PHYTOCHEMICAL CONSTITUENTS AND SOME BIOLOGICAL ACTIVITIES OF THE STEMS OF *ALLAMANDA CATHARTICA* L. (SHWEWA-PAN)

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Abstract

The aim of this research is to isolate bioactive compound of the stems of *Allamanda cathartica* L. (Shwewa-pan) and to investigate some biological activities. According to the phytochemical tests, alkaloids, flavonoids, terpenoids, steroids, glycosides, organic acids, phenolic compounds, saponins, tannins and carbohydrates were found to be present in the stems. The ethanol and watery extracts of total phenolic content (31.98 ± 0.94 mg GAE/g, 18.91 ± 0.53 mg GAE/g), total flavonoid contents (226.67 ± 8.50 mg QE/g, 100.67 ± 9.29 mg QE/g), total steroid contents (278.81 ± 7.70 mg CE/g , 154.72 ± 13.31 mg CE/g) and total tannin contents (317.5 ± 0.00 mg TAE/g, 25.83 ± 14.4 mg TAE/g) were observed respectively. By thin layer and silica gel column chromatographic methods, one compound, 2, 5, 7 - trihydroxy - 3 - (4 - hydroxyphenyl) - 4H - chromen - 4 - one (0.22 %, m.pt 151 °C), was isolated from ethyl acetate extract of the stems and identified by ^{1}H NMR, ^{13}C NMR, HMQC, HMBC and EI MS spectroscopies. The antioxidant activity of ethanol extract (IC_{50} =10.97 µg/mL) determined by DPPH radical scavenging activity assay was higher than that of watery extract (IC_{50} = 68.37 µg/mL). All of the extracts have mild antimicrobial activity (inhibition zone diameters = 12~18 mm) against all of the microorganisms. According to the results of brine shrimp cytotoxicity bioassay, both ethanol and watery extracts have cytotoxic effect. It was found that EtOAc, PE and EtOH extract exhibited the inhibition of tumor formation. Both ethanol and watery extracts did not show antiproliferative activity, however, 2, 5, 7 - trihydroxy - 3 - (4 -hydroxyphenyl) - 4H - chromen - 4 - one showed activity against A 549 (IC_{50} = 41.7 µg/mL), MCF 7 (IC_{50} = 47.9 µg/mL) and Hela (IC_{50} = 8.91 µg/mL) human cancer cell lines.

Keywords : *Allamanda cathartica* L., antioxidant activity, antimicrobial activity, cytotoxicity, antitumor activity, antiproliferative activity, 2, 5, 7-trihydroxy-3 - (4 hydroxyphenyl) - 4H - chromen - 4 - one

Introduction

Plants based drugs have been used worldwide in traditional medicines for the treatment of various diseases. Shwewa-pan scientifically known as *A.cathartica* is of the plant family Apocynaceae. Genus is *Allamanda* and species is *cathartica.* Myanmar name is Shwewa-pan and also called Shwe-pan-nwe. Other common names are Golden trumpet, Yellow bell, Buttercup flower and Angle's trumpet (Chandrasekhar *et al*., 2012). Allamanda species are apparently native to northern Brazil Guyana, Surinam and probably French Guiana. It is a genus of climbing shrubs. The distribution of this species is global but is mainly presented in subtropical to tropical (Uduak and Esther, 2015). In Myanmar it is cultivated as ornamental garden plants. *A.cathartica* has long been used in traditional medicine for treating malaria and jaundice. The leaf extract was found to promote wound healing. The flower is also used as a laxative. The chemical constituents are allamandin, allamandicin, allamdin, plumericin, isoplumericin, plumieride, ursolic acid, beta-amyrin, beta-sitosterol, fluvoplumeirin, lupeol, quercetin, kaempferol, glabridin and naringenin (Fah, 2013).

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A. cathartica (Shwewa-pan) has been chosen for this research because it has various biological activities and bioactive chemical constituents, and also due to the lack of scientific report on the locally grown *A. cathartica*. In this research work, screening of phytochemical constituents and investigation of antioxidant, antimicrobial and antitumor activities of the stems of *A.cathartica* (Shwewa-pan) were carried out on the respective various crude extracts.

Materials and Methods

Plant materials

The stems of *A. cathartica* were collected from Bago University Campus, Bago Township, Bago Region, during October, 2015. Some pharmacological activities such as antioxidant activity, antimicrobial activity, cytotoxicity, antitumor activity and antiproliferative activity of various crude extracts of the stems were determined in.

Phytochemical Screening

Preliminary phytochemical tests such as alkaloids, flavonoids, terpenoids, steroids, glycosides, organic acids, starch, phenolic compounds, saponins, tannins, carbohydrates, cyanogenic glycosides, reducing sugars and α- amino acids tests were carried out according to the appropriate reported methods (Sofowora, 2000).

Determination of Nutritional Values

The nutritional values such as moisture, ash, fiber, protein, fat and carbohydrate contents were determined by the respective AOAC method. (AOAC, 2000)

Preparation of Various Crude Extracts

(i) Preparation of pet-ether extracts

100 g of each dried powdered sample was extracted with 250 mL of pet-ether (60-80 °C) by using Soxhlet extractor. The filtrates were concentrated by removal of the solvent to give the respective pet-ether crude extract.

(ii) Preparation of ethyl acetate extracts

50 g of each dried powdered sample was extracted with 150 mL of ethyl acetate in similar manner mentioned in above procedure to yield the respective ethyl acetate extract.

(iii) Preparation of 95 % ethanol extracts

The marc from ethyl acetate extracts were refluxed with 150 mL of 95 % ethanol for 6 hours and filter under suction. Evaporation of the solvent was done under reduced pressure to get 95 % ethanol extract.

(iv) Preparation of watery extracts

Watery extract of each dried powdered sample was prepared by boiling 100 g of sample with 250 mL of distilled water for 6 hours and filtered. The filtrates were concentrated by removal of the water to give watery extract.

