Investigation of Antimicrobial Activity and Isolation of Phytoconstituents from Leaves of *Clausena excavata* Burm.f. (Pyin-daw-thein)

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Abstract

Clausena excavate Burm.f. (Pyin-daw-thein) has long been associated with medicinal benefits in folk medicine, particularly in the treatment of cancer and several ailments such as malaria, dysentery, tuberculosis, diarrhoea, wound and poisoning. The present work is concerned with investigation of antimicrobial activity and isolation of phytoconstituents from leaves of Pyin-daw-thein. The elemental analysis of leaves of Pyin-daw-thein was determined by EDXRF spectrometry. The crude extracts of Pyin-daw-thein were prepared with various solvents: pet-ether, ethyl acetate, ethanol and water by solvent extraction method. Antimicrobial activity of crude extracts of Pyin-daw-thein was investigated by agar well diffusion method with six species of microorganisms such as *Bacillus substilis, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus pumilus, Candida albicans* and *Escherichia coli*. In addition, two compounds: Stigmasterol, 0.21 %, mpt 168-170°C and Scopoletin (0.32 %, m.pt. 201- 203 ºC) were isolated from ethyl acetate extract of Pyin-daw-thein. They were identified by physicochemical determination and modern spectroscopic techniques such as UV, FT IR and also by comparing with the reported data.

Key words : *Clausena excavata* Burm.f. (Pyin-daw-thein), antimicrobial activity, agar well diffusion method, stigmasterol, scopoletin

Introduction

Clausena excavate Burm.f. (Pyin-daw-thein) is a wild shrub of the Rutaceae family. Pyin-daw-thein is a profusely branched, evergreen plant ranging in size from a shrub just 1-2 m tall to a small tree that is usually up to 10 m tall. The leaves are pinnate, 60 cm long, with 10 to 15 pairs of dark green narrowly oval oblique leaflets. They have a characteristic curry-like smell when crushed. Small white flowers occur in terminal clusters, followed by translucent pink berries 7 to 10 mm across, each containing 1 to 2 seeds. The fruits are ellipsoidal, 1.2-1.8 cm long, with persistent style, glabrous, transluscent pink to red. Pyin-daw-thein is extensively distributed throughout Southeast Asia, India and China. It is also distributed to southern Taiwan, Nepal, Bangladesh, Myanmar, Thailand, Cambodia, Malaysia, Indonesia and Philippines (Arbab *et al*., 2011).

Pyin-daw-thein leaves contain high moisture, ash and crude fibre. In addition, calcium, iron, magnesium, potassium, sodium, zinc, vitamin A, C and E are also present. It has been reported that carbazole alkaloids are a major component of this plant. The leaves consist of ferulic acid, safrole, sterol, scopoletin, steroidal glycosides, and carbazole alkaloids. The juice from the leaves is taken for intestinal worms or cough, and for fever, malaria or colds. A decoction of the roots, flowers or leaves is taken for bowel complaints, such as colic, dyspepsia and stomach-ache. The leaves are insecticidal and the pounded leaves may be applied to the head for headache. In Myanmar the leaves are taken for stomach troubles. Pyin-daw-thein has been reported to exhibit one of the highest beneficial biological activities among *Clausena* genus (Sripisut *et al*., 2012). Recent studies showed that Pyin-daw-thein also possessed anticancer, antioxidant, antimycobacteria, analgesic and antifungal activities as well as having anti HIV-1

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agents. These activities are attributed to its high phenolic compounds such as furanocoumarins and flavonoids (Sunthitikawinsakul, 2003)

The present work focused on investigation of antimicrobial activity, isolation and identification of some organic constituents from leaves of Pyin-daw-thein. The photographs of plant, flowers and fruits of Pyin-daw-thein are shown in Figure 1.

(a) (b) (c)

Figure 1. Photographs of Pyin-daw-thein (a) plant (b) flowers and (c) fruits

Materials and Methods

The chemicals used were reagent-grade ethanol, chloroform, methanol, pet-ether (60-80°C) and ethyl acetate. For isolation and identification of organic compounds, silica gel (40-60 μm, Wakogel), Melting point (Gallenkamp), UV lamp (365–254 nm), FT IR spectrometer (Perkin Elmer), UV-visible spectrometer-Shimadzu, Japan were used.

