniod compound, colourless crystals, 0.004 %, m.pt. = 242-243 ^{0}C) were isolated from pet-ether extract of fruits of *H. adenophyllum.* The structure of isolated compounds were classified by chemical reagent tests and identified by applying modern spectroscopic techniques such as UV and FT IR spectrometry (Finar, 1969).

Compound I

Compound I isolated as colourless needle shaped crystals in 0.033 % yield from PE extract of fruits of Phet-tham has the melting point of 213-214 °C. It was soluble in PE, EtOAc, 95 % EtOH, MeOH and $CHCl_3$ but insoluble in H_2O. The R_f value of compound I was found to be 0.53 in PE: EtOAc (10:1 v/v) solvent system and it was UV inactive. It gave violet spot on TLC chromatogram while spraying with anisaldehyde followed by heating, a yellow spot with iodine vapour.

The functional groups present in compound I were studied by UV and FT IR spectroscopy. FT IR spectrum is shown in Figure 4. The FT IR spectrum of compound I showed the bands at 3305 cm^{-1} due to O-H stretching vibration of alcoholic group. Absorption bands at 2921 cm^{-1} and 2849 cm^{-1} were due to asymmetric and symmetric C-H stretching vibration of $–CH_2$ and $–CH_3$ groups and 3067 cm^{-1} was due to =CH stretching vibration of vinylidene $=CH_2$ group. The C=C stretching vibration was observed at 1639 cm^{-1}. The bending vibration of C-H of $–CH_2$ and $–CH_3$ groups were noticed by the medium intense bands at 1450 cm^{-1} and 1379 cm^{-1}, respectively. The C-O stretching vibration of alcohol was shown as intense band at 1190 cm^{-1} and 1037 cm^{-1}. Absorption band at 880 cm^{-1} was due to the –C-H out of plane wagging of CH_2 group. According to the physiochemical properties, spectroscopic data and Co-TLC (Figure 3) with authentic lupeol, compound I was identified as lupeol.

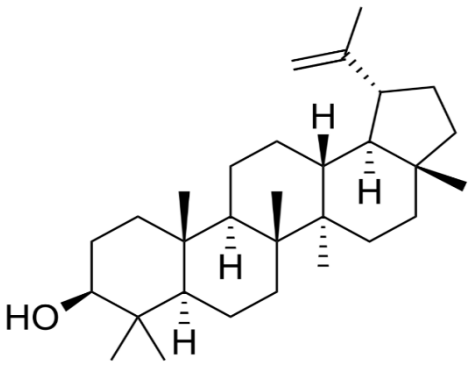

Figure 2 Structure of Lupeol ($C_{30}H_{50}O$)

Compound I
R_f value of compound A : 0.53
R_f value of authentic lupeol : 0.53
Solvent system : PE:EtOAc (10:1 v/v)
Spraing agent : 5 % H_2SO_4, heat

