PHENOLIC CONTENT, ANTIOXIDANT AND ANTITUMOUR ACTIVITIES AND ISOLATION OF A PHENOLIC COMPOUND FROM THE BARK OF *ALSTONIA SCHOLARIS* L. (TAUNG-MA-YO)

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

Abstract

The present work focused on the determination of total phenol content, antioxidant activity and screening of anti-tumour activity of some crude extracts of the bark of *Alstonia scholaris* L. (Taung-ma-yo). Moreover, the research deals with the isolation of a phenolic compound by column chromatography and identification of the isolated compound by UV and FT IR. The total phenol contents of crude extracts of 70 % ethanol (99.33 μg GAE/mg) and water (61.5 μg GAE/mg) were determined by spectrometric Folin-Ciocalteu reagent (FCR) method .The antioxidant activity of 70 % ethanol extract (IC_{50} value of 27.75 μg/mL) and water extract (IC_{50} value of 90.56 μg/mL) of the bark sample was determined by DPPH assay method. Preliminary screening of anti-tumour activity of crude extracts of ethyl acetate, 70 % ethanol and water was carried out by Potato Crown Gall test. It was observed that, of the three doses, i.e., 0.05, 0.1 and 0.15 g/disc each extract tested, the minimum dose showing activity was 0.15 g for ethyl acetate extract and 0.05 g for 70 % ethanol extract on day 5. On day 7, tumours appeared for the ethyl acetate extract but not for the 70 % ethanol extract as observed under microscope and by staining with iodine solution. Column chromatographic separation of ethyl acetate extract by gradient elution with pet ether, ethyl acetate and methanol yielded a compound (A_1) having R_f value of 0.45 on silica gel layer with pet ether : ethyl acetate (2.5 : 7.5, v/v). UV spectra of isolated compound (A_1) showed that it was to be a phenolic compound by alkaline red shift and also supported by FT IR spectrum.

Keywords *: Alstonia scholaris* L., phenol content, anti-oxidant activity, anti-tumour activity

Introduction

Herbal medicine contains natural substances that can promote health and reduce illness. There are many herbs, which are used to treat cardiovascular problems, liver disorders, central nervous system, digestive and metabolic disorder. The plant, *Alstonia scholaris* L., invites attention of the researcher worldwide for its pharmacological activities ranging from antimalarial to anticancer activities (Bhanu *et al*., 2013).

A.scholaris (Taung-ma-yo) belonging to family Apocynaceae, is a medium to large tree. It has wide occurrence in the Asia Pacific region from India, Srililanka, through mainland South East Asia and Southern China, throughout Malaysia to Northern Austria and Salomon island (Anubha and Yashwant, 2015). *A. scholaris* contains some of the important alkaloids such as echitamine, tubotaiwine, akuammicine, echitamidine, picrinine (Kalaria *et al*., 2012). The bark of the *A. scholaris* is used in Ayurvedic medicine to treat fever, malaria, troubles in digestion, tumors, ulcers, asthma and so forth.

Some biological properties of *A.scholaris* such as antimicrobial, antidiarrheal, antimalarial, anticancer, and antioxidant activities have been studied (Baliga, 2010). The present study was performed to investigate phenolic content, antioxidant and antitumour activities from bark of *A. scholaris*. The presence of alkaloids, phenolic compounds and flavonoids have also

[1] Lecturer, 1 PhD, Department of Chemistry, Dagon University
[2] Dr, Lecturer, Department of Chemistry, Dagon University
[3] Dr, Part time Professor, University of Yangon

been reported by many workers. A phenolic compound was also isolated and characterized especially in this study.

The report may be divided into five portions, (i) preparation of different crude extracts (ii) determination of phenolic content (iii) investigation of antioxidant activity and antitumour activity (iv) isolation of compounds (v) identification of an isolated compound.

Materials and Methods

Collection and Preparation of Plant material

The bark of *Alstonia scholaris* L. was collected from Kamayut Township, Yangon Region. The bark samples were transformed into powder and stored in air-tight container.

Preparation of Crude Extracts

The dried powdered sample (50 g) was percolated in 70 % ethanol about 3 days. After 70 % ethanol extract was filtered, again percolated and filtered in this solvent, until the extracted sample become faint.

The collected filtrates (70 % ethanol extract) obtained were concentrated by rotatory evaporator to get concentrated 70 % ethanol residue. Some ethanol extract was used to determine the activities. The other ethanol extract was then partitioned with ethyl acetate about five times. Resultant ethyl acetate extracts were concentrated and used to isolate some compounds.

Some dried powdered sample was also extracted in warm water and boiled in water bath for one hour. After boiling, water extract was filtered and dried. Watery extract were used to detect some activities.

