A STUDY ON THE PHYTOCHEMICALS, ANTIMICROBIAL ACTIVITY OF CRUDE EXTRACTS, AND IDENTIFICATION OF ECHITAMINE ALKALOID OF *ALSTONIA SCHOLARIS* L. (TAUNG-MA-YO) BARK

Khin Maw Maw[1], Swe Zin Myint[2], Saw Hla Myint[3]

Abstract

Alstonia scholaris (L.) R. Br., commonly known as Taung-ma-yo, is a traditional plant renowned for its diverse range of biological activities. The present research work focused on the investigation of phytochemical constituents, screening of the antimicrobial activity of crude extracts, and the isolation and identification of echitamine alkaloid of *A. scholaris* bark. Preliminary phytochemical tests revealed the presence of alkaloids, α-amino acids, carbohydrates, flavonoids, glycosides, phenolic compounds, reducing sugars, saponins, tannins, steroids, and terpenoids in the bark. The antimicrobial activity of the ethyl acetate, 70 % ethanol, and watery extracts of the bark of *A. scholaris* was evaluated using the agar well diffusion method against six different microorganisms. Both ethyl acetate and 70 % ethanol extracts exhibited significant antimicrobial activity, with inhibition zone diameters ranging from 18 to 24 mm on all the tested microorganisms. The watery extract showed inhibition zone diameters of 12-17 mm against four microorganisms but it was not active against *Pseudomonas aeruginosa* and *Escherichia coli*. One of the alkaloids, echitamine, was isolated from the ethyl acetate extract of the bark sample using column chromatography, and it was identified by FT IR, ^{1}H NMR, and ^{13}C NMR spectral data. This study also demonstrates that *A. scholaris* has high source of phytochemicals. Reported literatures showed that the isolated alkaloid, echitamine, does not protoplasmic poison and is used as an astringent herb.

Keywords: *Alstonia scholaris* bark, phytochemicals, antimicrobial activity, echitamine, agar well diffusion method

Introduction

Plants are not only a dietary source for both human beings and animals but also safer phytomedicines. Traditionally, phytomedicines have been used to treat various ailments and Ayurvedic systems of therapy (Twaij and Hasan, 2022). The ethnomedical information of medicinal plants has great potential for researchers to provide a scientific basis for their properties (Hassan, 2009). *Alstonia scholaris* (L.) R.Br. (Taung-ma-yo) has also long been used as a traditional medicine to cure human and livestock ailments. The plant, *A. schotaris*, attracts the attention of researchers worldwide for its pharmacological activities ranging from antimalarial to anticancer activities (Bhanu *et al*., 2013). *A. scholaris* possesses the family Apocynaceae and genus *Alstonia;* the English name is Devil tree or Dita bark, the Myanmar name is Taung-ma-yo (Figure 1). It has a wide occurrence in the Asia-Pacific region, from India and Sri Lanka through mainland Southeast Asia and China, throughout Malaysia to Northern Austria and the Salomon Islands (Anubha and Yashwant, 2015). Alkaloids, coumarins, flavonoids, leucoanthocyanins, reducing sugars, simple phenolics, steroids, saponins, and tannins were documented as the chief chemical constituents in the plant (Pankti *et al*., 2012). Various biological and pharmacological activities such as antimicrobial, antidiarrheal, antioxidant, antidiabetic, anticancer, analgesic, anti-inflammatory, hepato protective, CNS, wound healing, immune modulatory, antiasthematic, antifertility, and cytotoxicity properties of *A. scholaris* have

[1] Department of Chemistry, Yangon University of Education
[2] Department of Chemistry, Pathein University
[3] Department of Chemistry, University of Yangon

been reported by Khyade *et al. (*2014). The bark is also traditionally used by many ethnic groups in North East India and other parts of the world as a source of cure against bacterial infection, malarial fever, toothache, rheumatism, snakebite, dysentery, bowel disorder, etc. Also, the latex is used in treating coughs, sores, and fever (Bhattacharjee, 2004). The aim of this research work is to investigate some phytochemical constituents, to determine antimicrobial activity, and to isolate echitamine alkaloid from the bark of *A. scholaris*. The isolated compound was then identified by FT IR, ^{1}H NMR, and ^{13}C NMR.

