Investigation of Preliminary Phytochemical Constituents, Some Organic Compounds and Acute Toxicity of *Alstonia Scholaris* (L.) R.Br. (Taung-Mayo) Leaf

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Abstract

The aim of this research was to investigate preliminary phytochemical constituents, some organic compounds and acute toxicity of *Alstonia scholaris* (L.) R.Br. (Taung-mayo) Leaf. The preliminary phytochemical tests were carried out by standard procedure. From this tests, it was observed that steroids, terpenoids, phenolic compounds, flavonoids, glycosides, carbohydrates, alkaloids, α -amino acid, organic acid and saponins were present however cyanogenic glycosides and tannins were absent in *Alstonia scholaris* (L.) R.Br. (Taung-mayo) leaf. β-sitosterol (A, 0.0033%, m.pt =138~142℃) and betulin (B, 0.02%, m.pt = 236~240℃) were isolated from Taung-mayo leaf by column chromatographic separation. The isolated compounds were identified by physicochemical properties and modern spectroscopic methods. In addition, acute toxicity test of aqueous and 70 % EtOH extracts of Taung-mayo leaf was carried out on both sexes of albino mice of 25-35 g body weight. The maximum permissible doses of both extracts of Taung-mayo leaf (16 g/kg) were treated in mice. Both extracts of Taung-mayo leaf were free from acute toxicity of harmful effect. LD_{50} of both Taung-mayo leaf extracts were greater than (16 g/kg). The finding from the present work will contribute to the scientific development of Myanmar traditional medicine.

Keywords: *Alstonia scholaris* (L.) R.Br. (Taung-mayo) Leaf, β-sitosterol, betulin, acute toxicity, steroid

Introduction

During the past decade, traditional systems of medicine have become a topic of global importance. In many developing countries, a large proportion of the population relies heavily on traditional practitioner and medicinal plants to meet primary health care needs. Although modern medicine may be available in these countries, herbal medicines (phytomedicines) have often maintained popularity for historical and cultural reasons. Concurrently, many people in developed countries have begun to turn to alternative or complementary therapies, including medicinal herbs (WHO, 2005). Myanmar has a rich in variety of medicinal plants and people use various herbal medicine. But so far, there has not been full and systematic exploitation of this natural resource (May Aye Than, 2002).

Selected Myanmar Traditional Medicinal Plant (Taung-mayo)

Alstonia scholaris, commonly called blackboard tree, scholar tree, milkwood or devil's tree in English, is an evergreen tropical tree in the Dogbane Family (Apocynaceae). Myanmar name is Taung-mayo,Taung-me-oke. Plant parts used for medicine are leaf, bark and root (Boonchuay, 1976, Khan, 2003)

Description and distribution of Taung-mayo

It is a tree and is up to 40 m tall. The bole on older trees is strongly fluted; slash is cream coloured and with abundant, white latex. Leaves are in whorls, obovate or elliptic. Flowers are up to 1 cm long and cream or green, in branched, terminal panicles up to 120 cm long. Fruits are dehiscent, two-lobed follicle, somewhat body with 15-32 cm long and are with numerous seeds. Seeds are 4-5 mm

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long, brown, flat and oblong and with 7-13 mm long tufts of hair at the ends (Joker, 2000). Flowering times are from October to November. Fruiting periods are from December to April (Kyaw Soe, 2004). In Myanmar, it is widely distributed in all parts of country.

Figure 1. Photo of Taung-mayo tree, leaves, flower, fruits and bark

Chemical constituents of Taung-mayo

Some reported chemical constituents present in Taung-mayo leaf are picrinine, picalinal, narline, β-sitosterol, betulin, ursolic acid, oleanolic acid, alstonamine, rhazimanine, and echitamidine (Dutta, 1976).

Medicinal Uses of Taung-mayo

Taung-mayo leaf, roots and barks are used in the treatment of malarial fever. It has widely used in folk medicine as an astringent, alterative, tonic, asthma, anthelmintic and antiperiodic in fevers and has been claimed to be useful in restoring stomach muscle tone after debility due to fever and in the treatment of some forms of rheumatism and all parts of Taung-mayo exhibit improved and broader spectrum of antibacterial activity (Khan, 2003). Taung-mayo leaf is also used in the treatment of diabetes (Singh, 1997).

Toxicity Tests

Toxicology is the science of harmful effects of chemicals or drugs on living organism or is the science dealing with poison. To know the harmful effects of a new chemical or drugs, toxicity must be done the potential toxicity of new chemicals or drugs must be evaluated first on the laboratory animals. Animal toxicity experiments also prevent distinctly harmful drugs from becoming readily available to man.

