ACUTE TOXICITY OF THE ETHANOLIC PLANT EXTRACT AND STRUCTURE ELUCIDATION OF ISOLATED BIOACTIVE COMPOUND FROM THE STEM BARK OF *PROTIUM SERRATUM* (WALL.EX COLEBR.) ENGL.

Thin Yu Mar,[1] Arnt Win,[2] Aye Mon Thida Nyo[3]

Abstract

In this research work, one of Myanmar traditional medicinal plants, *Protium serratum* (Wall.ex Colebr.) Engl., Myanmar named Gati was selected for chemical analysis and pharmacological investigation. The stem bark of the selected plant was collected from Pyin Oo Lwin Township, Mandalay Region. Acute toxicity test of 95% ethanolic extract of the stem bark of the selected plant was examined by Organization of Economic Cooperation and Development (OECD) guideline 425. According to acute oral toxicity test of ethanolic extract of the stem bark of this plant, the test substance can be considered relatively safe to the dose level of 5000 mg/kg body weight. Furthermore, the bioactive compound was isolated from ethyl acetate portion *serratum* (Wall.ex Colebr.) Engl. by thin layer and column chromatographic method. The yield percent of this pure compound was found to be 1.21 based upon the ethyl acetate crude extract. The molecular formula of this isolated flavonoid compound was determined as $C_{15}H_{14}O_6$ by using some spectroscopic techniques, such as FT IR, ^{1}HNMR, ^{13}CNMR, DEPT, HSQC and DART-Mass spectrometry. The structure of this compound was elucidated by using DQF-COSY, ^{1}HNMR splitting patterns, coupling constant (*J* values) and HMBC spectroscopic data. IUPAC name of the isolated compound is C_2(*S*), C_3(*R*)-2-(3'-4'-dihydroxyphenyl)- 3,4,-dihydro-2*H*-chromene-3,5,7-triol.

Keywords: *Protium serratum* (Wall.ex Colebr.) Engl., acute toxicity, thin layer and column chromatography.

Introduction

Medicinal plants and plant-derived medicine are widely used in traditional cultures all over the world and there are becoming increasingly popular in modern society as natural alternatives to synthetic chemicals. Natural products and their derivatives represent more than 50% of all drugs in critical used in the world. Plant drugsare plant-derived medicines that contain a chemical compound or more usually mixtures of chemical compounds that act individually or combination on human body to prevent disorders and to restore or maintain health. Chemical entities are pure chemical compounds that are used for medicinal purposes (Van Wyk and Wink, 2004).In Myanmar, medicinal uses of *Protium serratum* (Wall.ex Colebr.) Engl. are less known. Thus, with the objectives of promotion of its medicinal uses, biologically active component from the stem bark of *serratum* is intended to be investigated.

Pure pale yellow needle-shaped compound was isolated from the stem bark of *serratum*, locally known as Gati, which is one of the indigenous medicinal plant. Local people in Pyin Oo Lwin Township use the stem bark *serratum* for the treatment of anti-inflammatory, hypertension, diabetic, dysentery and diarrhea (Figure 1). Pale yellow needle-shaped flavonoid compound was isolated from the stem bark of *serratum* by using thin-layer and column chromatographic methods. The molecular formula and the structure of this isolated compound were assigned by using advanced spectroscopic methods such as FT IR, ^{1}HNMR (500 MHz), ^{13}C NMR (125 MHz), DEPT, HSQC, DQF-COSY, HMBC and DART-MS spectral data.

