Investigation of Some Bioactivities' Screening on Fruit of *Haplophragma adenophyllum* (Wall.)P. Dop. (Phet-tham)

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Abstract

In this research paper, *Haplophragma adenophyllum* (Wall.)P. Dop., (Phet-tham fruits), Family-Bignoniaceae, were chosen for isolation of some phytoconstituents and bioactivities such as antimicrobial and antioxidant activities. From the phytochemical investigation, fruits of Phet-tham showed positive test for alkaloids, α-amino acids, carbohydrates, flavonoids, glycoside, phenolic compounds, reducing sugars, saponins, steroids, tannins, terpenoids and showed negative results for starch and cyanogenic glycoside. Elemental analysis by ED XRF method revealed that Phet-tham fruits contained K, Ca and Cl as major elements. By silica gel column chromatographic separation, three terpenoid compounds such as, compound A (lupeol, colourless needle shaped crystals, 0.033 %, m.pt. = 213-214 °C), compound B (colourless needle shaped crystals, 0.074 %, m.pt. = 121-122 °C), compound C (colourless crystals, 0.004 %, m.pt. = 242-243 °C) were isolated from pet-ether extract of fruits of *H. adenophyllum*. The identities of isolated compounds were studied by modern spectroscopic techniques such as UV and FT IR. Antimicrobial activities of pet-ether, ethyl acetate, 95 % ethanol, methanol and watery extracts from the fruits of *H. adenophyllum* 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. Although all tested crude extracts showed activity against all six tested microorganisms, EtOAc extract of fruit of Phet-tham exhibited the highest antimicrobial activity inhibition zone diameter in the range of (ID: 13~33 mm). According to DPPH free radical scavenging assay, H_2O extract (IC_{50} = 3.72 μg/mL) was found to be the highest antioxidant activity compared to the other extracts: EtOAc extract (IC_{50} = 4.52 μg/mL), PE extract (IC_{50} = 8.78 μg/mL) and 95 % EtOH extract (IC_{50} = 9.63 μg/mL). The total phenolic contents of the different crude extracts were calculated using the standard curve of Gallic acid. It was found that watery extract of Phet-tham has the highest total phenolic content (754.67 μgGAE/mg) by comparing with other extracts, (watery extract > EtOAc extract > PE extract > 95% EtOH extract) of fruits of Phet-tham.

Keywords: *Haplophragma adenophyllum* (Wall.)P. Dop., phytoconstituents, lupeol, antimicrobial activity, antioxidant activity, total phenolic content

Introduction

Haplophragma adenophyllum (Wall.)P. Dop. (Phet-tham fruit), 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.)P. Dop., have been used for various ailments treatment which includes cancer, gastrointestinal disorders, cholera, rheumatoid arthritis, hepatic disorders, leucorrhea and diabetes (Rahmatullah *et al*., 2010).

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In Myanmar, the plant is distributed in various regions and used as remedy for various medicinal purposes. Hnin Wutt Yee Win reported for her M.Sc thesis that 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, evaluation of antimicrobial and antioxidant activities.

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

Scientific classification

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

Figure 1 Phet-tham Fruits

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

The fruit of H. adenophyllum (Wall.), Dop, (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.

Aim and Objectives

The aim of this study is to investigate the phytoconstituents and screening the antimicrobial and antioxidant activities of fruit of *H. adenophyllum*. To fulfill this aim, the research was carried out according to the following objectives.

(1) To extract the sample with various solvents
(2) To determine the phytochemical tests and isolate some organic constituents
(3) To investigate the antimicrobial and antioxidant activities of fruit samples

Materials and Methods

Collection and Preparation of *H. adenophyllum* (Wall.)P. Dop. Extracts

The fruits of *H. adenophyllum* (Wall.),P.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 and air dried at room temperature for two weeks and dried fruits were ground into powder and then it was stored in air tight container.

Preparation of Crude Extracts by Direct Extraction Method for Screening of some Biological Activities

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.

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. Qualitative analyses of some elements in fruits of Phet-tham were measured by EDXRF method using EDX-700 instrument at the Universities' Research Center (URC), Yangon. Isolation of some organic constituents from pet-ether extract of the fruits of Phet-tham was performed by column chromatic separation technique.

(a) Screening of Antimicrobial Activity of Different Crude Extracts of Phet-Tham

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.