Determination of Total Phenol content by Folin-Ciocalteu reagent (FCR) method

The total phenolic content (TPC) in each sample was estimated by Folin-Ciocalteu method according to the procedure described by Song *et al.,* (2010). Each extract solution (1000 µg/mL) was mixed with 5 mL of F-C reagent (1:10) and incubated for about 5 min. To each test tube, 4 mL of 1 M sodium carbonate was added and the test tubes were kept at room temperature for 15 min and UV absorbance of reaction mixture was read at λ_{max} 765 nm. The blank solution was prepared as the above procedure by using distilled water instead of sample solution. Total phenol content was estimated as milligram gallic acid equivalent per gram (mg GAE/g) of extract.

Determination of Total Flavonoid Content by Aluminium chloride method

The total flavonoid content (TFC) in each sample was estimated by Aluminium Chloride method according to the procedure described by Song *et al.,* (2010). Each extract solution (1000 µg/mL) was mixed with 1.5 mL of methanol, 0.2 mL of 1 % $AlCl_3$ solution and 2.8 mL of distilled water. The absorbance of reaction mixture was at λ_{max} 415 nm. The blank solution was prepared as the above procedure by using distilled water instead of sample solution. Total flavonoid content was estimated as milligram quercetin equivalent per gram (mg QE/g) of extract.

Determination of Total Steroid Content by Zak's method

The total steroid content (TSC) in each sample was estimated by Zak's method according to the procedure described by Zak *et al.*, (1981). Each extract solution (1000 µg/mL) was prepared by ferric chloride diluting agent. The test sample solution (5 mL) was added 4.0 mL of concentrated sulphuric acid to each tube. After 30 minutes incubation, intensity of the colour was read at 450 nm. The blank solution was prepared as the above procedure by using ferric chloride diluting agent instead of sample solution. Total steroid content was estimated as milligram cholesterol equivalent per gram (mg CE/g) of extract.

Determination of Total Condensed Tannin by Broadhurst's method

The total condensed tannin contents were determined by method of Broadhurst and Jones (1978) with slight modification, using tannic acid as a reference compound. A volume of 0.4 mL of extract is added to 3 mL of a solution of vanillin and 1.5 mL of concentrated hydrochloric acid. After 15 min of incubation the absorbance was read at 500 nm. The blank solution was prepared as the above procedure by using methanol instead of sample solution. The condensed tannin was expressed as milligram of tannic acid equivalent per gram of extract.

Isolation and Identification of Phytochemical Constituent from EtOAc Extract of Stems of *A. cathartica* by Column Chromatography

Dried powdered stems sample (1000 g) were percolated in 1000 mL of 70 % EtOH for one week and filtered. This procedure was repeated for three times. Then the filtrate was concentrated by using a vacuum rotatory evaporator to get EtOH extract (23 g). Then the EtOH extract was defatted by using pet-ether and the defatted EtOH extract was successively partitioned between EtOAc and water. The EtOAc layer was concentrated under reduced

pressure using vacuum rotatory evaporator. Ethyl acetate crude extract (23 g) from the stems of *A. cathartica* was subjected to column chromatographic separation using silica gel (63-210 µm mesh). Gradient elution was performed successively with PE: EtOAc system in the ratios of 5:1, 3:1, 1:1, 1:2, 1:5 v/v followed by EtOAc only and MeOH only. Successive fractions obtained were combined on the basis of their behavior on TLC. Finally, seven main fractions F-I to F-VII was obtained.

After the solvents have been evaporated, fraction F-I (f_{1-2}), F-II (f_{3-4}). F-IV (f_{8-27}), F-V (f_{28-29}), F-VI (f_{30-31}) and F-VII (f_{32-33}) were obtained. The fraction F-III was evaporated and washed with pet-ether, giving yellow powder of the Compound A in 121.1 mg (0.22 % of yield).

The isolated compound was then identified by using joint application of its physicochemical properties and modern spectroscopic techniques such as UV, FT IR, NMR and Mass spectroscopies, and compared with the reported data. The NMR and Mass spectra of the isolated compound were measured at Division of Natural Product Chemistry, Institute of Natural Medicine, and University of Toyama, Japan.

Screening of Antioxidant Activity of Ethanol and Watery Extracts of the Stems of *A. cathartica*

In this experiment, DPPH (2 mg) was thoroughly dissolved in EtOH (100 mL). This solution was freshly prepared in the brown coloured reagent bottle and stored in the fridge for no longer than 24 h. Each tested samples (2 mg) and 10 mL of EtOH were thoroughly mixed by shaker. The mixture solution was filtered and the stock solution was obtained. By adding with EtOH, the sample solutions in different concentrations of 200, 100, 50, 25, 12.5 and 6.25 µg/mL were prepared from the stock solution. The effect on DPPH radical was determined by using the method of Marinova and Batchvarov (2011). The control solution was prepared by mixing 1.5 mL of 50 µM DPPH solution and 1.5 mL of EtOH using shaker. The test sample solution was also prepared by mixing thoroughly 1.5 mL of 50 µM DPPH solutions and 1.5 mL of each sample solution. The mixture solutions were allowed to stand at room temperature for 30 min. Then, the absorbance of each solution was measured at 517 nm by using UV-1650 spectrophotometer. Absorbance measurements were done in triplicate for each concentration and then mean values so obtained were used to calculate percent inhibition of oxidation. The capability to scavenge the DPPH radical was calculated by using the following equation:

$$\% \text{ RSA} = \frac{A_c - (A - A_b)}{A_c \times 100}$$

Where, %RSA = Radical Scavenging Activity
A_c = absorbance of the control (DPPH only) solution
A_b = absorbance of the blank (EtOH + Test sample solution) solution
A = absorbance of the test sample solution

Screening of Antimicrobial Activity of Various Crude Extracts of the Stems of *A. cathartica* by Agar Disc Diffusion Method

The screening of antimicrobial activity of various crude extracts such as PE, EtOAc, EtOH, watery extracts of the stems of *A. cathartica* were carried out by agar disc diffusion method at Fermentation Laboratory, Pharmaceutical Research Department, Ministry of Industry,

Yangon, Myanmar. Six microorganisms namely *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus pumilus, Candida albicans* and *Escherichia coli* were used for this test (Perez *et al*., 1990).