[1] Dr, Lecturer, Department of Chemistry, University of Magway
[2] Dr, Associate Professor, Department of Chemistry, Kyaukse University
[3] Dr, Associate Professor, Department of Chemistry, University of Mandalay

Botanical Description


Figure 1 Leaves, Fruits and Stem Bark of *Protium serratum* (Wall.ex Colebr.) Engl**.**

Family : Burseraceae
Genus : *Protium*
Species : *P.serratum*
Botanical name : *Protium serratum* (Wall. ex Colebr.) Engl.
Myanmar name : Gati
Part used : Stem bark
Medicinal uses : Hypertension, antibacterial, antifungal, diuretic, antidiabetes, dysentery, diarrhea and mouth ulcers (Localpeople in Pyin Oo Lwin Township, Myanmar)

Materials and Methods

The advanced instruments were used in the characterization of sample and structural elucidation of organic compound. These are UV lamp (Lambda 40, Perkin Elmer Co. England), FT IR spectrometer (Shimadzu, Japan), ^{1}H NMR spectrometer (500 MHz, Japan), ^{13}C NMR spectrometer (125 MHz, Japan), and DART-MS spectrometer, Japan.

Analar grade reagents and solvents were used throughout the experiment. Analytical preparative thin-layer chromatography was performed by using aluminium coated sheets silica gel (Merck. Co. Inc. Kiesel gel 60 F_{254}) and silica gel (70 to 230 mesh ASTM) was used for column chromatography.

Plant material

The stem bark of *Protium serratum* (Wall.ex Colebr.) Engl. (Burseraceae), Myanmar name Gati was collected from Pyin Oo Lwin Township, Mandalay Region in Myanmar.

Preliminary Phytochemical Test of the Stem Bark of *Serratum*

The phyotochemical tests of the selected plant were carried out by usual method.

Determination of Acute Toxicity

Acute toxicity study was performed on the ethanolic extract of the stem bark of *serratum* using mice as the experimental model. The acute toxicity test on 95% ethanolic extract of the stem bark of selected plant could be carried out according to OECD (Organization of Economic Co-operation and Development) guidelines 425 (OECD, 2008; Dixon, 1965, 1991; Bruce, 1985; Gallagher, 2003).

Extraction and Isolation

The air-dried stem barks of *serratum* (1000 g)were extracted with ethanol at room temperature for two months. The ethanol extract was concentrated in air. The ethanol extract was then re-extracted with ethyl acetate and evaporated to dryness at room temperature. The ethyl acetate crude sample extract (2.5 g) was obtained. The ethyl acetate crude extract was fractionated by column chromatography over silica gel with various ratios of *n*-hexane and ethyl acetate from non-polar to polar. Totally 280 fractions were collected. Then each fraction was checked on TLC using iodine as visualizing agent. The fraction with same R_f values were

combined. Major combined fraction gave only one spot on TLC and it was UV active. Pale yellow needle-shaped crystal, flavonoid compound (30.2 mg) was obtained.

Flow Sheet for Isolation of Organic Compound from EtOAc Extract

Ethyl acetate extract (2.5 g)
- Separated by column chromatography
  Adsorbent = SiO_2 (70-230 mesh)
  Eluent = *n* hexane: EtOAc (various volume ratio)
  (non polar to polar)

Totally (280) fractions
- Checked by TLC, I_2vapour and UV lamp
- Combined

(15) Combined fractions
- Checked by TLC

major combined fraction — Other fractions

major combined fraction
- checked by TLC
- one spot on TLC
- UV active

pale yellow solid compound
- re-crystallized by 30% EtOAc in *n*-hexane for two times

pale yellow needle-shaped crystal (30.2 mg)
R_f = 0.32
n-hexane: EtOAc1 :1 (v/v)1.21% yield based upon EtOAc extract

Spectroscopic Studies and Structure Elucidation of the Isolated Compound

The isolated organic compound was subjected to analyze by FT IR, ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz) DEPT, HSQC, DQF-COSY, DART-MS and NOESY spectroscopic techniques (Silverstein *et al.*, 2005). FT IR spectrum of the isolated organic compound was measured at the Department of Chemistry, Monywa University. The remaining spectral data were measured at the Department of Natural Resources Chemistry, Faculty of Pharmacy, Meijo University, Nagoya, Japan.

Conformational Phytochemical Test of the Isolated Compound

Phytochemical test for the isolated compound gave positive for flavonoid and phenolic tests. Therefore, compound is a flavonoid compound.