(b) *In vitro* Screening of Antioxidant Activity of some Crude Extracts from Fruit of Phat-tham

DPPH (1, 1-Diphenyl -2 –picrylhydrazyl) free radical scavenging assay was chosen to access the antioxidant activity of sample materials. Antioxidant activities of 95% EtOH, PE, EtOAc, H_2O extracts from fruit of Phet-tham were evaluated.

Procedure

DPPH radical scavenging activity was determined by UV spectrophotometic method. The control solution was prepared by mixing 1.5 mL of 60 μM DPPH solution and 1.5 mL of 95% EtOH. The sample solution was also prepared by mixing thoroughly 1.5 mL of 60 μM DPPH solution and 1.5 mL of test sample solution. The solutions were allowed to stand at room temperature for 30 minutes. After 30 minutes, the absorbance of these solutions was measured at 517 nm by using UV spectrophotometer. Absorbance was measured in triplicate for each solution and mean values obtained were used to calculate percent inhibition of oxidation by the following equation.

$$\%\,\text{Oxidative Inhibition} = \frac{A_{DPPH} - (A_{Test\,sample} - A_{Blank})}{A_{DPPH}} \times 100$$

A_{DPPH} = absorbance of DPPH in 95% EtOH solution

$A_{Test\ sample}$ = absorbance of (sample + DPPH) solution

A_{Blank} = absorbance of (sample + 95% EtOH solution)

Average, $\overline{X} = \frac{X_1 + X_2 + X_3 + \cdots + X_n}{n}$

Standard deviation (SD) = $\sqrt{\frac{(\overline{x} - x_1)^2 + (\overline{x} - x_2)^2 + (\overline{x} - x_3)^2 + \cdots + (\overline{x} - x_n)^2}{n-1}}$

where, $\overline{X}$ = average % inhibition of oxidation, n = number of times

$x_1, x_2, x_3, \ldots, x_n$ = % inhibition of test sample solution

Then, IC_{50} (50% oxidative inhibitory concentration) values were also calculated by linear regressive excel programme.

(c) Determination of Total Phenolic Content of Fruit of Phat-tham by Folin- Ciocalteau Methods

Total phenolic content (TPC) was determined using Folin- Ciocalteau methods (Rekha *et al*., 2012). Total phenolic content of PE, EtOAc, 95% EtOH and watery extracts of Phet-tham fruits were measured by using PD 303 UV spectrophotometer at Pathein University.

Procedure

(i) Construction of Gallic acid standard curve

First, 0.5mL each of different concentration of Gallic acid solutions (1000, 500, 250, 125, 62.5, 31.25 μg/mL) was mixed with 5mL of 10% FC reagent in the test tubes and incubated for 5 minutes. 4 mL of 1M Na_2CO_3 was added to each tube and the tubes were kept at room temperature for 15 minutes and the absorbance of reaction mixture was read at 765 nm.

(ii) Determination of Gallic acid equivalent in crude extract samples

The total phenolic content in the crude extract was estimated by Folin- Ciocalteau method. Each crude extract sample (0.5mL) was added into 5mL of 10% FC reagent and incubated for 5 minutes. To each tube, 4 mL of 1 M Na_2CO_3 was added and the tubes were kept at room temperature for 15 minutes and the absorbance of reaction mixture was read at 765 nm. The blank solution was prepared as above procedure by using distilled water instead of sample solution. Total phenolic content was estimated as μg of Gallic acid equivalent per milligram (μg GAE/mg) of crude extracts (Table 4 and Figure 4).

Results and Discussion

From preliminary photochemical analysis of the fruit 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 were 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 A (lupeol, colourless needle shaped crystals, 0.033 %, m.pt. = 213-214^{0}C), compound B (a terpenoid compound, colourless needle shaped crystals, 0.074 %, m.pt. = 121-122^{0}C) and compound C (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).

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 PE, EtOAc, 95% EtOH, MeOH and H_2O 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 Table 1. Among the tested crude extracts of Phet-tham, EtOAc extracts showed highest antimicrobial activity against all six microorganism (18 ~33 mm). PE extract of Phet-tham fruits exhibited antimicrobial activity against six tested microorganisms (ID: 14 ~ 18 mm). 95% EtOH extracts of Phet-tham (ID: 15 ~ 19 mm), MeOH extract (ID: 16 ~ 20 mm) and H_2O extract (ID: 11 ~ 15 mm) exhibited activity against all six tested microorganisms, respectively. Therefore, it may be inferred that EtOAc extract of fruits of Phet-tham processes the highest antimicrobial activity.