Determination of Cytotoxicity

The cytotoxicity of crude ethanol and watery extracts of the sample was investigated by using brine shrimp lethality bioassay according to the procedure described by Dockery and Tomkins, 2000. The brine shrimp (*Artemia salina*) was used in this study for cytotoxicity bioassay (Ali *et al*., 2016). Brine shrimp cysts (0.5 g) were added to the 1.5 L of artificial sea water bottle. This experiment was carried out at the Department of Chemistry, Yangon University, Myanmar. The suspension was aerated by bubbling air into the funnel and kept for 24 h at room temperature. After aeration had been removed, the suspension was kept for 1 h undisturbed, whereby the remaining unhatched eggs dropped. In order to get a higher density of larvae, one side of the separating funnel was covered with aluminium foil and the other illuminated with a lamp, whereby the phototropic larvae were gathering at the illuminated side and could be collected by pipette. The shrimp larvae were transferred to an agar well filled with 9 ml of salt water and the dead larvae counted (number N). A solution of crude extract (1, 10, 100, 1000 ppm) (1 mL) was added and the plate kept at room temperature in the dark. After 24 h, the dead larvae were counted in each well under the microscope (number A). The still living larvae were killed by addition of ca. 0.5 mL methanol so that subsequently the total number of the animals could be determined (number G).The control solution was prepared as the above procedure by using distilled water instead of sample solution. The mortality rate M was calculated in %. Each test row was accompanied by a brine solution (number B). The mortality rate M was calculated by using the following formula:

$$M = \left[\frac{(A - B - N)}{(G - N)} \right] \times 100$$

M = percent of the dead larvae after 24 h

A = number of the dead larvae after 24 h

B = average number of the dead larvae in the brine solution after 24 h

N = number of the dead larvae before starting of the test.

G = total number of brine shrimps

Screening of Antitumor Activity of the Various Crude Extracts from the Stems of *A. cathartica*

The antitumor activity of ethanol, ethyl acetate and petroleum ether extracts from the stems of *A. cathartica* was examined by Potato Crown Gall (PCG) or Potato Disc Assay (PDA) method (Coker *et al.,* 2003) at the Fermentation Laboratory, Pharmaceutical Research Department, Ministry of Industry, Yangon, Myanmar.

Fresh disease-free potatoes were purchased from a local market. Tubers of moderate size were surface sterilized by immersion in 0.1 % sodium hypochlorite for 20 min. Ends were removed and the potatoes were soaked an additional 10 min. A core of the tissue was extracted from each and discarded. The remainder of the cylinder was cut into 1.0 cm thick discs with a

surface sterilized scalpel. The discs were then transferred to agar plates (1.5 g of agar dissolved in 100 mL deionized distilled water, autoclaved for 20 min at 121 ºC, 20 mL poured into each Petri dish). Each plate contained four potato discs and 4 plates, were used for each of the sample solution.

Sample (0.05, 0.10, 0.15 g) was individually dissolved in DMSO (1 mL) and filtered through Millipore filters (0.22 μm) into sterile tube. This solution (0.5 mL) was added to sterile distilled water (1.5 mL), and broth culture of *A. tumefaciens* in PBS (2 mL) was added. Controls were made in this way; DMSO (0.5 mL) and sterile distilled water (1.5 mL) were added to the tube containing 2 mL of broth culture of *A. tumefaciens.* By using a sterile disposal pipette, 1 drop (0.05 mL) each from these tubes was used to inoculate each potato disc by spreading it over the disc surface. After inoculation, Petri dishes were sealed by film and incubated at 27~30 ºC for 3 days. Observation was made on appearance of tumors on potato discs after 3 days under stero-microscope followed by staining with Lugol's iodine (10 % KI and 5 % I_2) after 30 min and compared with control. The antitumor activity was examined by observation of tumor produced or not on the potato discs. The results are shown in Table 4.

Determination of Antiproliferative Activity

Antiproliferative activity of ethanol and watery extracts were investigated in *in vitro* by using cancer cell lines at Division of Natural Product Chemistry, Institute of Natural Medicine, and University of Toyama, Japan. The cell lines used were Hela (human cervix cancer), A 549 (human ung cancer) and MCF 7 (human breast cancer). K562 α-Minimum essential medium with L-glutamine and phenol red (α -MEM, Wako) were used for cell cultures. All media were supplemented with 10 % fetal bovine serum (FBS, sigma) and 1 % antibiotic antimycotic solution (Sigma). For MCF 7 cell, 1 % 0.1 M non-essential amino acid (NEAA, Gibco) and 1 % 1 mM sodium pyruvate (Gibco) were also supplemented. The *in vitro* antiproliferative activity of the crude extracts was determined by the procedure described by Win *et al.* (2015). Briefly, each cell line was seeded in 96-well plates (2×10^3 per well) and incubated in the respective medium at 37 °C under 5 % CO_2 and 95 % air for 24 h. After the cells were washed with PBS (Nissui Pharmaceuticals), serial dilutions of the tested samples were added. After 72 h incubation, the cells were washed with PBS and 100 μL of medium containing 10 % WST-8 cell counting kit (Dojindo; Kumamoto, Japan) solution was added to the wells. After 2 h incubation, the absorbance at 450 nm was measured. The concentrations of the crude extracts were 200, 100, 10 μg/ mL and 10, 1, 0.1 mM for positive control were prepared by serial dilution. Cell viability was calculated from the mean values of the data from three wells using the equation below and antiproliferative activity was expressed as the IC_{50} (50 % inhibitory concentration) value. 5-fluorouracil (5FU) was used as positive control.