There are three basic types of toxicity tests on animals detecting toxic effect. They differ primarily in their duration. They are acute test (single dose, 24 hours test and survivors followed for 7 days), subacute or prolong test (daily dose for 3 months) and chronic test (daily dose for 1-2 years) (Litchfield & Wilcoxon, 1949).

Materials and Methods

Collection of Plant Sample

This sample was collected from Kamayut Township, Yangon Region and it was identified as *Alstonia scholaris* (L.) R.Br. at the Department of Botany, University of Yangon.

The collected plant sample was cleaned by washing thoroughly with water and air-dried at room temperature. The dried sample was cut into small pieces and ground into powder by grinding machine. The dried sample was used for chemical and biological investigation.

Preliminary Phytochemical Investigation of Taung-mayo Leaf

In order to find out the types of organic compounds present in the sample, the preliminary phytochemical investigation was carried out according to the appropriate reported methods.

Preparation of Crude Extract from Taung-mayo Leaf

300g of dried Taung-mayo leaf powder were percolated with 900 cm^3 of petroleum ether (60-80℃) for about one week followed by filtration. This procedure was repeated another two times. The combined 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 900 cm^3 of 70% ethanol for about 1 month at room temperature and filtered. This procedure was also repeated another two times. The defatted 70% ethanol extract was obtained by concentrating the filtrate, using rotary evaporator. Then defatted 70% ethanol extract was partitioned between EtOAc and H_2O. After removal of the solvent from EtOAc layer, the concentrated EtOAc extract was then eluted with $CHCl_3$,$CHCl_3$ soluble portion and $CHCl_3$ insoluble portion were obtained, and they were then separately concentrated. The resultant crude extracts such as PE and 70% EtOH extract, $CHCl_3$ soluble and $CHCl_3$ insoluble portions of EtOAc extract were obtained and kept for separation.

Isolation of Compound 'A' from PE extract from Taung-mayo Leaf

PE extract of Taung-mayo leaf (5g) was fractionated by silica gel column chromatography using 100 ml of PE, PE:EtOAc in the ratio of 40:1v/v (100 cm^3), 20:1v/v (100 cm^3), 9:1v/v (200 cm^3) and 3:1v/v (100 cm^3) as eluent. Colorless needle shaped crystal 0033% (10 mg) Compound 'A' was isolated.

The isolated compound 'A' was identified by some physico-chemical properties and FT-IR spectral data.

Isolation of Compound 'B' from Chloroform Soluble portion of EtOAc Extract from Taung-mayo Leaf

Isolation of chemical constituents of chloroform soluble portion (1.5g) of EtOAc extract of Taung-mayo leaf was carried out by column chromatographic method. Gradient elution was performed successively with PE: EtOAc (20:1, 10:1, 3:1 v/v). 0.02% (60 mg) of compound 'B' as a white crystal was obtained.

The isolated compound 'B' was identified by some physico-chemical properties, FT-IR spectral data and 1H NMR spectral data.

Acute Toxicity Study of Taung-mayo Leaf

Acute toxicity test was carried out on mice by treating with aqueous extract and 70 % ethanol extract of Taung-mayo leaf.

Preparation of extract

Different weights of the 70 % EtOH extract and aqueous extract from Taung-mayo leaf were separately dissolved in distilled water to get required doses as follows: Different doses of Taung-mayo leaf aqueous and 70 % EtOH extracts: 6 g/kg, 8 g/kg, 12 g/kg and 24 g/kg

Materials

Albino mice (body weight 25-35 g), Mice cages, Aqueous and 70 % ethanol extracts Taung-mayo leaf, Animal balance, Intragastric needle, Syringes

Methods

In this study, 160 albino mice, both sexes with the body weight ranging between 25-35 g were used. They were divided into 8 groups (IX to XVI) and each group contained 10 mice. Since the route of administration selected should be the intended route for administration of the tested drug given to human during therapy, the oral route was chosen. Groups IX to XII mice were treated with aqueous extract Taung-mayo leaf and Groups XIII to XVI mice with 70 % EtOH extract of Taung-mayo leaf in the doses of 6, 8, 12, 24 g/kg body weight, respectively. The extracts were dissolved in distilled water and required dose was administrated orally with intragastric needle (Figure 3(i)), to every mice. After administration of test agent, each group of mice was housed separately (Figure (ii)), and food and water were

supplied. They were observed carefully for 24 hours. Any mortality within the group was recorded within 24 hours, survivors were observed daily for 7 days.