Results and Discussion

Preliminary Phytochemical Test

Preliminary phytochemical tests were carried out by standard methods and the observed results are shown in **Table 1.**

Table. 1 Preliminary Phytochemical Test of the Stem Bark of *Protium serratum* (Wall.ex Colebr.) Engl.

No.	Test	Reagent used	Observation	Result
1.	Alkaloid	Dragendorff's reagent	Orange ppt	+
2.	Flavonoid	EtOH, Conc: HCl, Mg ribbon	Pink colour solution	+
3.	Glycoside	10 % Lead acetate	White ppt	+
4.	Phenolic	10 % $FeCl_3$	Green colour solution	+
5.	Polyphenol	1 % $K_3[Fe(CN)_6]$, 1 % $FeCl_3$	Greenish blue colour solution	+
6.	Lipophilic	0.5 N KOH solution	Deep blue colour solution	+
7.	Saponin	Shaked with H_2O	Frothing	+
8.	Sugar	Benedict's solution	Brick red ppt	+
9.	Steroid	$(CH_3CO)_2O$, Conc.H_2SO_4	Blue colour solution	+
10.	Terpene	$(CH_3CO)_2O$, $CHCl_3$, Conc.H_2SO_4	Reddish brown colour solution	+
11.	Tannin	1% $FeCl_3$	Yellowish brown ppt	+

(+) = present of constituents

Acute Toxicity of the Stem Bark of *serratum*

Different groups of mice administered with 4 different doses of ethanolic extract of the selected plant and vehicle (distilled water) 10 mL/kg body weight (control) were kept under observations for two weeks. The results based on daily body weight record are shown in Table 2.

Table 2 Acute Toxicity Study of the Stem Bark of *Protium serratum* (Wall.ex Colebr.) Engl. Based on Daily Body Weight Record (in grams)

Groups	Marking	Sex	Dose in mg/kg Body Weight	Weight of Mice/kg		
				1st day	7th day	14th day
I	Head	Female	175	23.7	24.6	29.8
	Head	Male		23.5	24.8	28.3
	Back	Male		28.0	29.2	32.4
			Mean value	25.1	26.2	30.2
II	Back	Female	550	23.3	25.4	27.7
	Tail	Male		24.4	24.9	25.9
	R.Hand	Male		26.8	27.1	27.8
			Mean value	24.8	25.8	27.1
III	Tail	Female	2000	25.6	26.5	26.8
	R.Hand	Female		22.5	22.3	23.0
	L.Hand	Male		22.5	23.5	24.6
			Mean value	23.5	24.1	24.8
IV	Head	Female	5000	23.2	24	25.2
	Back	Female		22.0	22.6	24.6
	Head-Back	Female		28.04	29.5	30.6
			Mean value	24.4	25.4	26.8
Control	R-Hand	Female	Distilled Water	24.0	24.9	24.3
	R-Leg	Male		27.4	26.9	28.0
	L-Leg	Male		26.5	26.9	30.0
			Mean value	25.9	26.2	27.4

Body weight is an important factor to monitor the health of an animal. Loss of body weight is the first indicator of an adverse effect (Gallagher, 2003). According to the daily body weights record, all the animals from treated groups increased body weight for all the 14 days as compared with the 0 day body weights values. Hence, the test substance, ethanolic extract of the stem bark of *Protium serratum* indicating no sign of toxicity and lethality.

After two weeks, all the mice were alive and did not show any toxic symptoms such as body weight loss, diarrhea, inactivity, aggressiveness, restlessness, etc. and no death when compared with that of the control group. The results of mortality records and cage side observations are described in Tables 3 and 4.