(a) (b) (c)

Figure 1. Photographs of *A. scholaris* (a) trees (b) leaf and (c) trunk bark

Materials and Methods

Sample Collection and Preparation

In this research work, the bark of *A. scholaris* (Taung-ma-yo) was collected from Kamayut Township, Yangon Region, in March 2018. The scientific name of this selected plant was confirmed by the Department of Botany, Dagon University. The collected bark sample was washed with water and dried in the air at room temperature. The dried bark sample was cut into small pieces and then ground into powder using a grinding machine. The powdered samples were stored in air-tight containers to prevent moisture changes and other contamination.

Preparation of Crude Extracts

Various crude extracts of ethyl acetate, 70 % ethanol, and water were prepared for screening antimicrobial activity using the percolation method in their respective solvents. The following crude extracts were also prepared for the isolation of some constituents from the bark sample. The dried powdered sample (500 g) was percolated in petroleum ether (60–80 °C) for 5 days, followed by filtration. This procedure was repeated four more times. The combined pet ether extracts were evaporated under reduced pressure by means of a rotary evaporator to get petroleum ether crude extract. The defatted marc was then extracted with ethyl acetate for 3 days and filtered. This procedure was also repeated four more times. The defatted ethyl acetate extract was obtained by concentrating the filtrate using a rotary evaporator. The remaining marc was then percolated with ethanol for five days and filtered. Ethanol percolation was repeated four times until the extract became faint. Ethanol crude extract was concentrated by using a rotary evaporator. The crude extracts: pet ether, ethyl acetate (EA-1), and ethanol crude extracts were obtained. A portion of the ethanol crude extract was then dissolved in 1 M hydrochloric acid and partitioned with further ethyl acetate. After evaporating the solvent, an **ethyl acetate** extract **(EA-2)** was obtained. Crude extracts were kept for separation.

Investigation of Phytochemical Constituents

In order to classify the types of organic constituents present in the sample, preliminary phytochemical tests for alkaloids, α-amino acids, carbohydrates, flavonoids, glycosides, phenolic compounds, reducing sugars, saponins, starch, steroids, tannins, and terpenoids on the bark sample were carried out according to the appropriate reported methods (Marini-Bettolo *et al*., 1981; Shriner *et al*., 1980; Robinson, 1983; Raymond, 1982; Vogel, 1966; Harborne, 1994).

Screening of Antimicrobial Activity

In vitro antimicrobial activity of ethyl acetate, 70 % ethanol, and watery extracts from the bark of *A. scholaris* was investigated by using the agar well diffusion method (Anibijuwon and Udeze, 2009) at the Pharmaceutical Research Department (PRD), Ministry of Industry, Yangon Region. The test microorganisms were *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus pumilus, Candida albicans*, and *Escherichia coli.* The inhibition zone (clear zone) appeared around the agar well (10 mm diameter), indicating the presence of antimicrobial activity. The antimicrobial activity of crude extracts was determined by the diameter of inhibition zone.

Isolation and Purification of Isolated Organic Compound

The isolation of the pure compound was carried out from 3 g of ethyl acetate crude extract (EA-2) of the bark of *A. scholaris* by column chromatography. Column chromatography was performed by successive gradient elution with *n*-hexane (150 mL), followed by *n*-hexane:ethyl acetate (9:1 v/v,150 mL), *n*-hexane: ethyl acetate (1:1 v/v, 150mL), ethyl acetate (150 mL), ethyl acetate: methanol (9:1 v/v, 150 mL), ethyl acetate: methanol (1:1 v/v, 150 mL), and methanol (300 mL) solvent systems. The eluate was collected with 150 mL per fraction to provide a total of seven fractions (F_1-F_7). A colourless solid was observed in the fraction F_6. After decantation of F_6, the remaining solid was washed with *n*-hexane and then methanol. The solid compound was purified by recrystallization using ethyl acetate: methanol (1:1) solvent system. Then the purified crystal (51 mg, 0.01 %) was isolated from EA-2 extract.