For this investigation, the selected medicinal plant was *Clausena excavate* Burm.f. (Pyin-daw-thein). It was identified at the Department of Botany, University of Myitkyina. The leaves of Pyin-daw-thein were collected from University of Myitkyina Campus, Kachin State. The collected fresh leaves were washed and air dried at room temperature for two weeks and then they were made into powder by a blender. The dried powdered sample was stored in the air-tight containers to prevent the moisture and other contaminations.

Preliminary Phytochemical Tests of Pyin-daw-thein

In order to classify the types of phytoconstituents such as alkaloids (Robison,1983), α- amino acids, carbohydrates, flavonoids, glycosides, organic acids, phenolic compounds (Marini *et al.*, 1981), reducing sugars, saponin glycosides, starch, tannins, steroids and terpenoids were tested according to the standard phytochemical methods.

Elemental Analysis of Pyin-daw-thein by Energy Dispersive X-ray Fluorescence (EDXRF) Spectrometry

Energy dispersive X-ray fluorescence spectrometer (Shimadzu EDX-720) can analyze the elements from Na to U under vacuum condition. The individual element in sample is detected by using semiconductor [Si-Li] that permits multi-elements and simultaneous analysis. In this way, EDX-720 spectrometer determines elements that are present in the sample. Analysis of some elements in Pyin-daw-thein was measured by EDXRF method using EDX-720 instrument at West Yangon University, Yangon.

Determination of Soluble Matter Contents by Direct Solvent Extraction Method

The dried powdered sample (25 g) was percolated with 150 mL of pet-ether (60-80 ^{o}C) in conical flask. The flask was shaken at 4 hr intervals for 24 hr at room temperature and then filtered. The filtrates were placed in a weighed porcelain basin and then evaporated to dryness on a water-bath until it was completely dried. The residue with the basin was weighed. The difference in weights of basin before and after was taken to be the pet-ether soluble matter content. Similarly, ethyl acetate and ethanol extracts were prepared according to the above procedure. Watery extract of dried powdered sample was prepared by boiling the sample (25 g) with 150 mL of distilled water for 6 hr and filtered. The filtrate was placed in a weighed porcelain basin and then evaporated to dryness on a water-bath until it was completely dried. The residue with the basin was weighed. The difference in weights of basin before and after was taken to be the watery soluble matter content.

Screening of Antimicrobial Activity of Leaves of Pyin-daw-thein

The antimicrobial activity of different crude extracts such as pet-ether, ethyl acetate, ethanol and watery extracts from leaves of Pyin-daw-thein was determined against six strains of microorganisms such as *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus pumilus*, *Candida albicans* and *Escherichia coli* by employing agar well diffusion method at Fermentation Department, Development Centre of Pharmaceutical Technology, Yangon.

Meat extract (0.5 g), peptone (0.5 g) and sodium chloride (0.25 g) were mixed with distilled water and the solution made up to 100 mL with distilled water. The pH of this solution was adjusted at 7.2 with 0.1 M sodium hydroxide solution and 1.5 g of agar was added. The nutrient agar medium was put into sterilized conical flask and plugged with cotton wool and then autoclaved at 121°C for 15 min. After cool down to 40 °C, one drop of suspended strain was inoculated to the nutrient agar medium with the help of a sterilized disposable pipette near the burner. About 20 mL of medium was poured into the sterilized petri dishes and left 10-15 min in order to set the agar. After that the agar wells were made with a 10 mm sterilized cork bore and the wells were filled with 0.1 mL of each extract sample to be tested. And the plates were incubated at 37 °C for 24 hr. After incubation, the diameters of inhibition zones including 10 mm wells were measured.