$$(\%)\ \text{Cell viability} = 100 \times \frac{\left\{\text{Abs}_{(\text{test samples})} - \text{Abs}_{(\text{blank})}\right\}}{\left\{\text{Abs}_{(\text{control})} - \text{Abs}_{(\text{blank})}\right\}}$$

Results and Discussion

Types of Phytochemicals Present in the Stems of *A. cathartica*

In order to find out the types of phytochemical constituents present in the stems of *A. cathartica*, the phytochemical tests were preliminary carried out according to the reported procedure. From the data findings, it was observed that various secondary metabolites such as alkaloids, flavonoids, terpenoids, steroids, glycosides, organic acids, starch, phenolic compounds, saponins and tannins together with starch and carbohydrates were present, however cyanogenic glycosides, reducing sugars and α- amino acids were not detected in the samples. According to these results, it can be seen that the roots samples might contain potent bioactive secondary metabolites.

Some Nutritional Values of the Stems of *A. cathartica*

The nutritional values were determined by AOAC method resulting 6.91 % of proteins, 2.04 % of moisture, 2.71 % of ash, 62.42 % of fiber and 10.33 % of fat and 5.7 % of carbohydrate in the stems of *A. cathartica.* According to these results, the root samples were found to be high in fiber content. Fiber is a type of carbohydrate that the body cannot digest. Though most carbohydrates are broken down into sugar molecules, fiber cannot be broken down into sugar molecules, and instead it passes through the body undigested. Fiber helps to regulate the body's use of sugars, helping to keep hunger and blood sugar in check.

Total Phenol Contents (TPCs) of the Crude Extracts of the Stems of *A. cathartica*

The total phenol content of the ethanol and watery extracts of the stems were determined with spectrophotometric method by using Folin-Ciocalteu reagent. The total phenol content of the ethanol extract was 31.98 ± 0.94 mg GAE/g those of watery extract was 18.91± 0.53 mg GAE/g. The results are shown in Table 1.

Total Flavonoid Contents (TFCs) of the Crude Extracts of the Stems of *A. cathartica*

The total flavonoid content of the ethanol and watery extracts of the stems were determined with spectrophotometric method by aluminium chloride reagent. The total flavonoid contents of ethanol and watery extracts of the stems were found to be 226.67 ± 8.50 mg QE/g and 100.67 ± 9.29 mg QE/g respectively. The results are shown in Table 1.

Total Steroid Contents (TSCs) of the Crude Extracts of the Stems of *A. cathartica*

The total steroid content of the ethanol and watery extracts of the stems were determined by Zak's method were found to be 278.81 ± 7.70 mg CE/g and 154.72 ± 13.31 mg CE/g of crude extracts, respectively. The results are shown in Table 1.

The Total Condensed Tannin Contents (TCTCs) of the Crude Extracts of the Stems of *A.cathartica*

The total condensed tannin in the ethanol and watery extracts estimated by Broadhurst's method were found to be the same content of 317.5 ± 0.00 mg TAE/g and 25.83 ± 14.4 mg TAE/g, respectively. The results are shown in Table 1.

Table 1 Total Phenol Contents (TPCs), Total Flavonoid Contents (TFCs), Total Steroid Contents (TSCs) and Total Condensed Tannin Contents (TCTCs) of Crude Extracts

Types of compounds	EtOH extracts	Watery extracts
TPCs (mg GAE ± SD)/g of extract	31.98 ± 0.94	18.91 ± 0.53
TFCs (mg QE ± SD)/g of extract	226.67 ± 8.50	100.67 ± 9.29
TSCs (mg CE ± SD)/g of extract	278.81 ± 7.70	154.72 ± 13.31
TCTs (mg TAE ± SD)/g of extract	317.5 ± 0.00	25.83 ± 14.40

Identification of the Compounds Isolated from the EtOAc Extract from the Stems of *A. cathartica*

From the silica gel column chromatographic separation of EtOAc extract of the stems, one compound was isolated; compound A as white crystal in 1.51 % of yield based on EtOAc extract. Melting point of compound A was found to be 151°C and its R_f value was observed to be 0.6 (n-hexane: EtOAc, 3:1 v/v). Compound A is soluble in chloroform, methanol and ethyl acetate but insoluble in pet-ether, ethanol and acetone. It was isolated as a flavonoid compound containing phenolic -OH group.

Compound A was then structurally identified by using 1D and 2D NMR and EI-MS spectroscopies. The integration of ^{1}H NMR (500 MHz, $CDCl_3$) spectrum (Figure 1) indicated the presence of only six protons in the compound A. The two doublet signals with coupling constant of 10 Hz appeared at δ_H 8.00 ppm and the two doublet signals with coupling constant of 10 Hz appeared at δ_H 6.89 ppm showed that there were four methine groups. The singlet signals appeared at δ_H 6.15 ppm and 6.39 ppm indicated the presence of two methine groups.

According to the ^{13}C NMR (125 MHz, $CDCl_3$) spectrum (Figure 2) of the isolated compound A, it was found that there were fifteen carbons including six methine carbons at the chemical shifts of δ_C 130.04, 130.04, 115.96, 115.96, 98.72 and 94.05 ppm, eight quartenary carbons at δ_C 164.4, 161.25, 159.71, 156.71, 147.32, 136.19, 122.21 and 103.58 ppm and one carbonyl group at δ_C 176.42 ppm.

The C and H ($^{1}J_{C\text{-}H}$) one bond correlation of A is described in HMQC spectrum (Figure 3) and Table 2.