Thin-layer chromatography Screening of Isolated Compound

The isolated compound was subjected to TLC analysis, and the R_f value was determined. Silica gel GF_{254} percolated aluminium plate (Merck, England) was employed, and the chromatogram was developed in the appropriate solvent system. The chromatogram was viewed under UV-254 nm and UV-365 nm light, and treated with visualizing agents. The observed R_f value for the isolated compound was then recorded.

Identification of Isolated Compound by Modern Spectroscopic Techniques

The Fourier Transform Infrared Spectrum of isolated compound was recorded by an FT IR (8400) spectrophotometer (Shimadzu, Japan), at the Chemistry Department, University of Mandalay, in order to identify the functional groups. The nuclear magnetic resonance NMR spectra for the isolated compound were recorded to examine the types and numbers of hydrogen and carbon present. The NMR spectra of the isolated compound were recorded by a Bruker NMR spectrophotometer with 400 MHz for ^{1}H NMR spectra and 100 MHz for ^{13}C NMR spectra at the Institute of Natural Medicine, University of Toyama, Japan. The spectra of the isolated compound were measured using pyridine-d_6 solvent.

Results and Discussion

Phytochemical Constituents of the *A. scholaris* Bark

Phytochemical constituents of the bark of *A. scholaris* investigated by chemical test method were alkaloids, α-amino acids, carbohydrates, flavonoids, glycosides, phenolic compounds, reducing sugars, saponins, steroids, tannins, and terpenoids. The results are shown in Table 1.

Table 1. Phytochemical Constituents in the Bark of *A. scholaris*

No.	Tests	Extracts	Reagents used	Observation	Remark
1	alkaloids	1 % HCl	Dragendorff's reagent	brick red ppt.	+
			Wagner's reagent	white ppt.	+
			Mayer's reagent	yellow ppt.	+
			Sodium picrate	yellow ppt.	+
2	α-amino acids	H_2O	Ninhydrin reagent	purple colour spot	+
3	carbohydrates	H_2O	10 % α-naphthol, conc. H_2SO_4	red ring	+
4	flavonoids	EtOH	Mg ribbon, conc. HCl	pink colour	+
5	glycosides	H_2O	10 % lead acetate solution	white ppt.	+
6	phenolic compounds	EtOH	5 % $FeCl_3$ solution, 1 % $K_4Fe(CN)_6$	dark blue colour	+
7	reducing sugars	2 M H_2SO_4	Benedict's solution	brick red ppt	+
8	saponins	H_2O	distilled water	frothing	+
9	starch	H_2O	I_2 solution	no deep blue colour	-
10	steroids	pet ether	acetic anhydride, conc.H_2SO_4	red colour solution	+
11	tannins	EtOH	1 % Gelatin, 5 % $FeCl_3$ solution	green colour solution	+
12	terpenoids	$CHCl_3$	acetic anhydride, conc.H_2SO_4	pink colour	+