Lupeol **Compound I**

Figure 3 Co-TLC chromatogram of Lupeol and Compound I

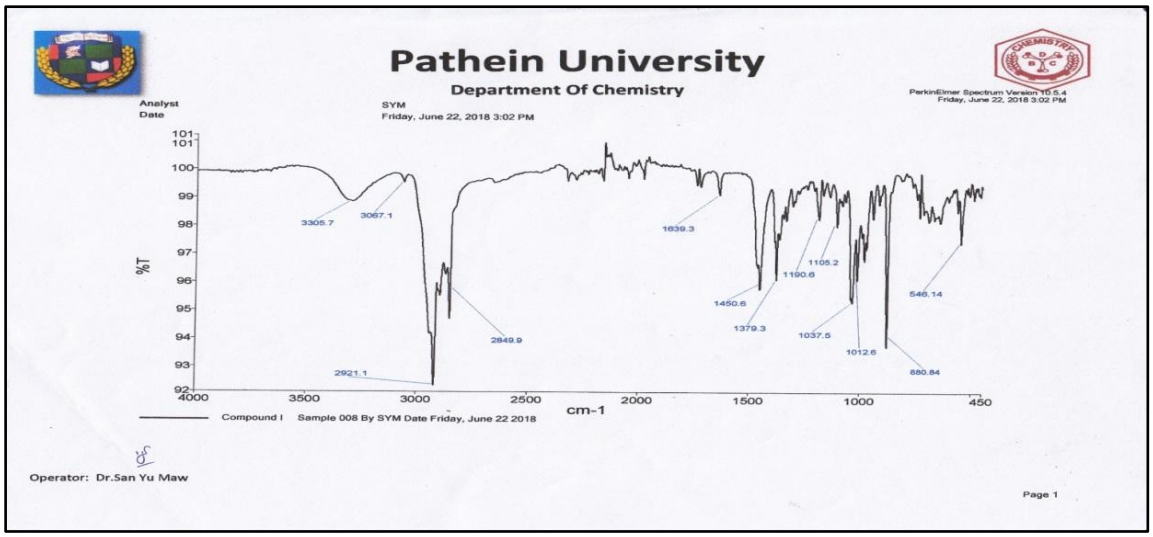

Figure 4 FT IR spectrum of the isolated compound A

Bioactivities

In vitro antimicrobial activity of some crude extracts of fruits of Phet-tham by agar well diffusion method

In vitro antimicrobial activity of various crude extracts such as pet ether, ethyl acetate, 95% ethanol, methanol and watery extracts were investigated by employing agar well diffusion method against six species of microorganisms such as *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aureginosa*, *Bacillus pumilus*, *Candida albicans* and *Escherichia coli*. The inhibition zone diameter (ID) showed the degree of the antimicrobial activity. The larger the inhibition zone diameters, the higher the antimicrobial activity. The photographs illustrating the inhibition zones provided by crude extracts against six species of microorganisms and the observed data are summarized in Figure 4 and Table 1. Among the tested crude extracts of Phet-tham, ethylacetate extracts showed highest antimicrobial activity against all six microorganism (18 ~33 mm). Pet ether extract of Phet-tham fruits exhibited antimicrobial activity against six tested microorganisms (ID: 14 ~ 18 mm). 95% ethanol extracts of Phet-tham (ID: 15 ~ 19 mm), methanol extract (ID: 16 ~ 20 mm) and watery extract (ID: 11 ~ 15 mm) exhibited activity against all six tested microorganisms, respectively. Therefore, it may be inferred that ethyl acetate extract of fruits of Phet-tham pocesses the highest antimicrobial activity.

Table 1 Results of *in vitro* Antimicrobial Activity Screening of Phet-tham Fruits by Agar Well Diffusion Method (at PRD)

No.	Microorganisms	Inhibition well diameter (mm) of various crude extracts				
		PE	EtOH	EtOAc	H_2O	MeOH
1	*Bacillus subtilis*	18(++)	17(++)	28(+++)	13(+)	18(++)
2	*Staphylococcus aureus*	15(++)	15(++)	18(++)	14(+)	17(++)
3	*Pseudomonus aeruginosa*	15(++)	15(++)	30(+++)	15(++)	17(++)
4	*Baccilus pumilus*	14(+)	19(++)	18(++)	13(+)	16(++)
5	*Candida albicans*	15(++)	17(++)	32(+++)	11(+)	20(+++)
6	*Escherichia coli*	15(++)	15(++)	33(+++)	12(+)	17(++)

Agar well diameter = 10 mm
10mm~14 mm = (+) (low activity)
15mm~19 mm = (++) (medium activity)
20mm and above = (+++) (high activity)
(-) = no zone of inhibition

PE= Pet ether extract
EtOH= Ethyl alcohol extract
EtOAc= Ethyl acetate extract
H_2O= Watery extract
MeOH Methanol extract