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

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. *Pseudomonas aeruginosa*	15	15	30	15	17
4. *Bacillus 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
10 mm ~ 14 mm = (+)
15 mm ~ 19 mm = (++)
20mm and above = (+++)
(-) = no zone of inhibition

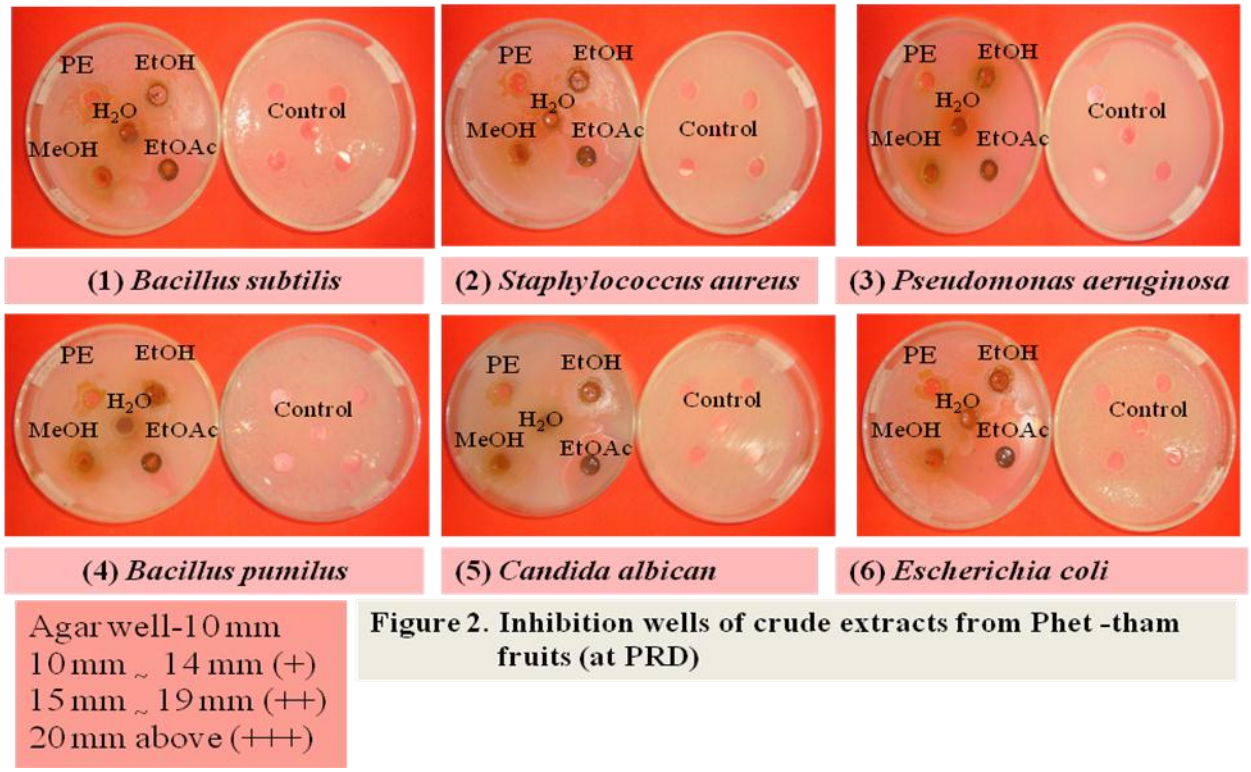

Figure 2. Inhibition wells of crude extracts from Phet -tham fruits (at PRD)

***In virto* antioxidant activity of some crude extracts of fruit of Phet-tham by DPPH assay**

The antioxidant activities of four crude extracts from fruit of Phet-them were studied by DPPH (1, 1-diphenyl-2-pricrylhydrazyl) free radical scavenging UV spectrophotometric assay method.