(+) presence (-) absence (ppt) precipitate

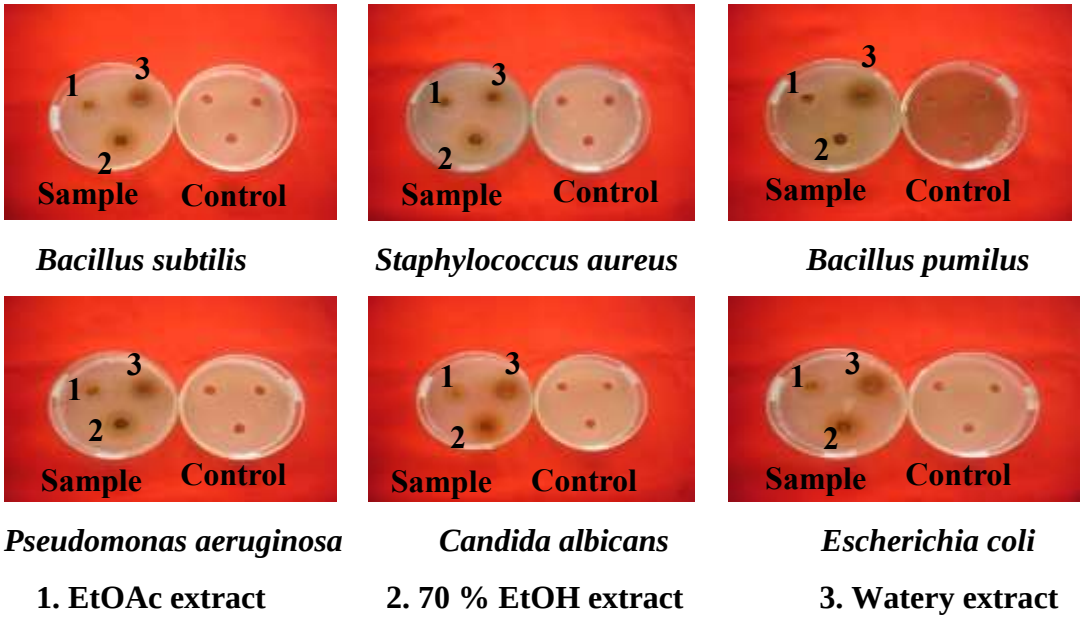

Figure 2. Antimicrobial activity of various crude extracts of bark of *A. scholaris* L.against six test microorganisms

Table 2. Antimicrobial Activity of Crude Extracts from Bark of *A.scholaris* L. by Agar Well Diffusion Method

Organisms used	Diameter of inhibition zone (mm) in various crude extracts		
	EtOAc	70 % EtOH	H_2O
B. subtilis	21	24	17
S. aureus	18	20	14
P. aeruginosa	20	22	-
B. pumilus	18	20	14
C. albicans	19	19	12
E. coli	21	20	-

agar well diameter 10 mm moderate activity 15 - 19 mm
lower activity 10 - 14 mm highest activity 20mm - above

Screening of Antimicrobial Activities

Antimicrobial activities of various crude extracts such as ethyl acetate, 70 % ethanol, and watery of bark were investigated by employing the agar well diffusion method. The advantages of this method are simplicity and low cost that have contributed to its common use for the antimicrobial screening of plant extracts. In this study, the samples were tested on six different strains of microorganisms, namely, *B. subtilis*, *S. aureus*, *P. aeruginosa*, *B. pumilus*, *C. albicans* and *E. coli*. This method is based on inhibition zone diameter that shows the degree of the antimicrobial activity. The larger the zone diameter, the more effective against tested microorganisms. The antimicrobial studies of various crude extracts of bark are shown in Figure 2. The results showed that a larger zone of inhibition was observed in ethyl acetate and

70 % ethanol extracts (inhibition zone diameter ranged from 18 to 24 mm) against six different strains of microorganisms. The watery extract exhibited lower activity (inhibition zone diameter ranged from 12 to 17 mm) than the other extracts, and it exhibited antimicrobial activity only for four types of microorganisms except *P. aeruginosa* and *E. coli*. The observed data are described in Table 2.

Isolation and Identification of an Alkaloid from Ethyl Acetate Extract

In this study, the crude extracts of bark samples were successively percolated with different solvents: pet ether, ethyl acetate, and ethanol. A dried bark sample (500 g) was extracted by the percolation method with pet ether to get pet ether crude extract (8.4 g), containing non-polar constituents. Then all of the polar components were extracted with ethyl acetate from the defatted residue to obtain moderately polar constituents. A 5.1 g of ethyl acetate extract was obtained from the extraction. The remaining marc was successively percolated with ethanol, so 8.2 g of ethanol extract was obtained. Some of the ethanol extracts were partitioned again with ethyl acetate to obtain more ethyl acetate extracts containing more polar chemical compounds.

One of the polar compounds from ethyl acetate extract, an alkaloid, was isolated by column chromatography and purified by a suitable solvent system. A colourless needle-shaped crystal of compound (51 mg, 0.01 %) was formed after evaporating the solvent. It was soluble in methanol, ethanol, ethyl acetate and chloroform but insoluble in pet ether and *n*-hexane. It was UV inactive under UV lamp (254 nm and 365 nm) and the R_f value was found to be 0.18 in EtOAc : MeOH : H_2O (150 : 26 : 19 v/v). The photograph of the isolated compound and the TLC chromatograms are shown in Figure 3. The isolated compound gave brown colouration on TLC after spraying and heating with 5 % H_2SO_4 solution. Besides, it showed an orange spot on TLC by spraying with Dragendorff 's reagent. It also provided a light pink colour on TLC when treated with anisaldehyde-sulphuric acid reagent followed by heating. From the observed TLC behaviour, and the FT IR spectral data as shown in Figure 4 and Table 3, the isolated compound may be an alkaloid.