(i) (ii)

Figure 3. (i) Administration of plant extracts to mice
(ii) ddY mice housed in separate cages for the acute toxicity tests

Results and Discussion

Preliminary Phytochemical Tests on Taung-mayo Leaf

In order to find out the types of phytoorganic constituents present in the samples, the preliminary phytochemical tests were carried out according to the procedures as mentioned in above section. From these experiments, it was observed that streoids, terpenoids, phenolic compounds, glycosides, carbohydrates, alkaloids, α -amino acids, organic acid and saponins were present however cyanogenic glycosides and tannins were absent in Taung-mayo leaf. The results obtained from these experiments were summarized in Table 1.

Table 1. Results of Phytochemical Examination on Taung-mayo leaf

Sr.No.	Tests	Extract	Test Reagents	Observations	Remark
1.	Terpenoids	$CHCl_3$	Acetic anhydride and conc: H_2SO_4	Pink colouration	+
2.	Steroids	$CHCl_3$	Acetic anhydride and conc: H_2SO_4	Blue-green colouration	+
3.	Phenolic compounds	EtOH	1 % $FeCl_3$	Deep blue colouration	+
4.	Flavonoids	EtOH	HCl/Mg turning	Pink colouration	+
5.	Glycoside	EtOH	10 % lead acetate	White ppt	+
6.	α-amino acid	H_2O	Ninhydrin reagent	Pink	+
7.	Carbohydrate	H_2O	10 % α–naphthol conc: H_2SO_4	Red ring	+
8.	Tannins	H_2O	2 % NaCl, Gelatin	White ppt	-
9.	Cyanogenic glycosides	H_2O	conc:H_2SO_4,Sodium picrate paper	No brick red	-
10.	Organic acid	H_2O	Bromocresol blue	Blue	+
11.	Saponins	H_2O	Distilled water	Frothing	+
12.	Alkaloids	1 % HCl	Mayer's reagent Dragendroff's reagent Wagner's reagent	White ppt Orange ppt Brown ppt	+

Identification of Isolated Compound 'A' by Modern Spectroscopic Technique

Table 2. Physico-chemical Properties of Isolated Compound 'A' and β-sitosterol

Experiment	Observation	
	Compound 'A'	β-sitosterol
Physical State	White needle shaped crystal	White needle shaped crystal
R_f (PE: EtOAc-5:1)	0.54	0.54
m.pt (EtOH)	138-142℃	138-141℃*
Libermann-Burchard	Green	Green
Anisaldehyde - H_2SO_4	Purple	Purple
Vanillin - H_2SO_4	Blue	Blue
I_2 –vapour	Brown	Brown
5% H_2SO_4	Cherry red	Cherry red

Merck-Index (2001)

According to FT-IR spectrum (Figure 4), the broad band at 3435cm^{-1} indicated the presence of hydroxyl group. The peaks at 2945, 2928, 2870cm^{-1} showed the C-H stretching. The bands at 1450 and 865 cm-1 indicated the C-H bending and that at 1383cm^{-1} (doublet) confirmed the presence of isopropyl group. The band at 1645cm^{-1} showed C=C stretching and 1056 cm^{-1} indicated stretching of C-OH.

From the observation of physic-chemical properties and FT-IR spectral data, it can be inferred that the compound 'A' was β-sitosterol, molecular formula $C_{29}H_{50}O$, one of the reported constituents of Taung-mayo leaf.