According to mass spectrum (Figure 4), the molecular weight of the isolated compound A was found to be m/z 286. From NMR spectral data, it could be assumed that there were 15 carbons, 6 protons, one carbonyl group and hydroxyl group (more than one). If this compound contains two hydroxyl groups, the partial molecular formula is $C_{15}H_8O_4$ with molecular weight of m/z 252. The remaining molecular weight of m/z was 34. Consequently, the isolated compound must contain another two oxygen atoms as two hydroxyl groups. The complete structural formula of this isolated compound must be assigned as $C_{15}H_{10}O_6$ with the molecular weight 286.

The correlation between the protons directly attached to the carbons, i.e., types of carbons (methyl, methine and quarternary carbons) was studied by HMQC spectrum (Figure 3). Furthermore, the long range proton-carbon correlation was also examined by using 2 D HMBC spectrum (Figures 5, 6, 7 and 8).

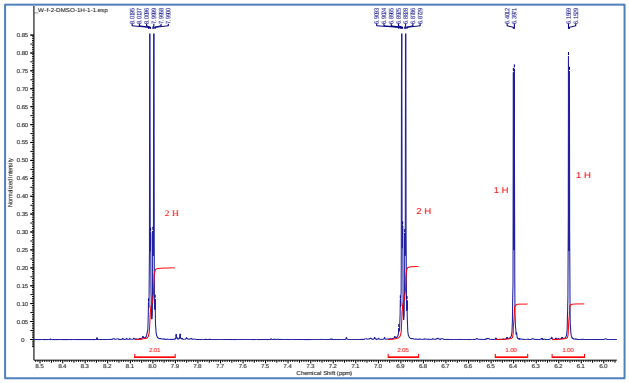


Figure 1 ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of the isolated compound