(a) (i) (ii) (iii)

(b)

Solvent system = EtOAc: MeOH: H_2O (150:26:19 v/v), R_f = 0.18

(i) Under UV_{254} nm (ii) Spraying Dragendorff's Reagent (iii) Spraying Anisaldehyde

Figure 3. (a) Crystal of isolated compound from ethyl acetate extract

(b) Thin layer chromatograms of isolated compound

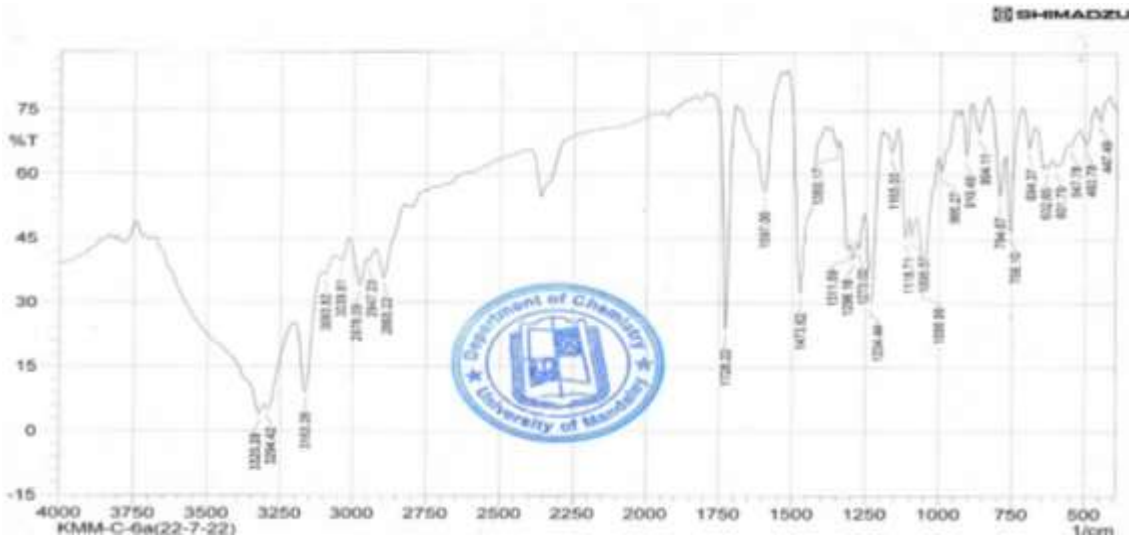

Figure 4. FT IR spectrum of isolated compound from the bark of *A. scholaris*

Table 3. FT IR Spectral Data of Isolated Compound

Wavenumber (cm^{-1})	Vibrational mode	Remark
3325, 3294	O-H stretching	OH group
3163	N-H stretching	-NH in heterocyclic ring
3093, 3039	=C-H stretching	sp^2 CH group
2978, 2947, 2893	C-H as- and s-stretching	sp^3 CH group (-CH_3, -CH_2-)
1728	C=O stretching	Carbonyl group stretching
1620	C=C stretching	C-C in aromatic compound
1473, 1375	C-H bending	-CH_2, -CH_3 group
1254	C-O stretching	COO group
794, 756	=CH oop bending	aromatic ring and alkene

Identification of Isolated Compound by NMR

The ^{1}H NMR spectrum (400 MHz, pyridine) of the compound was compared with the ^{1}H NMR spectrum (600 MHz) of echitamine predicted by the ACD (Advanced Chemistry Development) Lab. In the ^{1}H NMR spectrum of the isolated compound, there is a solvent effect at chemical shift values of 5.1, 7.2, 7.6, and 8.7 ppm. The remaining signals of the ^{1}H NMR spectrum of the isolated compound were matched with the ^{1}H NMR spectrum of echitamine. Chemical shift values at 3.625 ppm showed the proton signals from N-H, O-H at positions 1, 19, and 21. These chemical shift values were found to be similar to those of the predicted values of echitamine.