This method is based on the reduction of colored free radical DPPH in ethanolic solution by different concentrations of the sample. The antioxidant activity was expressed as 50% oxidative inhibitory concentration (IC_{50}). In this experiment, ascorbic acid was used as standard. The antioxidant activity was determined for five different concentrations 0.625 µg/mL, 1.25 µg/mL, 2.5 µg/mL, 5µg/mL and 10µg/mL of each sample in 95% EtOH solvent. The absorbance of control solution (DPPH in EtOH solvent), blank solution (sample in EtOH) and sample solution (sample + DPPH in EtOH) were measured at maximum wavelength of 517 nm using UV spectrophotometer. From the average values of % inhibition, IC_{50} values (50% inhibition concentration) were calculated by linear regressive excel program. Table 2 shows percent oxidative inhibition of various crude extracts of the fruits of Phet-tham at various concentrations in comparison with standard ascorbic acid. From these experimental results, it was found that as the concentrations increased the absorbance values were found to decrease and the antioxidant activity increased. In the fruits of Phet-tham, the antioxidant activity were found to be H_2O extract (IC_{50} = 3.72 µg/mL) > EtOAc extract (IC_{50} = 4.52 µg/mL) > PE extract (IC_{50} = 8.78 µg/mL)> 95 % EtOH extract (IC_{50} = 9.63 µg/mL).

Table 2. Percent Oxidative Inhibition and IC_{50} values of Crude extracts of Fruit of Phet-tham and Ascorbic acid

Extracts	Percent Oxidative Inhibition (%) (mean ±SD) in different concentrations (µg/mL)					IC_{50} (µg/mL)
	0.625	1.25	2.5	5	10	
PE	1.34±0.15	1.05±3.76	18.24±0.68	39.58±1.57	51.51±0.53	8.77
EtOAc	19.39±0.23	34.38±0.23	45.17±0.23	64.21±0.31	70.66±0.31	4.52
EtOH	2.69±0.15	2.24±0.15	8.19±2.21	22.34±2.48	53.32±0.43	9.62
H_2O	21.43±0.30	38.13±0.23	46.57±0.23	68.51±0.15	79.71±0.23	3.72
Ascorbic acid	14.04±2.09	54.83±2.48	72.44±3.83	77.13±1.47	87.4±2.37	1.17

Total phenol content of Fruits of Phet-tham by FC method

In this study, the total phenolic content of Phet-tham fruits sample was estimated by Folin-Ciocalteu method. Gallic acid was used to construct standard calibration curve for total phenol. Total phenolic content (TPC) was expressed as microgram of Gallic acid equivalent per milligram of crude extract (µg GAE/mg). Standard calibration curve of Gallic acid was prepared by varying the amount of Gallic acid in the range of 1000 to 31.25 µg/mL. As shown in Table 3 and Figure 3, the prepared Gallic acid standard curve gave a straight line, which obeyed Beer's Lambert law with $y = 0.0037x + 0.3815$, $R^2 = 0.9798$. From the standard curve it was found that the increase in concentration of Gallic acid, the complexation would also increase and as a result increase in the absorbance is observed. It is clear from the standard curve that absorbance is directly proportional to Gallic acid concentration. The results of total phenolic content of various crude extracts from Phet-tham fruits were presented in Table 4. Bar graphs of total phenolic content of various crude extracts from Phet-tham are shown in Figure 4. Among the crude extracts of Phet-tham fruits, the TPC was the highest in the watery extract (754.67 µg GAE/mg), followed by EtOAc extract (638.00 µg GAE/mg), PE extract (624.67 µg GAE/mg) and finally 95 % EtOH extract (43.33 µg GAE/mg).

Table 3. The Absorbance of Gallic Acid Standard Solution at λ_{max}765 nm

Sr. No.	Concentration (µg/mg)	Absorbance at λ_{max}765 nm
1	31.25	0.29
2	62.5	0.51
3	125	0.85
4	250	1.67
5	500	2.30
6	1000	4

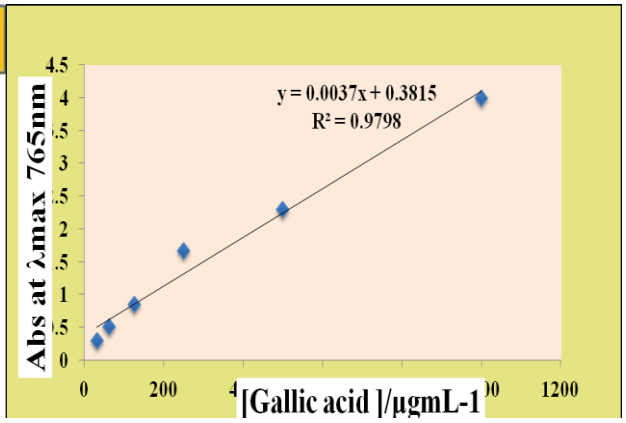

Figure 3. A plot of gallic acid standard curve

Table 4. Total Phenolic Content (TPC) of Various Crude Extracts from the Fruits of Phet-tham by Folin-Ciocalteau Method