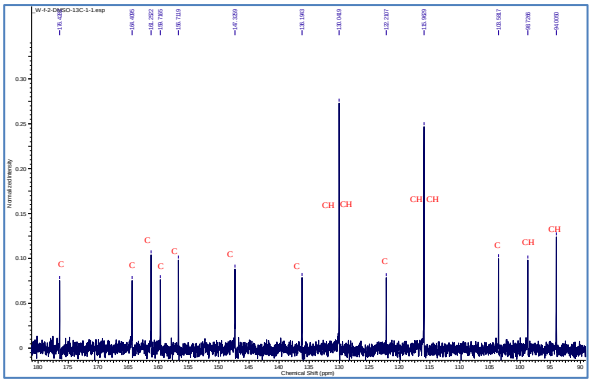


Figure 2 ^{13}C NMR spectrum (125 MHz, $CDCl_3$) of the isolated compound

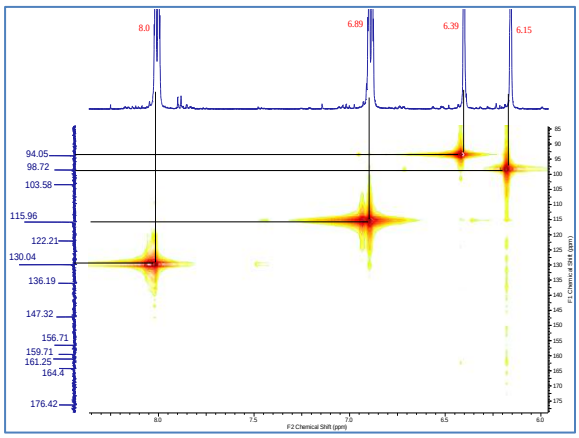


Figure 3 HMQC spectrum (500 MHz, $CDCl_3$) of the isolated compound A

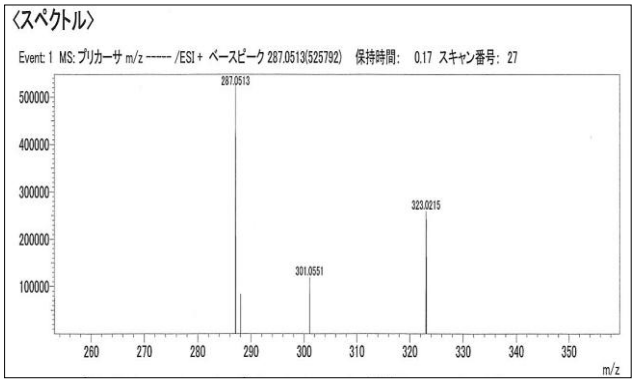


Figure 4 Mass spectrum of the isolated compound YYT-2

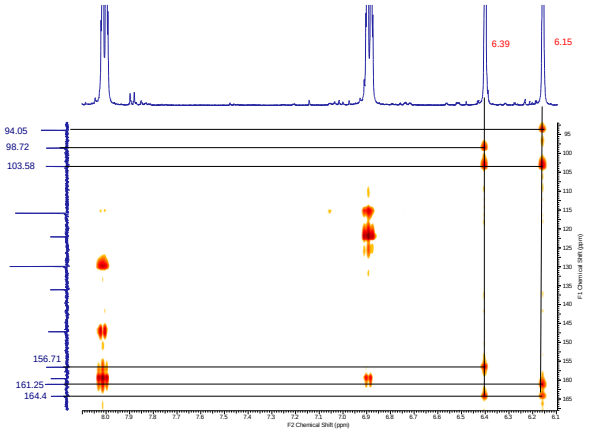


Figure 5 MBC spectrum of the isolated compound A

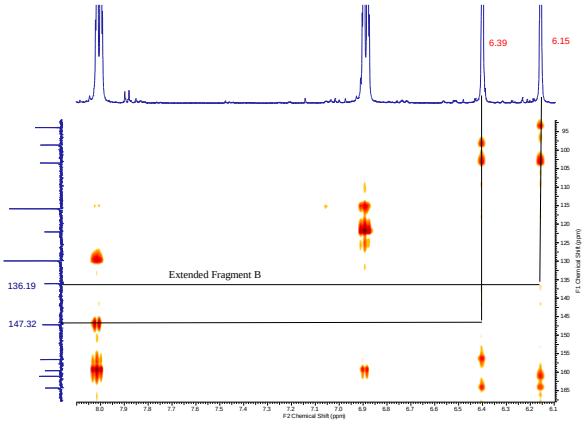


Figure 6 HMBC spectrum of the isolated compound A

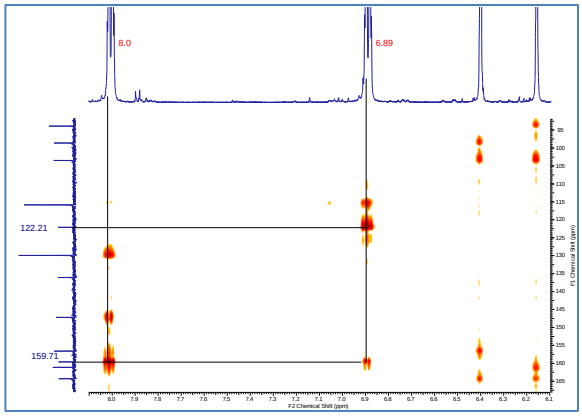


Figure 7 HMBC spectrum of the isolated compound A

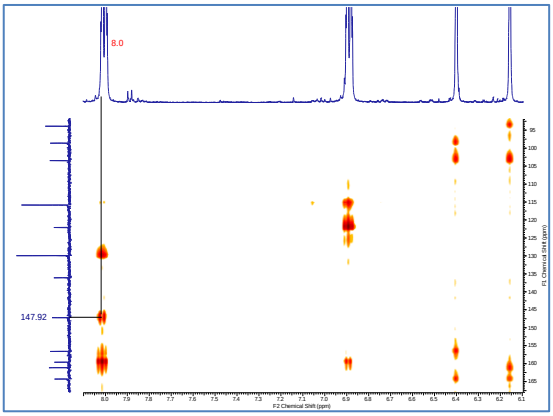
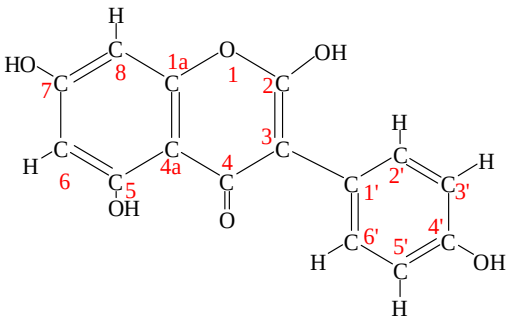


Figure 8 HMBC spectrum of the isolated compound A

Table 2 ^{1}H and ^{13}C NMR (500 MHz, $CDCl_3$ and 125 MHz, $CDCl_3$) and HMQC Assignment of Isolated Compound A

δ_C, type	δ_H, (*J* in Hz)
176.42, C=O	-
164.4, C-OH	-
161.25, C	-
159.71. C-OH	-
156.71, C-OH	-
147.32, C	-
136.19, C-OH	-
130.04, CH	8.00, d (10)
130.04, CH	8.00, d (10)
122.21	-
115.96, CH	6.89, d (10)
115.96, CH	6.89, d (10)
103.58	-
98.72, CH	6.15, s
94.05, CH	6.39, s

Finally, the structure of the isolated compound A was assigned as 2, 5, 7 - trihydroxy - 3 - (4 - hydroxyphenyl) - 4H - chromen - 4 – one.



2, 5, 7 - trihydroxy - 3 - (4 - hydroxyphenyl) - 4H - chromen - 4 – one

Antioxidant Activity of the Stems of *A. cathartica*

The antioxidant activity was measured in terms of hydrogen donating or radical scavenging ability of the EtOH and watery extracts of the samples by using the stable radical DPPH. The radical scavenging activity of standard gallic acid is shown in Table 3 and Figure 9. The results are shown in Table 4, Figure 10 and 11. From these observations, the radical scavenging activity of EtOH extracts (IC_{50} = 10.97 μg/mL) was found to be greater than watery extracts (IC_{50} = 68.37 μg/mL).

Table 3 Radical Scavenging Activity of Standard Gallic Acid

Sample	% RSA ± SD at Different Concentration (μg/mL)					IC_{50} (μg/mL)
	0.625	1.25	2.5	5	10	
Gallic Acid	25.20 ± 1.40	53.58 ± 0.88	65.53 ± 1.13	74.82 ± 0.59	94.59 ± 0.48	1.17

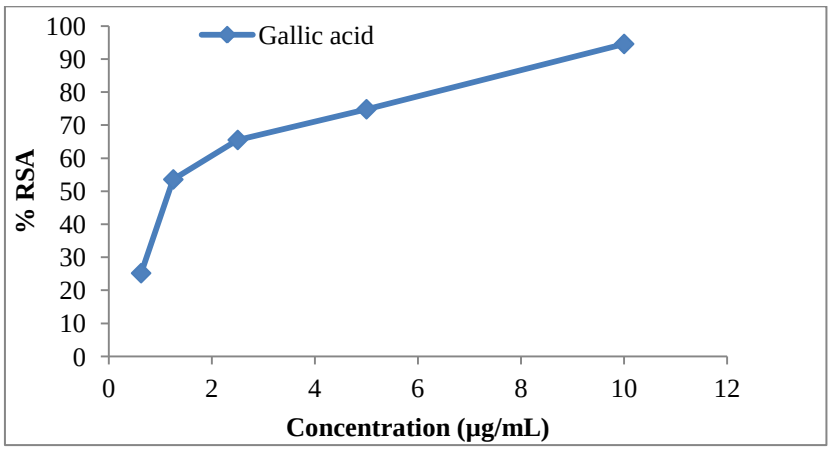


Figure 9 A plot of % DPPH free radical scavenging activity *vs* different concentrations of standard gallic acid