Isolation of Organic Constituents from Active Crude Extract

The ethanol extract was extracted with 250 mL of pet-ether (60-80 °C) by using separating funnel. The soluble matter of pet-ether was obtained after evaporating the pet-ether layer. The defatted alcohol portion was then partitioned between ethyl acetate and water by using separating funnel. After removal of the solvent, ethyl acetate soluble extract was obtained. The ethyl acetate extract (3 g) was separated by column chromatographic separation techniques. Gradient elution was performed successively with PE : EtOAc (9:1, 4:1, 2:1, 1:2, 1:4, 1:9 v/v) solvent systems, and successive fractions obtained were combined on the basis of their behaviours on TLC. Six main fractions (F I to F VI) were collected. After removal of the solvents, fraction F II (f_6 - f_{10}) and F IV (f_{16} - f_{20}) were obtained as solid substances. From fraction F II, compound A was isolated as colorless needle crystal. This isolated compound A was purified by washing with pet-ether followed by crystallization from ethyl acetate to give compound A (0.21 %) as colorless needle crystal. The solid compound B was obtained from fraction F

IV. Compound B was washed with pet ether followed by ethyl acetate and then purified by recrystallized from methanol. Compound B (0.32 %) was obtained as pale yellow crystal.

Characterization of Isolated Compounds

The isolated compounds (A and B) from ethyl extract of Pyin-daw-thein were characterized by determination of physicochemical properties: melting point, R_f values, and some chemical tests as well as the modern spectroscopic techniques such as UV-visible and FT IR spectrophotometers at Universities' Research Centre, Yangon.

Results and Discussion

Preliminary Phytochemical Tests

The powdered sample was subjected to preliminary phytochemical tests in order to find out the types of organic constituents such as alkaloids, α- amino acids, carbohydrates, flavonoids, glycosides, phenolic compounds, saponin glycosides, starch, steroids, tannins and terpenoids according to appropriate reported methods. But, organic acids and reducing sugars were not detected in sample.

Elemental Analysis of Pyin-daw-thein

In the research work, relative abundance of elements present in leaves of Pyin-daw-thein was determined by EDXRF spectrometry. The EDXRF spectral data of Pyin-daw-thein is shown in Table 1. Leaves of Pyin-daw-thein were found to contain Ca and K as major elements. In addition, S was found to present as minor element and Fe, Mn, Sr, Zn, Cu, Rb as trace elements in Pyin-daw-thein. Among the elements, calcium is the most abundant in leaves of Pyin-daw-thein. The health benefit of calcium is the most widely known bone health. Another benefit of calcium is that it has been shown in scientific studies to prevent certain forms of cancer, such as breast cancer and ovarian cancer. The health benefits of potassium include relief from stroke, blood pressure, heart and kidney disorders.

Soluble Matter Contents by Direct Extraction Method

In this experiment, the crude extracts of leaves of Pyin-daw-thein were extracted with different polarity of pet-ether, ethyl acetate, ethanol and water by employing direct extraction method. The resultant soluble matter contents of Pyin-daw-thein are shown in Table 2. From the results, it was observed that ethanol soluble matter content was found to be highest, followed by watery, ethyl acetate and pet-ether soluble matter contents. It indicated that the polar constituents were higher than that of nonpolar constituents in leaves of Pyin-daw-thein

Table 1. Relative Abundance of Elements in Leaves of Pyin-daw-thein

No.	Elements	Relative abundance (%)
1.	Calcium (Ca)	3.429
2.	Potassium (K)	1.580
3.	Sulphur (S)	0.420
4.	Iron (Fe)	0.015
5.	Manganese (Mn)	0.004
6.	Strontium (Sr)	0.004
7.	Zinc (Zn)	0.003
8.	Copper (Cu)	0.002
9.	Rubidium (Rb)	0.001

Table 2. Soluble Matter Contents of Leaves of Pyin-daw-thein

No.	Extract	Yield (%)
1.	Pet-ether	2.36
2.	Ethyl acetate	5.39
3.	Ethanol	7.34
4.	Watery	5.78