Sr. No.	Samples	TPC(µgGAE/mg ±SD)
1	PE	624.67 ± 0.001
2	EtOAc	638.00 ± 0.070
3	EtOH	43.33 ± 0.012
4	H_2O	754.67 ± 0.228

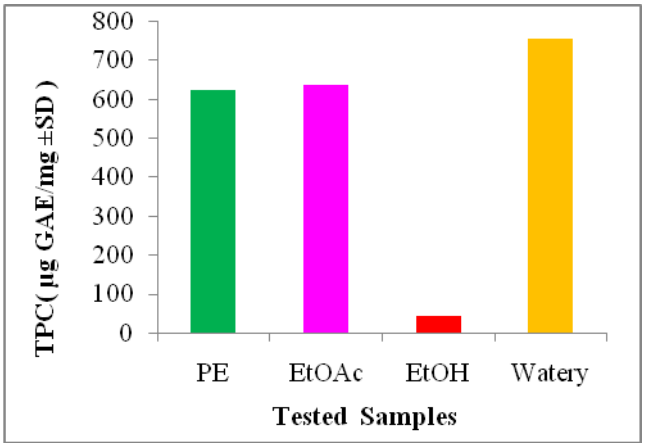

Figure 4. A bar graph of total phenolic content (TPC) of various crude extract from the fruits of Phet-tham by Foli-Ciocalteu method

Conclusion

In Myanmar, no scientific study was carried out to assess antimicrobial and antioxidant activities of the fruits of *H. adenophyllum*. Therefore, present study was conducted to determine the bioactivities of various crude extract of fruits of *H. adenophyllum*.

The preliminary phytochemical investigation revealed the presence of alkaloids, α-amino acids, carbohydrates, flavonoids, and 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.

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 by the agar well diffusion method, EtOAc extract showed highest antimicrobial activity (ID: 18~33 mm) and H_2O extract showed lowest antimicrobial activity (ID: 11~15 mm).

In vitro antioxidant activity screening by using DPPH free radical scavenging assay method, H_2O extract of the fruits of Phet-tham (IC_{50}= 3.72 μg/mL) was found to have the highest antioxidant activity than that of other extracts of the fruits of Phet-tham.

The total phenolic contents of the different sample extracts from *H. adenophyllum* were determined by Folin-Ciocalteau (FC) method. Gallic acid was used to construct standard calibration curve for total phenol. TPC was expressed as microgram of Gallic acid equivalent per milligram of crude extracts (μgGAE/mg). From the study of total phenolic content from Phet-tham, H_2O extracts of Phet-tham fruits was found to be the highest total phenolic content (754.67 μgGAE/mg) than the other extracts. According to the experimental studies, fruits of Phet-tham contain some bioactive compounds and extract may have good antimicrobial and antioxidant activities. Isolated compounds A, Lupeol, is a non-toxic, highly potent chemopreventive and chemotherapeutic agent. Moreover, various *in vitro* and preclinical animal studies suggest that lupeol has a potential to act as an anticancer, anti-inflammatory, antimicrobial, antiprotozoal, antiproliferative, antiinvasive and antiangiogenic. From this study, it could be inferred that Phet-tham fruit have valuable medicinal properties.

Acknowledgements

We would like to thank to the Department of Botany, Dagon University, for inviting us to present research paper. We would like to thank the Department of Higher Education, Ministry of Education in Myanmar, for giving us the opportunity to do this research. Our deepest gratitude is expressed to Dr Si Si Hla Bu, Rector of Pathein University, for her encouragement, kind guidance, and kind help to do this research. We wish to thank Dr Than Tun and Dr Nilar Myint, Pro-Rectors of Pathein University for their invaluable advices and encouragement. Thanks are also extended to Professor Dr Ye Myint Aung (Head of Department) and Dr Than Than Oo (Professor), Department of Chemistry, Pathein University, for their helpful advice, precious suggestions and provision of research facilities at the Department of Chemistry, Pathein University, Myanmar.

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