Table 3 Acute Toxicity Study on the Ethanolic Extract of the Stem Bark of *Protium serratum* (Wall.ex Colebr.) Engl. Based on Mortality Record

Extracts	Groups	Number of mice/group	Dose of extract (mg/kg)	Observed period	Ratio of dead and tested	Death %
Ethanol	I	3	175	Two weeks	0/3	0
	II	3	550	Two weeks	0/3	0
	III	3	2000	Two weeks	0/3	0
	IV	3	5000	Two weeks	0/3	0
	Control	3	D/W 10mL/kg	Two weeks	0/3	0

Table. 4 Acute Toxicity Study on Ethanolic Extract of the Stem Bark of *Protium serratum* (Wall.ex Colebr.) Engl. Based on Cage Side Observations

Parameters	Observations
Condition of the fur	Normal
skin	Normal
Subcutaneous swelling	Nil
Abdominal distention	Nil
Eyes - dullness	Nil
Eyes - opacities	Nil
Pupil diameter	Normal
ptosis	Nil
Colour and consistency of the faces	Normal
Wetness or soiling of the perineum	Nil
Condition of teeth	Normal
Breathing abnormalities	Nil
Gait	Normal

Mortality is the main criteria in assessing the acute toxicity of any drug (Lalitha, 2012). The acute toxicity (Table 3) on mortality recorded was found nil. The dose level (LD_{50} value) of the test substance was found to be more than 5000 mg/kg. From the cage-side observation, the tested animals at all dose levels showed no significant changes in behaviors after oral administration.

According to the results of daily body weight recorded and cage side observation the test substance, ethanolic extract of the stem bark of *serratum* can be considered safe. Moreover, the acute toxicity mortality is found nil, so the test substance can be considered free from toxic effect up to the dose level of 5000 mg/kg for oral administration.

Molecular Formula Determination of the Isolated Pure Compound

The isolated compound was obtained as pale yellow needle-shaped crystal. The structure of the isolated compound was elucidated by modern instrumental techniques (Figures 2 -9). The molecular formula was determined to be $C_{15}H_{14}O_6$ from the observation of DART-MS spectrometry (Figure 9). The FT IR spectrum showed absorption bands at 3330, 3020, 2921, 2851, 1619, 1520, 1464, 1289, 1145, 1095, 979 and 766 cm^{-1} ascribable to hydroxyl, sp^2 H/C, sp^3 H/C, aromatic ring and ether functional group respectively (Figure 2). The ^{1}H NMR spectrum (Figure 3 and Table 5) revealed two sp^3 methylene protons at δH (2.73, 2.88 ppm), two sp^3 methine protons δH at (4.18, 4.82 ppm) and five sp^2 methine protons at δH (5.91, 5.94, 6.78, 6.80, 6.97 ppm).

The FT IR and DEPT spectral data (Figure 2 and Figure 6) show the presence of one sp^3 methylene carbon, two sp^3 methine carbons, seven sp^2 quaternary carbons, five sp^2 methine carbons, one hydroxyl group and one ether group.

Table. 5 ^{1}H NMR Spectral Data of the Isolated Compound (500 MHz) Solvent used = Deuterated chloroform

No.	Chemical shift (δ/ppm)	No. of protons	Splitting pattern	Coupling constant (*J* values Hz)	Proton assignment
1	2.73 2.88	2H	dd dd	2.8, 16.7 4.23, 16.7	sp^3 methylene proton
2	4.18	1H	dt	4.23, 2.8	sp^3 methine proton
3	4.82	1H	-	-	sp^3 methine proton
4	5.91	1H	d	2.3	sp^2 methine proton
5	5.94	1H	d	2.3	sp^2 methine proton
6	6.78	1H	d	8.1	sp^2 methine proton
7	6.80	1H	dd	2.01, 8.1	sp^2 methine proton
8	6.97	1H	d	2.01	sp^2 methine proton
Total number of protons = 9					

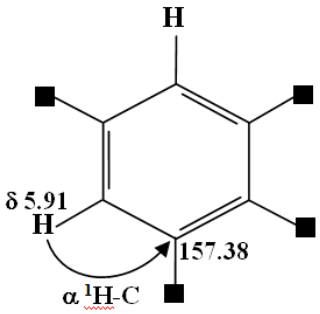
Table 6 ^{1}H-^{13}C Correlation in HSQC spectrum of the Isolated Compound