^{13}C NMR spectrum (100 MHz, pyridine-d_6) of the isolated compound indicated that it contained 22 carbons. The ^{13}C NMR spectrum of this compound was also matched with the ^{13}CNMR spectrum (150 MHz) of echitamine predicted by ACD Lab. Chemical shifts near 120, 135, and 150 ppm in the compound were solvent peaks due to solvent pyridine. The chemical

shift values in the ^{13}C NMR spectral data of the compound were nearly the same as those of the predicted data of echitamine. Comparative NMR spectra are illustrated in Figures 5 and 6, and comparative NMR spectral data are also shown in Table 4. Thus, the isolated compound was confirmed to be an alkaloid, namely echitamine (molecular formula $C_{22}H_{29}N_2O_4$), based on these results.

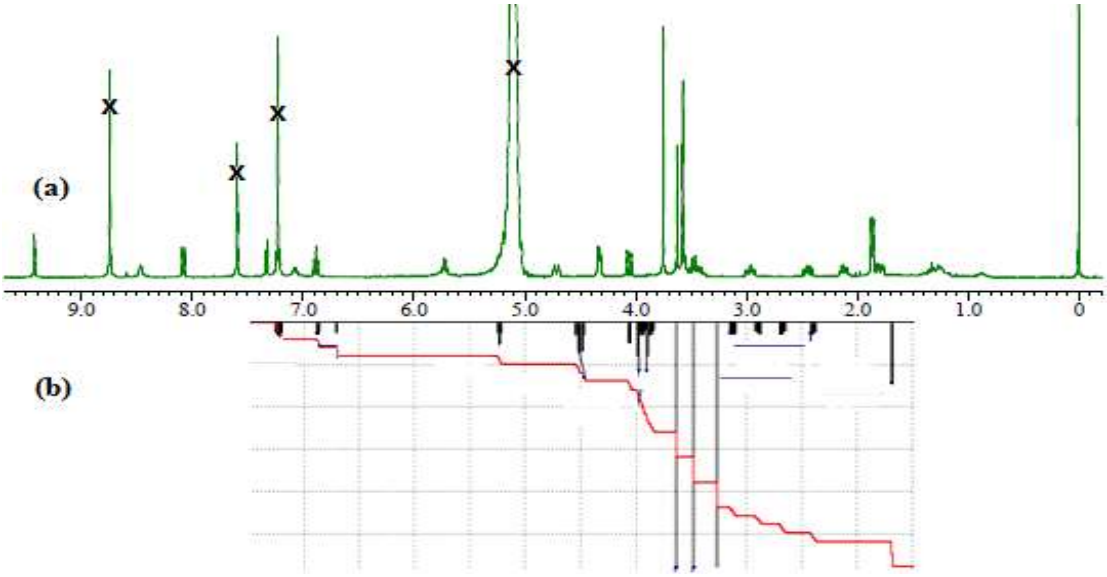

Figure 5. Comparison of (a) ^{1}H NMR spectrum of isolated compound (400 MHz, pyridine-d_6 and (b) the predicted ^{1}H NMR (600 MHz) spectrum of echitamine by ACD Lab