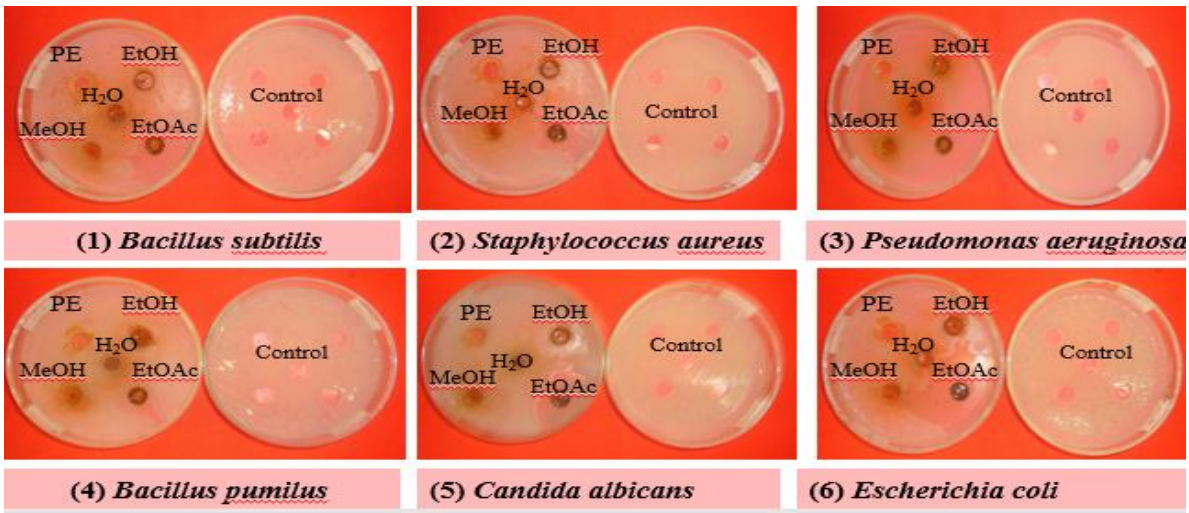

Figure 4. Inhibition wells of crude extracts from Phet -tham fruits against six microorganisms (at PRD)

Conclusion

From the overall assessment of the research work, the following inferences could be deduced. The preliminary phytochemical investigation revealed the presence of alkaloids, α-amino acids, carbohydrates, flavonoids, glycosides, phenolic compounds, reducing sugars, saponins, steroids, tannins and terpenoids in the fruits of Phet-tham. But, starch and cyanogenic glycisides were found to be absent in the fruits of Phet-tham. ED XRF elemental analysis revealed that the fruits of Phet-tham contain K, Ca and Cl as major elements, S, Br and Mn as minor elements and Cu, Rb, Zn and Br as trace elements.

By silica gel column chromatographic separation, compound A (lupeol), colourless needle shaped crystals, 0.033 %, mpt = 213-214 ^{0}C), compound B (a terpenoid compound, colourless needle shaped crystals, 0.074 %, mpt = 121-122 ^{0}C) and compound C (a terpeniod compound, colourless crystals, 0.004 %, mpt = 242-243 ^{0}C) were isolated from pet-ether extract

of fruits of *H. adenophyllum*. The structure of isolated compounds were classified by chemical reagent tests and identified by applying modern spectroscopic techniques such as UV and FT IR spectrometry.

Moreover, the antimicrobial activities of PE, 95 % EtOH, EtOAc, MeOH and H_2O extracts of the fruits of Phet-tham were screened on *Bacillus pumilus, Bacillus subtilis, Candida albicans, Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* by agar well diffusion method. From this investigation, EtOAc extract showed highest antimicrobial activity (ID: 18~33 mm) and H_2O extract showed lowest antimicrobial activity (ID: 11~15 mm).

According to the experimental studies, fruits of Phet-tham were found to contain some bioactive compounds and extract may have potent antimicrobial activities. The compound I, Lupeol, a non-toxic, highly potent chemo preventive and chemotherapeutic agent was isolated. From this study, it could be inferred that Phet-tham fruits have valuable medicinal properties.

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