No.	^{13}C NMR chemical shift (δ/ppm)	^{1}H NMR chemical shift (δ/ppm)	Carbon Assignment
1	30.74	2.73, 2.85	sp^3 methylene carbon
2	67.51	4.18	sp^3 methine carbon
3	79.90	4.82	sp^3 methine carbon
4	95.90	5.94	sp^2 methine carbon
5	96.32	5.91	sp^2 methine carbon
6	100.09	–	sp^2 quaternary carbon
7	115.34	6.97	sp^2 methine carbon
8	115.92	6.80	sp^2 methine carbon
9	115.96	6.78	sp^2 methine carbon
10	132.31	–	sp^2 quaternary carbon
11	145.80	–	sp^2 quaternary carbon
12	145.97	–	sp^2 quaternary carbon
13	157.38	–	sp^2 quaternary carbon
14	157.70	–	sp^2 quaternary carbon
15	158.01	–	sp^2 quaternary carbon
Total no. of carbons = 15			

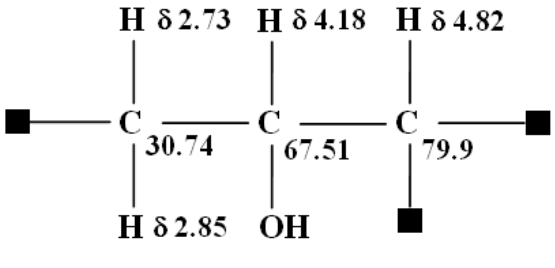
Structure Elucidation of the Isolated Compound

The structure of pure compound could be determined by ^{1}H NMR, DQF-COSY, HSQC and HMBC spectral data, respectively (Figures 5,7 and 8).

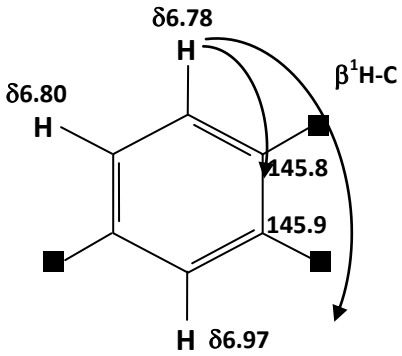
In the structure elucidation, the tri-substituted benzene ring fragments a, b and d could be assigned by DQF-COSY, ^{1}H NMR splitting patterns, coupling constant (*J*-values), HSQC and HMBC spectra.



a

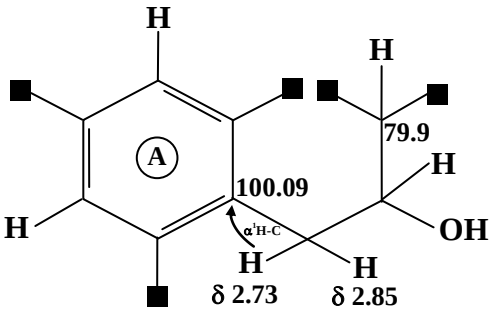


b



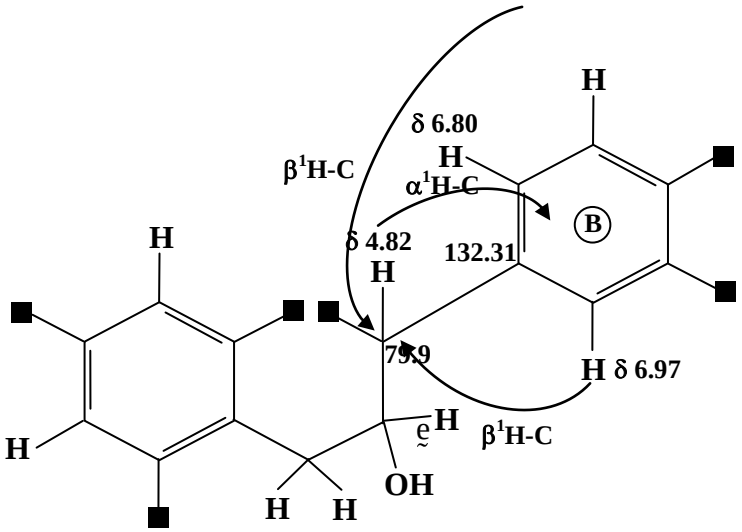
d

Fragments a and b could be connected according to the HMBC spectrum which gives rise to fragment c.