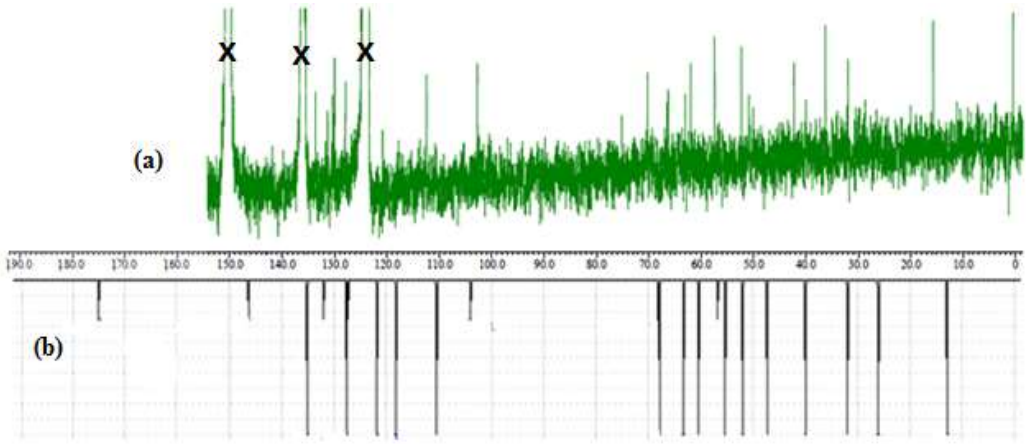

Figure 6. Comparison of (a) ^{13}C NMR spectrum of compound (100 MHz, pyridine-d_6) and (b) the predicted ^{13}C NMR (150 MHz) spectrum of echitamine by ACD Lab

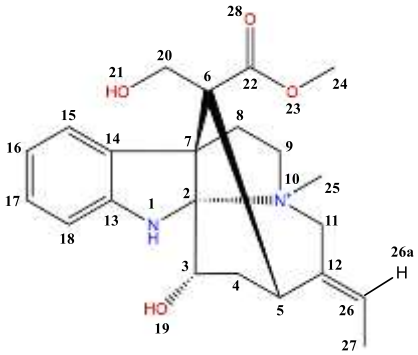

Figure 7. Structure of echitamine

Table 4. Comparison of ^{1}H and ^{13}C NMR Spectral Data of Isolated Compound and Echitamine*

Position	Chemical Shift (δ_H) (ppm)		Chemical Shift (δ_C) (ppm)	
	Compound	Echitamine	Compound	Echitamine
1	3.625	3.479	-	-
2	-	-	102.706	103.91
3	-	-	62.986	60.41
4	-	-	36.278	31.99
5	-	-	42.333	40.01
6	-	-	70.288	68.12
7	-	-	62.031	56.78
8	-	-	32.007	26.12
9	-	-	57.465	55.27
11	-	-	75.117	63 23
12	-	-	127.760	127.19
13	-	-	147.356	146.22
14	-	-	131.260	131.9
15	-	-	120.762	121.71
16	-	-	117.729	118.11
17	-	-	129.860	127.52
18	-	-	112.421	110.39
19	3.625	3.479	-	-
20			66.255	67.82
21	3.625	3.479	-	-
22	-	-	-	174.93
24	3.750	3.639	52.354	
25	3.575	3.265	50.855	47.28
26	-	-	133.515	135.08
27	1.865	1.683	15.750	12.93

***Predicted by ACD (Advanced Chemistry Development) Lab

Conclusion

The preliminary phytochemical tests on the bank of A scholars revealed the presence of alkaloids, α-amino acids, carbohydrates, flavonoids, glycosides, phenolic compounds, reducing sugars, saponins, tannins, steroids, and terpenoids. The antimicrobial activity of EtOAc and 70 % EtOH extracts. Exhibits to the (inhibition zone diameter ranged from 18 mm to 24 mm) against six different strains of microorganisms. The result showed the efficiency of plant extracts as natural antimicrobial compounds against microorganisms and suggested the possibility of employing them in drugs for the treatment of infectious diseases caused by the tested microorganisms. A compound from the ethyl acetate extract of bark sample was isolated by column chromatographic technique. It was structurally identified by FT IR, ^{1}H NMR and ^{13}C NMR techniques. This isolated compound (0.01% yield) was found to be one of the alkaloids according to TLC and FT IR results and was identified as echitamine from ^{1}H NMR and ^{13}C NMR results. The isolated compound has also pointed to effective natural product herbal medicines which could be contributed to the health programme with the scientific evidence for Myanmar indigenous medicinal plants.

Acknowledgements

The authors would like to thank the Department of Higher Education, Ministry of Education, Yangon, Myanmar, for the permission of doing this research and for allowing to write this paper. Our deepest gratitude is expressed to Dr Kay Thwe Hlaing, Rector, Yangon University of Education, for her encouragement and Dr Nay Mar Soe, Professor and Head, Department of Chemistry, Yangon University of Education for her helpful advice, valuable suggestions and provision of research facilities at the Department of Chemistry, Yangon University of Education.

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