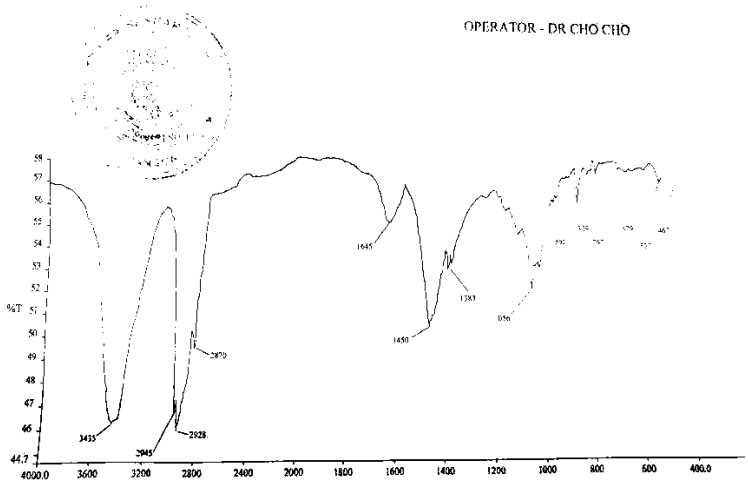

Figure 4. FT-IR spectrum of Isolated compound **A**

Identification of Isolated Compound 'B' by Modern Spectroscopic Technique

Table 3. Physico-chemical Properties of Isolated Compound 'B' and Betulin

Experiment	Observation	
	Compound 'B'	Betulin
Physical State	White crystal	White crystal
R_f ($CHCl_3$: MeOH-1:1)	0.46	0.46
m.pt (EtOH)	236-240℃	235-237C*
Libermann-Burchard	pink	Pink
Anisaldehyde - H_2SO_4	Purple	Purple
Vanillin - H_2SO_4	Brown	Brown
1%$FeCl_3$	Not detected	Not detected

I_2 –vapour	Yellow	Yellow
5% H_2SO_4	Red	Red

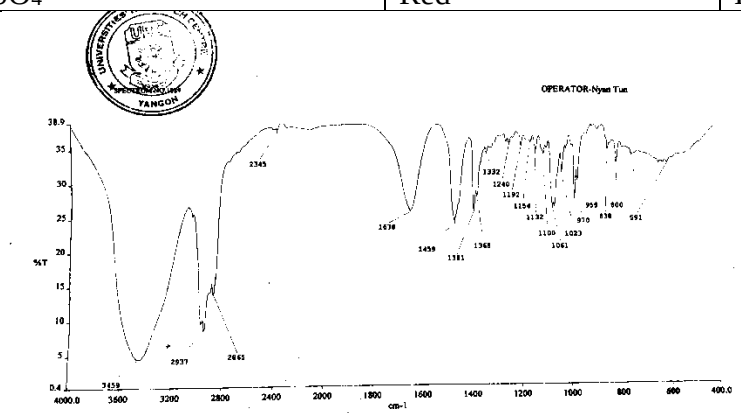

Figure 5. FT-IR spectrum of Isolated compound **B**

FT-IR spectrum (Figure 5) of 'B' was taken with KBr pellet sample and the following assignment of functional groups was done. The broad absorption occurred at 3459 cm^{-1} indicated the presence of hydroxyl group. The weak band at the 3010 cm^{-1} indicated the presence of =CH group and it appeared due to =CH stretching. The bands appeared at 2937 cm^{-1} due to CH asymmetric stretching and 2865 cm^{-1} presence of CH2 and CH3 groups, and the corresponding CH bending absorption occurred at 1459 cm^{-1}. The bands at 1132 cm^{-1} and 1061 cm^{-1} were respectively formed by C-O stretching vibration of 2˚cyclic alcohol (-CHOH) and 1˚ alcohol (CH_2OH). The presence of C=C group was confirmed by the absorption band at 1638 cm^{-1}due to C=C stretching vibration.

The ^1H NMR spectrum (400NHz, $CDCl_3$) of isolated compound 'B' is described in Figure 6. Figure 7 represents the expended 1H spectrum of 'B' between 0.5~2.05 ppm. From these spectral data, the numbers of protons and their environments could be assigned as shown in Table (4). There were six methyl groups present in 'B', confirmed by the singlet signals at δ 0.94, 0.76, 0.82, 1.02, 0.98 and 1.69 ppm. A multiplet signal at 3.17 ppm represented the H-3 proton, i.e., 2˚cyclic alcoholic –CHOH proton at position 3 of betulin. Two doublet signals at 3.32 and 3.81 ppm were attributed to two protons (H-28) of 1˚ CH_2OH alcohol. In addition, two doublet peaks at 4.58 and 4.68 ppm confirmed the presence of terminal methylene protons (=CH, H-29). On the study of ^1H NMR spectral data of 'B' by comparing with those of betulin (William, 1992) Table (4), these ^1H NMR spectral data were found to be the same. Therefore compound 'B' was assigned as betulin, molecular formula $C_{30}H_{50}O_2$.