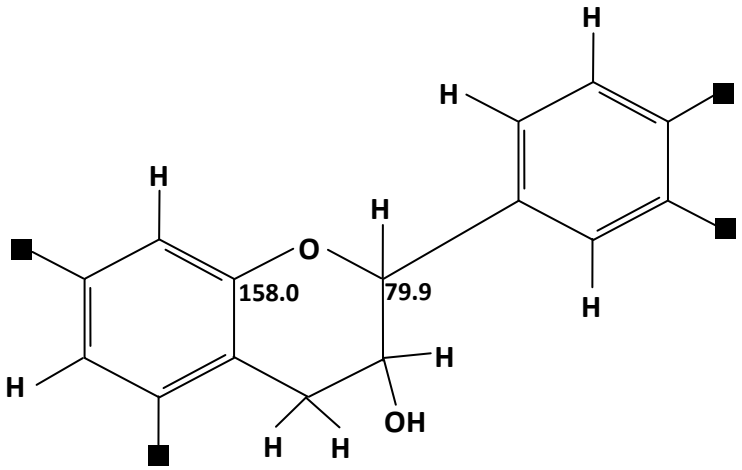


c

Fragments c and d could be connected by HMBC spectrum which leads to fragment e.



In this state, elucidated HDI is 8. Remaining HDI one must be one ring. The ether oxygen atom flanked between the reasonable down filed chemical shifts of aromatic quaternary carbon (δ 158.01 ppm) and sp^3 methine carbon (δ 79.9 ppm) which established the following fragment f .



f

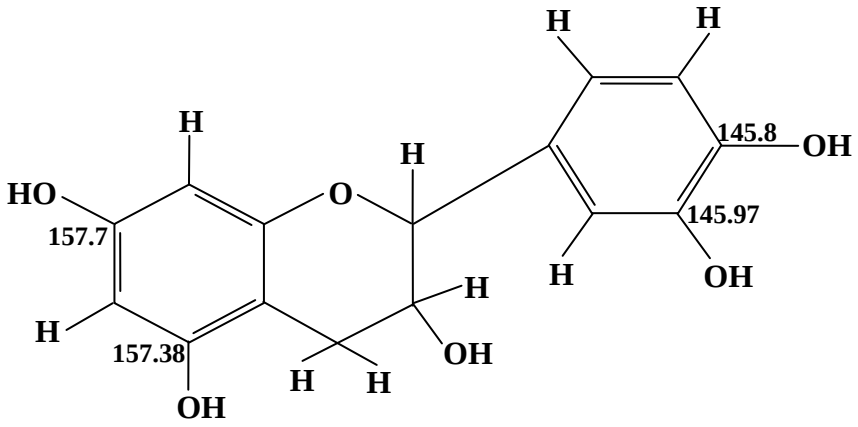
In the above fragment f , elucidated molecular formula is $C_{15}H_{10}O_2$.

The remaining molecular formula = $C_{15}H_{14}O_6 - C_{15}H_{10}O_2$

= H_4O_4,

$\therefore$ It must be four – OH groups:

The remaining four hydroxyl groups could be connected to the four downfield chemical shift carbons (δ 145.8 ppm, δ 145.97 ppm, δ157.38 ppm and δ157.7ppm) which accomplished the following complete structure of compound.