Antimicrobial Activity of Crude Extracts of Pyin-daw-thein

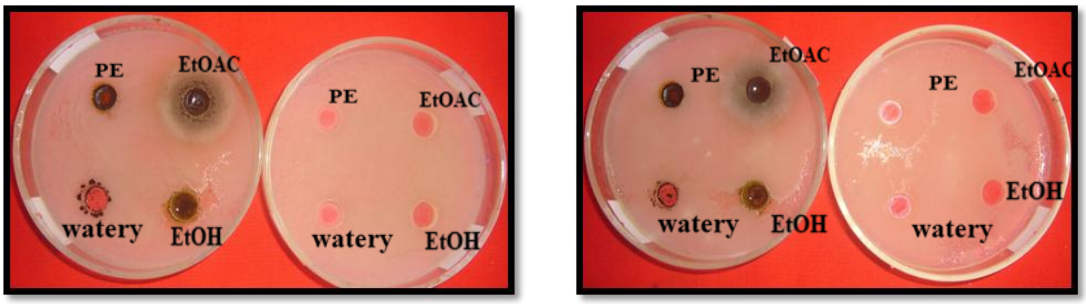
Screening of antimicrobial activity of various crude extracts such as pet-ether, ethyl acetate, ethanol and watery extracts from leaves of Pyin-daw-thein was investigated by employing agar well diffusion method. In this study, the samples were tested on six species of microorganisms such as *Bacillus substilis, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus pumilus, Candida albicans* and *Escherichia coli* species. The inhibition zone diameter shows the degree of the antimicrobial activity. The larger the inhibition zone diameters indicate the higher antimicrobial activity. The inhibition zone diameters of crude extracts against six microorganisms tested are summarized in Table 3 and the observed inhibition zones are shown in Figure 2.

From these results, it was found that pet-ether extract of Pyin-daw-thein did not exhibit antimicrobial activity against all tested microorganisms except *Bacillus pumilus*. Among the crude extracts, ethyl acetate extract of leaves of Pyin-daw-thein showed the most pronounced antimicrobial activity against six microorganisms with inhibition zone diameter range in 13 mm to 19 mm. According to the results, all crude extracts except pet-ether extract of leaves of Pyin-daw-thein showed significant antimicrobial properties against both Gram (+) ve and Gram (-) ve microorganisms tested.

Table 3. Inhibition Zone Diameters of Various Crude Extracts of Leaves of Pyin-daw- thein against Six Microorganisms by Agar Well Diffusion Method

Microorganisms	Types of Microorganisms	Inhibition Zone Diameters (mm)			
		PE	EtOAc	EtOH	Watery
Bacillus substilis	Gram (+)ve	-	19	13	12
Staphylococcus aureus	Gram (+)ve	-	15	17	14
Pseudomonas aeruginosa	Gram (-)ve	-	13	13	11
Bacillus pumilus	Gram (+)ve	14	19	17	16
Candida albicans	Gram (+)ve	-	18	14	12
Escherichia coli	Gram (-)ve	-	17	15	16

Agar well diameter - 10 mm



Bacillus substilis ***Candida albicans***

Figure 2. Images of inhibition zones of various crude extracts of Pyin-daw-thein against *Bacillus substilis and Candida albicans*

Characterization of Isolated Compounds from Leaves of Pyin-daw-thein

Compound A

Compound A was isolated as colourless needle crystals in 0.21 % yield from EtOAc crude extract of Pyin-daw-thein. The observed melting point was 168-170 °C (EtOAc). R_f value of compound A was found to be 0.6 in PE : EtOAc - 4:1v/v solvent system. It was soluble in $CHCl_3$, EtOAc, EtOH and MeOH. Compound A was UV inactive suggesting the absence of conjugated double bond. According to the results from 2,4-DNP test, carbonyl group was absent in it. The C = C bond was present due to decolourization of 10 % $KMnO_4$ solution test. Since it gave green colourization when treated with Libermann burchard test, it was classified as steroidal compound. The R_f value of compound A was found to be identical to that of stigmasterol in any solvent system and gave the same behaviour as stigmasterol on TLC. The melting point of compound A was also identical to that of stigmasterol (melting point 169-170°C, EtOAc). Compound A was also studied by co-TLC with authentic stigmasterol, visualizing with anisaldehyde H_2SO_4 followed by heating. The two compounds migrate with the same R_f values and gave the same purple colour (Figure 3).

According to FT IR spectrum (Figure 4) and the interpreted spectral data (Table 4), the absorption bands appeared at 3425 cm^{-1}, 1655 cm^{-1} and 1064 cm^{-1} confirmed that the compound A contained the respective functional groups: O-H, C=C and C-O groups. Asymmetric and symmetric stretching vibrations of C-H gave the bands at 2947 cm^{-1} and 2877 cm^{-1}, indicating the presence of $–CH_2$ and $–CH_3$ groups and their C-H bending occurred at 1458 cm^{-1}. The FT IR spectral data of compound A were found to be similar to those of stigmasterol (Goad and Akihisa, 1997). All of the information; physicochemical properties, melting point, R_f value and FT IR spectral data of compound A were found to be consistent with those of authentic stigmastreol. Thus, compound A could be assigned as stigmasterol.