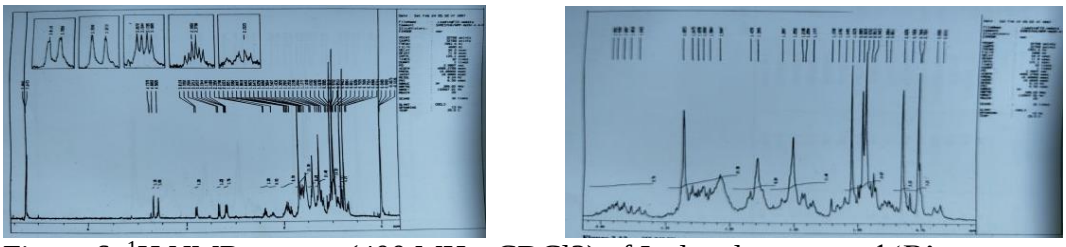

Figure 6. ^1H NMR spectra (400 MHz, CDCl3) of Isolated compound 'B'

Table 4. Comparison of ^{1}H NMR Spectral Data of Compound 'B' and Betulin

Compound'B' δ/ppm	Betulin * δ/ppm	Integration	Multiplicity
4.68, 4.58	4.68,4.58 (H-29)	2H	D
3.81, 3.32	3.77,3.31 (H-28)	2H	D
3.17	3.18 (H-3)	1H	M
1.95, 1.42	1.95, 1.40 (H-21)	2H	M
1.84, 1.02	1.86, 1.02 (H-22)	2H	M
1.64	1.64 (H-13)	1H	M
1.58	1.57 (H-18)	1H	M
0.67	0.67 (H-5)	1H	M
1.64, 0.89	1.65, 0.89 (H-1)	2H	M
0.94	0.96 (H-23)	3H	S
0.76	0.76 (H-24)	3H	S
0.82	0.82 (H-25)	3H	S
1.02	1.02 (H-26)	3H	S
0.98	0.98 (H-27)	3H	S
1.68	1.68 (H-30)	3H	S

*William, 1992

Acute Toxicity Test

The acute toxicity of 70 % EtOH and aqueous extracts from Taung-mayo leaf were examined by Litchfield and Wilcoxon (Litchfield, 1949) method. From these acute toxicity tests on albino mice, even with the maximal permissible dose of aqueous extract of Taung-mayo leaf was 16 g/kg body weight, the mice were found to be alive and healthy during the observation period of 7 days. The results obtained were shown in Table 5 and 6. Aqueous and 70% EtOH extracts of Taung-mayo leaf did not show acute toxic effect in the concentrations ranged from 6 g/kg to 16 g/kg body weight. Both extracts of aqueous and 70% EtOH extracts were found to be free from acute toxic of harmful effect on the concentration ranged from 6 g/kg to 16 g/kg body weight.

Table 5. Results of Acute Toxicity Tests of Aqueous Extract from Taung-mayo leaf on Albino Mice

Group	Dose (g/kg)	No.of Dead per test	Observed % Dead	Correct value	Expected % Dead	Observed minus expected	Contribution to Chi^2 (C)
IX	16	0/10	0	0	0	0	0
X	12	0/10	0	0	0	0	0
XI	8	0/10	0	0	0	0	0
XII	6	0/10	0	0	0	0	0

Table 6. Results of Acute Toxicity Tests of 70% EtOH Extract from Taung-mayo leaf on Albino Mice

Group	Dose (g/kg)	No.of Dead per test	Observed % Dead	Correct value	Expected % Dead	Observed minus expected	Contribution to Chi^2 (C)
XIII	16	0/10	0	0	0	0	0
XIV	12	0/10	0	0	0	0	0
XV	8	0/10	0	0	0	0	0
XVI	6	0/10	0	0	0	0	0

Conclusion

From these observations, it was found that steroids, terpenoids, phenolic compounds, glycosides, carbohydrates, alkaloids, α -amino acid, organic acid and saponins were present however cyanogenic glycosides and tannins were absent in *Alstonia scholaris* L.R.Br. (Taung-mayo) leaf. One steroid compound : β-sitosterol (A, mpt 138-148℃, 0.0033%) and one terpenoid compounds: betulin (B, mpt 236-240℃, 0.02%) were isolated from selected medicinal plant. Acute toxicity test of aqueous and 70% EtOH extracts of Taung-mayo leaf was carried out on both sexes of albino mice of 25-35g body weight. The maximum permissible doses of both extracts of Taung-mayo leaf (16g/kg) were treated in mice. Aqueous and 70% EtOH extracts of Taung-mayo leaf was free from acute toxicity effect. LD_{50} of aqueous and 70% EtOH extracts of Taung-mayo leaf was greater than 16g/kg. This finding from the present work will contribute to the scientific development of Myanmar traditional medicine.

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