IUPAC name of the isolated compound is C_2(*S*), C_3(*R*)-2-(3',4'-dihydroxyphenyl) - 3,4-dihydro-2*H*-chromene-3,5,7-triol

Confirmation of Molecular Formula of Compound

Molecular formula of compound could be confirmed by DEPT and FT-IR spectra.
The partial molecular formula = $C_{15}H_{11}O_3$
The partial molecular mass = 239
According to DART-mass spectrum, M + H (M + 1) peak m/z = 290.0852
Therefore, molecular mass = 290
The remaining molecular mass = 290 – 239 = 51
It must be three hydroxyl groups.
Therefore, the real molecular formula of isolated compound = $C_{15}H_{14}O_6$

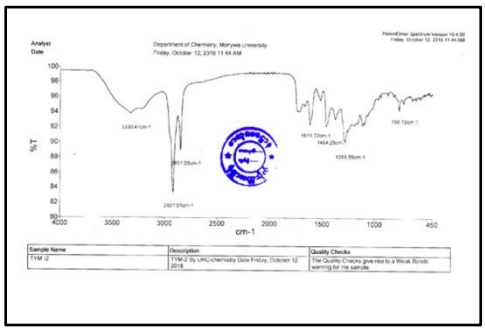


Figure 2 FT IR Spectrum of Isolated Compound

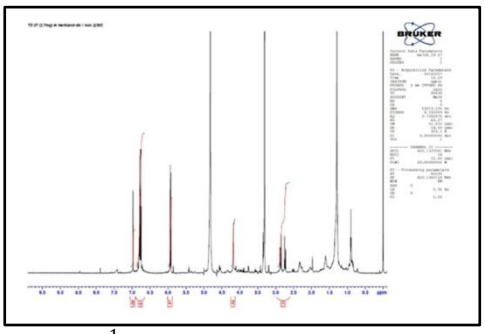


Figure 3 ^{1}H NMR Spectrum of Isolated Compound

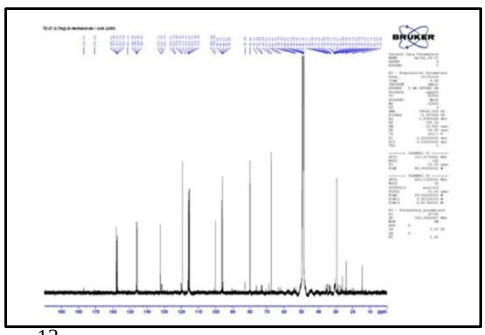


Figure 4 ^{13}C NMR Spectrum of Isolated Compound

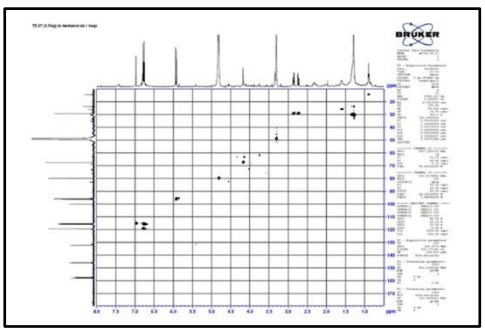


Figure 5 HSQC Spectrum of Isolated Compound

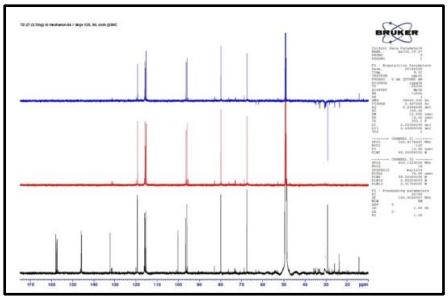


Figure 6 DEPT Spectrum of Isolated Compound

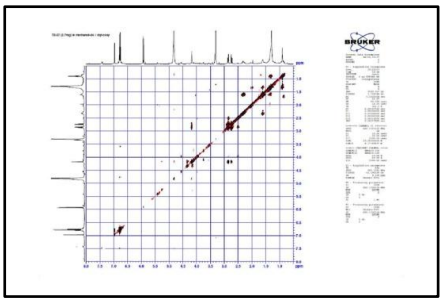


Figure 7 DQF-COSY Spectrum of Isolated Compound

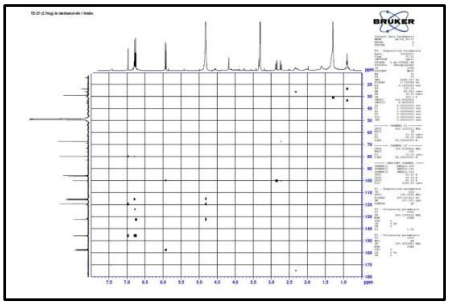


Figure 8 HMBC Spectrum of Isolated Compound

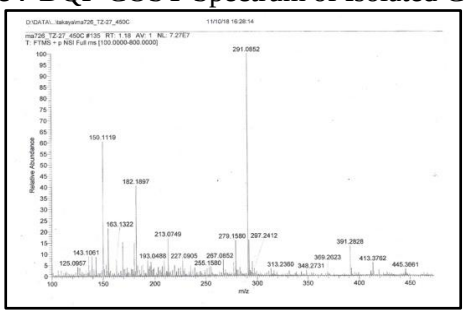


Figure 9 DART-Mass Spectrum of Isolated Compound

Conclusion

In the present investigation, acute toxicity and isolation of flavonoid compound [C_2(*S*),C_3(*R*)-(3',4'-dihydroxyphenyl)-3,4-dihydro-2*H*-chromene-3,5,7-triol] which has so many biological activities, especially anti-inflammatory and anti-diabetic activities from the stem bark of *serratum* were evaluated. From the determination acute toxicity, all the mice from treated groups increased body weight for all the 14 days as compared with 0 day body weight values. In addition the acute toxicity mortality recorded was found nil. The dose level of tested substance was found to be