Solvent system - PE : EtOAc (4 : 1 v/v)
Spraying agent - Anisaldehyde - H_2SO_4, Δ
Observation - purple
A - Compound A
I - Authentic Stigmasterol
II - Compound A + Authentic Stigmasterol

Figure 3. Co- TLC of isolated compound A and authentic stigmasterol

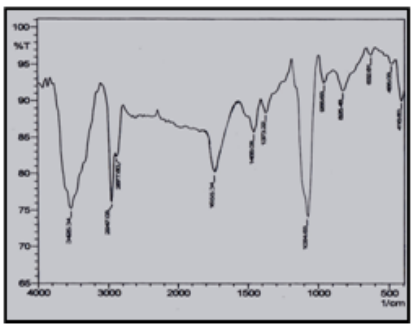


Figure 4. FT IR spectrum of isolated compound A

Table 4. FT IR Spectral Data of Isolated Compound A Compared with Reported Data of Stigmasterol

Wave number (cm^{-1})		Assignment
observed data	*Stigmasterol	
3425	3428	ν_{OH} of alcoholic O-H
2947, 2877	2936, 2867	$\nu_{asym-CH}$ and υ_{sym-CH} of $-CH_2$ and $–CH_3$
1655	1658	$\nu_{C=C}$ of olefinic group
1458	1458	δ_{C-H} of CH_2 and CH_3
1373	1382	δ_{C-H} of isopropyl
1064	1061	δ_{C-O-H} of cyclic alcohol
956	970	δ_{oop} of C-H

* Goad and Akihisa, 1997

Compound B

Compound B isolated as pale yellow crystal in 0.32 % yield from EtOAc extract of Pyin-daw-thein and the melting point was 201-203 °C (MeOH). It was soluble $CHCl_3$, EtOAc, and MeOH but insoluble in PE. Its R_f value was found to be 0.5 in PE : EtOAc- 1:2 v/v solvent system. It gave a blue fluorescence under UV light on TLC as well as bright blue spot on TLC while spraying with NH_3 vapour. Compound B was classified as phenylated coumarin due to the greenish blue occured when it was treated with 5% KOH solution. The UV spectra recorded in MeOH and MeOH/ NaOH, are illulstrated in Figure 5. The maximum absorption wavelengths λ_{max} in methanol of compound B were found to be 228 nm, 252 nm, 297 nm and 345 nm due to π- π* and n-π* transitions indicating the presence of conjugated double bond. In MeOH/NaOH, the absorption bands were shifted to 228 nm, 257 nm, 302 nm and 351 nm, indicating the presence of free-OH group in compound B. The respective maximum absorption wavelengths are also summarized in Table 5. The UV spectral data were found to be similar to those of scopoletin (Ferdinal *et al.*, 2015).

The functional groups present in compound B were studied by FT IR spectroscopy. The FT IR spectrum is shown in Figure 6 and the interpreated spectral data are shown in Table 6. The FT IR spectrum of compound B showed the band at 3325 cm^{-1} due to the OH-stretching vibration of alcoholic or phenolic-OH group. The band at 1705 cm^{-1} appeared due to the lactone carbonyl (C=O) group. The bands at 1612, 1566 and 1504 cm^{-1} attributed the stretching vibrations of C=C of aromatic group. The bands at 1288 and 1134 cm^{-1} appeared due to the stretching vibration of C-O-C in Ar-O group and C-OH stretching. The FT IR spectral data were observed to be consistent with those of scopoletin (Ferdinal *et al.*, 2015). From physicochemical properties, melting point, UV, FT IR and comparing reported spectral data, the compound B could be identified as scopoletin.