Table 4 % Radical Scavenging Activity and IC_{50} Values of Crude Extracts of the Stems of *A. cathartica* by DPPH Radical Scavenging Assay

Extracts	% RSA ± SD at Different Concentration (μg/mL)						IC_{50} (μg/mL)
	6.25	12.5	25	50	100	200	
Ethanol (Stems)	22.39 ± 0.00	51.49 ± 0.00	58.96 ± 0.02	79.10 ± 0.00	81.34 ± 0.00	95.52 ± 0.00	10.97
Watery (Stems)	4.25 ± 0.63	12.53 ± 1.42	23.04 ± 3.32	39.15 ± 0.16	68.68 ± 1.58	98.66 ± 0.95	68.37

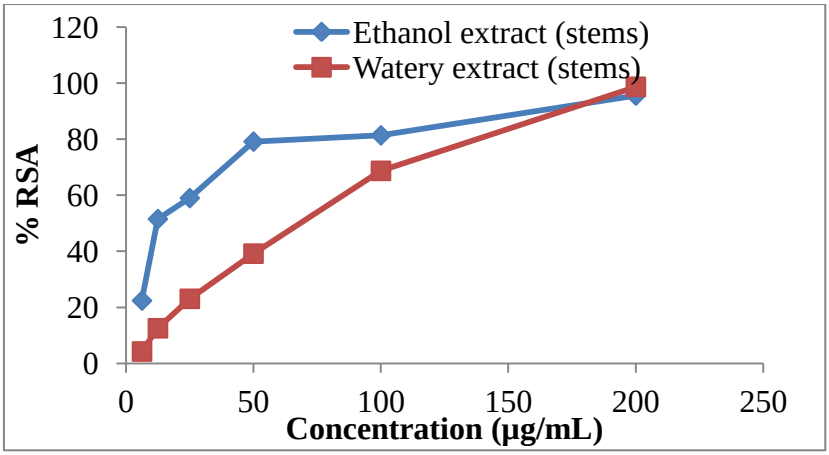


Figure 10 A plot of % DPPH free radical scavenging activity *vs* different concentrations of crude extracts of the stems of *A. cathartica*

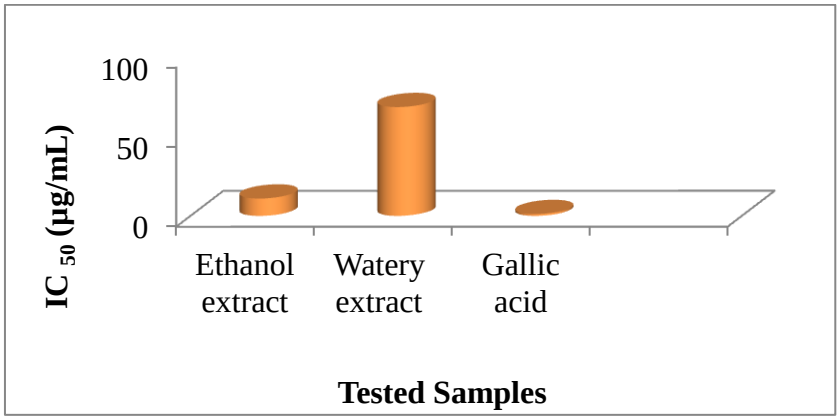


Figure 11 A bar graph of IC_{50} values of stems of *A. cathartica* (DPPH free radical scavenging assay)

Antimicrobial Activity of Crude Extracts of the Stems of *A. cathartica*

Four crude extracts such as PE, EtOAc, EtOH and water extracts from the stems of *A. cathartica* were subjected to screening of antimicrobial activity against six different pathogenic microbes such as *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus pumilus, Candida albicans and Escherichia coli* using agar well diffusion method. This method is based on zone diameter including the well diameter, in millimeter (mm). The larger the zone diameter, the higher the activity is. According to the results, PE, EtOH and EtOAc extracts of stems showed mild antimicrobial activities against all tested microorganisms whereas watery extract of the stems did not show antimicrobial activity against *P. aeruginosa* and *E. coli*. The resulted data are shown in Table 5.

Table 5 Inhibition Zone Diameters of Various Extracts of the Stems of *A. cathartica* against Six Microorganisms by Agar Well Diffusion Method

No.	Microorganisms	Inhibition Zone Diameters (nm) of Different Crude Extracts			
		PE	EtOAc	EtOH	Watery
1	*Bacillus subtilis*	13 (+)	14 (+)	16 (++)	13 (+)
2	*Staphylococcus aureus*	13 (+)	15 (++)	14 (+)	14 (+)
3	*Pseudomonas aeruginosa*	18 (++)	18 (++)	15 (++)	-
4	*Bacillus pumilus*	12 (+)	13 (+)	14 (+)	15 (++)
5	*Candida albicans*	12 (+)	14 (+)	15 (++)	15 (++)
6	*Escherichia coli*	14 (+)	14 (+)	14 (+)	-

Agar well – 10 mm, 10 mm ~ 14 mm (+), 15 mm ~ 19 mm (++), 20 mm and above (+++)

Cytotoxicity of the Ethanol and Watery extracts of the Stems of *A. cathartica*

The cytotoxicity of water and ethanol extracts from the sample was evaluated by brine shrimp cytotoxicity bioassay. This bioassay is general toxicity screening for bioactive phytoconstituents and their derivatives. A model animal that has been used for this purpose is the brine shrimp, *Artemia salina* (Tawaha, 2006). The cytotoxicity of crude extracts was expressed in term of mean ± SEM (standard error mean) and LD_{50} (50% Lethality Dose) and the results are shown in Table 6. In this experiment, standard potassium dichromate ($K_2Cr_2O_7$) and caffeine were chosen because $K_2Cr_2O_7$ is well-known toxic in this assay (Salinas and Fernendez, 2006) and caffeine is a natural product. According to the results of brine shrimp cytotoxicity bioassay, all of the tested samples have cytotoxic effect.