more than 5000 mg/kg based on body weight. After two weeks, all the mice were alive and did not show any toxic symptoms. Therefore the ethanolic extract of the stem bark of *serratum* can be considered relatively safe. Moreover, the pale yellow needle-shaped compound could be illustrated by using sophisticated spectroscopic method and confirmed by DART-MS spectroscopy. This studies support that the stem bark of *serratum* could be safely used for anti-inflammatory and anti-diabetic drug without harmful effect. Further studies are required and in progress here.

Acknowledgements

The authors would like to thank the Myanmar Academy of Art and Science for allowing to present this paper and Professor and Head Dr Thida Aung, Department of Chemistry, Magway University, for her kind encouragement.

References

Bruce, R. D. (1985). "An Up-and-Down Procedure for Acute Toxicity Testing". *Journal of Fundamental Apply Toxicity*, vol. 5, pp. 151-157

Dixon, W. J. (1965). "The Up-and-Down Method for Small Samples". *Journal of the American Statical Association*, vol. ~~60,~~ pp. 967-978

Dixon, W. J. (1991). "Staircase Bioassay: The Up-and-Down Method". *Journal of Neuroscience and Biobehavioral Review*, vol. 15, pp.47-50

Gallagher, M. E. (2003). "Toxicity Testing Requirements, Methods and Proposed Alternatives". *Environ.*, vol. 26, pp.257-258

Lalitha, P., Sripathi, S. K. and Jayanthi, P. (2012)." Acute Toxicity Study of Extracts of *Eichhorinacrassipes* (Mart.) Solms". *Asian Journal of Pharmaceutical and Clinical Research*, vol.5(4), pp.59-61

OECD. (2008).*OECD Guidelines for the Testing of Chemicals.*

Silverstein, R. M., Webster, F. X. and Kiemle, D. J. (2005), *Spectrometric Identification of Organic Compounds*, Singapore: 7thed, John Wiley & Sons, Inc.

Van Wyk, B. E. and Wink, M. (2004). *Medicinal Plants of the world*. Singapore: Selangor Darul Ehsan Alam, Times edition, pp.1-22