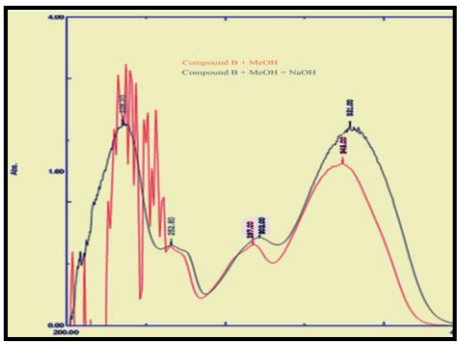


Figure 5. UV spectrum of isolated compound B

Table 5. UV Spectral Data of Isolated Compound B Compared with Reported Data of Scopoletin

Reagent	λ_{max} (nm)		Remark
	Compd B	Scopoletin*	
MeOH	228, 252, 297, 345	228, 252, 295, 344	Double bond conjugation
MeOH+ NaOH	228, 257, 302, 351	-	phenolic OH group (red shift)

Figure 6. FT IR spectrum of isolated compound B

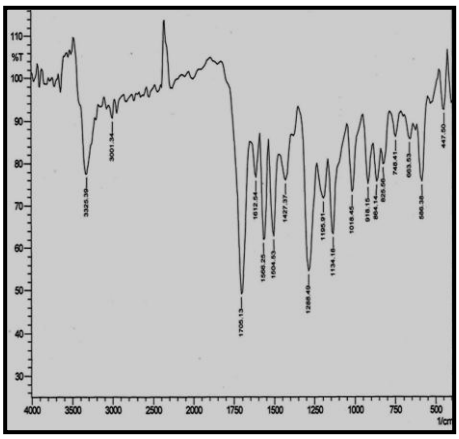


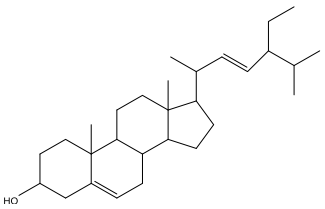
Table 6. FT IR Data of Isolated Compound B Compared with Reported Data of Scopoletin

Wave number(cm^{-1})		Assignment
Compd B	Scopoletin *	
3325	3327	ν_{OH} of alocohotic group
3001	-	$\nu_{=CH}$ stretching
2848	2850	ν_{C-H} stretching of CH_3
1705	1702	$\nu_{C=O}$ stretching
1612, 1566, 1504	1608,1565, 1510	$\nu_{C=C}$ stretching of cumarone and benzene
1427	-	δ_{C-H} of CH_3 group
1288, 1134	-	ν_{C-O} stretching of OH
864	861	Disubstitution of benzene

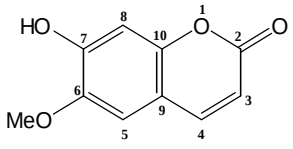
* Ferdinal *et al.*, 2015

Conclusion

From the overall assessment of the present work, the following inferences could be deduced. Preliminary phytochemical screening revealed the presence of alkaloids, α- amino acids, carbohydrates, flavonoids, glycosides, phenolic compounds, saponin glycosides, starch, steroids, tannins and terpenoids in leaves of Pyin-daw-thein. From elemental analysis, calcium was the highest content in leaves of Pyin-daw-thein determined by EDXRF spectrometer. By solvent extraction method, ethanol soluble matter content was found to be highest. Moreover, from the results of antimicrobial activity by agar well diffusion method, ethylacetate extract of Pyin-daw-thein exhibited the most pronounced antimicrobial activity against six microorganisms. On silica gel chromatographic technique, two compounds: Stigmasterol (0.21 %, mpt 168-170 °C) and Scopoletin (0.32 %, m.pt. 201-203 ºC) were isolated from ethyl acetate extract of Pyin-daw-thein. The isolated compounds were identified by physicochemical determination and modern spectroscopic techniques such as UV and FT IR and also by comparing with the reported data. Consequently, Pyin-daw-thein can be used as traditional medicine in preventing many major diseases such as bacteria and fungus infections, inflammatory, tumor and malaria. It may also help to maintain blood pressure, heart and kidney disorders and bone health.



Stigmasterol



Scopoletin

Acknowledgements

The author acknowledges to Dr Aung Khin Myint (Rector) and Dr Su Su Win (Pro-rector), Kyaukse University for allowing to carry out this research programme. Thanks are also extended to Dr Nu Nu Yee and Dr Nay Thwe Kyi, Pro-rectors, Dagon University and Dr Myat Myat Moe (Professor and Head), Department of Botany, Dagon University for permitting to present this research paper. And also thanks to Dr Khin Mar Yee (Professor and Head) and Dr Aye Aye Cho (Proessor), Department of Chemistry, Kyaukse University for their kind encouragement and allowing to submit this research paper.

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