Table 6 Cytotoxicity of Ethanol and Watery Crude Extracts of the Stems Samples

Crude extracts	Dead % by using different concentrations (μg/mL) of samples				LD_{50} (μg/mL)
	1	10	100	1000	
EtOH	40.00 ±17.32	90.30 ± 0.53	100±0.00	100 ± 0.00	2.79
Watery	48.83 ±14.95	80.65 ± 6.01	82.35 ±10.04	84.83 ± 5.29	1.34
*Caffeine	0 ± 0	0 ± 0	9.582 ±0.917	12.73 ±4.103	>1000
*$K_2Cr_2O_7$	48.63 ±19.19	73.13 ±4.076	74.67 ±11.8	100 ± 0	1.5

*****standard

Antitumor Activity of the Stems of *A. cathartica*

Antitumor activity in this study was investigated by potato crown gall (PCG) assay as it is a valuable tool that indicated antitumor activity of the tested samples by their inhibition of the crown gall formation that was induced in wounded potato tissues by *Agrobacterium tumefaciens*.

Table 7 Tumor Inhibition by the Crude Extracts from the Stems of *A. cathartica*

Test Samples	Extracts/Compound	Concentrations of Samples (g/mL/disc)	Tumor Inhibition Activity
Control	-	0.00	-
Stem	PE	0.05	+
	PE	0.10	+
	PE	0.15	+
	EtOAc	0.05	+
	EtOAc	0.10	+
	EtOAc	0.15	+
	EtOH	0.05	+
	EtOH	0.10	+
	EtOH	0.15	+

(+) antiumor activity present, (-)antiumor activity absent

It could be clearly seen from the Table 7 that, all of the samples inhibited tumor growth in a concentration dependent manner. Significant tumor inhibition was observed at the concentrations of 0.05, 0.10 and 0.15 g/mL/disc.

Since, tumor inhibition significantly occurred by the extracts of the stems of *A. cathartica* on potato discs, it could be concluded that *A. cathartica* might be used as a potential source of antitumor agent.

Antiproliferative Activity of the Stems of *A. cathartica*

Antiproliferative activity was studied in vitro using human cancer cell lines. Screening of antiproliferative activities of ethanol and watery extracts from the stems of *A. cathartica* was done by using three human cancer cell lines such as A 549 (human lung cancer), MCF7 (human breast cancer) and Hela (human cervix cancer). Antiproliferative activity was expressed as the IC_{50} (50 % inhibitory concentration) value. 5-Fluorouracil was used as positive control. The antiproliferative activity of crude extracts is summarized in Table 8. Ethanol and watery extracts of stems did not show antiproliferative activity. Compound A was found to possess antiproliferative activity against lung cancer (A 549) (IC_{50} = 41.7 µg/mL), breast cancer cell (MCF 7) (IC_{50} = 47.9 µg/mL) and cervix cancer cell (Hela) (IC_{50} = 8.91 µg/mL).

Table 8 Antiproliferative Activities of the Crude Extracts and the Isolated Compound A of *A. cathartica*

Samples	IC_{50} (µg/mL) of Various Samples against Tested Cell Lines		
	A 549	MCF 7	Hela
EtOH extract	>100	>100	>100
Watery extract	>100	>100	>100
Compound A	41.7	47.9	8.91
*5FU	10.2	11.5	6.93

A 549 = Lung cancer cell lines; MCF = Breast cancer cell lines; Hela = Cervix cancer cell lines; *5FU = 5 Fluorouracil

Conclusion

From the overall assessment concerning with the investigation of phytochemicals and biological activities on the stems of *A. cathartica*, the following inferences could be deduced. One organic compound, 2, 5, 7 - trihydroxy - 3 - (4 - hydroxyphenyl) - 4H - chromen - 4 – one (0.22 %, mpt. 151 °C) was isolated from ethyl acetate crude extract of the stems by using silica gel column chromatographic separation technique. The ethanol extract possessed greater antioxidant activity than that of the watery extract. According to the antimicrobial activities, PE, EtOH and EtOAc extracts of stems showed mild antimicrobial activities against all tested microorganisms whereas watery extract of the stems did not show antimicrobial activity against *P. aeruginosa* and *E. coli.* Furthermore EtOAc, PE and EtOH extracts of stems were also found to inhibit the formation of tumor. However, ethanol and watery extracts did not show antiproliferative activitiy, compound A showed antiproliferative activity against A 549 (41.7 µg/mL), MCF 7 (47.9 µg/mL) and Hela (8.91 µg/mL).

In conclusion, antimicrobial, antioxidant and antitumor activities of different extracts obtained from the stems of *A. cathartica* grown in Myanmar could be evaluated. In conclusion, the chemical constituents in ethanol extract of the stems were found to be rich comparing with other extracts. Among the chemical constituents, total condensed tannin contents were observed to be highest in ethanol extract. The second and third highest chemical constituents were found to be the steroid and flavonoid compounds. Flavonoid and tannin compounds can prevent the development of bacteria and can be converted to inactive substances. Among the nutritional constituents of both of the sample powder, the fiber content was observed in highest amount. Fiber is a type of carbohydrate that the body cannot digest. Though most carbohydrates are broken down into sugar molecules, fiber cannot be broken down into sugar molecules, and instead it passes through the body undigested. Fiber helps to regulate the body's use of sugars, helping to keep hunger and blood sugar in check. The ethanol extract of stems showed the distinct higher activities such as antioxidant activity than the watery extract. Therefore, the present study will contribute that the stems of *A. cathartica* can be used in the traditional medicinal formulation for the treatment of many diseases.

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