Preliminary Phytochemical Test

A few grams of dried bark powder of *A. scholaris* was subject to the tests of alkaloids, α-amino acids, carbohydrates, flavonoids, glycosides, phenolic compounds, reducing sugars, saponins, starch, steroid, tennis and terpenoids according to the standard procedures (Marini-Bettolo *et al*.,1981).

Preparation of Solution and Determination of Total Phenol Content

The amount of total phenolics in ethanol and water extracts was determined by the spectrometric Folin-Ciocalteu reagent method. Gallic acid was used as a standard and the total phenolics were expressed as μg/mL gallic acid equivalents. For this purpose, the calibration curve of gallic acid was drawn (Figure 1).Standard solution having concentrations (3.125, 6.25, 12.5, 25, 50, 100 μg/mL) of gallic acid were prepared. The sample solutions (70 % EtOH and water extract) were also prepared by dissolving 10 mg of crude extracts in 10 mL of ethanol. Each sample (1 mL) was introduced into test tube and mixed with 5 mL of a 10 fold dilute Folin-Ciocalteu reagent 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 2 h. Absorbance of standard solution and sample solutions was measured at λ_{max} 765nm (Vance *et al*., 2003).

Screening of Antioxidant activity

DPPH (1,1-diphenyl-2-picryl-hydrazyl) radical scavenging assay was chosen to assess the antioxidant activity of plant materials. This assay has been widely used to evaluate the free radical scavenging effectiveness of various flavonoids and polyphenol in food systems (Lee and Shinbamoto, 2001 ; Thaw Thaw Zin, 2016).

Different concentrations (3.125, 6.25, 12.5, 25, 50, 100 and 200 μg/mL) of Standard Ascorbic Acid solution and sample (crude extracts of 70% ethanol and water) solutions were prepared. The concentrations of the solutions were serially diluted with appropriate amount of ethanol. DPPH (0.002 %) solution was also prepared by dissolving DPPH (0.002 g) in 100 mL ethanol.

The control solution was prepared by mixing 1.5 mL of 0.002 % DPPH solution and 1.5 mL of 95 % ethanol using vortex mixer. The sample solutions and standard solution were also prepared by mixing thoroughly 1.5 mL of 0.002 % DPPH solution and 1.5 mL for each concentration of test sample solutions and standard solution respectively. The solutions were allowed to stand at room temperature for 30 min. After 30 min, the absorbance of these solutions was measured at 517 nm by using UV spectrophotometer. Absorbance measurements were done in triplicate for each solution and then mean values so obtained were used to calculate percent inhibition of oxidation by the following equation (Tomoko, 1998).

$$\text{Percent Inhibition} = \frac{A_{control}(A_{sample} - A_{blank})}{A_{control}} \text{ x } 100\,\%$$

Preliminary Screening of Anti-tumour Activity

The antitumour activity screening of different crudes such as ethyl acetate, 70 % ethanol, and watery extracts of bark of *A. scholaris* was carried out against *Agrobacterium tumefacien* by Potato Crown Gall test of Potato Disc Assay method at the Pharmaceutical Research Department Ministry of Industry, Yangon, Myanmar. Fresh, disease free potato tubers were obtained from local market and transferred within 48 h to the laboratory. Tubers of moderate sizes were surface-sterilized by immersion in 50 % sodium hydrochlorite (Clorox) for 20 min. The ends were removed and soaked for 10 min in Clorox. A core of the tissue was extracted from each tuber by using surface-sterilized (ethanol and flame) 1.5 cm wide cork borer. And, 2 cm pieces were removed from each end and discarded and the remainder of the cylinder is cut into 0.5 cm thick discs with a surface-sterilized cutter. The discs were then transferred to 1.5 % agar plates (1.5 g of petri dish). Each plate contained three discs. The procedure was done in the clean bench in the sterile room, 50 mL, 100 mL and 150 mL of this solution of each extract was separately dissolved in 2 mL of dimethyl sulphoxide (DMSO); the solution was filtered through Millipore filters into a sterile tube 0.5 mL of this solution was added to 1.5 mL of sterile distilled water and 2 mL of broth culture of *A. tumefaciens* strain (48 h culture containin 3.5×10^9 cells/mL) were added aseptically. Controls were made as mentioned above procedure.

Using a sterile disposable pipette, 1 drop (0.05 mL) from these tubes was used to inoculate each potato disc, spreading it over the disc surface. The process of cutting the potatoes and incubation must be conducted within 30 min. the plates were sealed with tape to minimize moisture loss and incubated at room temperature for 12 days. After incubation, Lugol's solution (I_2-KI) was added and the tumors were counted with a microscope and compared with control.

The antitumor activity was examined by observation of tumor produced or not within 5 days and 7 days (Priestman and Edwards, 1953).

Extraction and Isolation of Bioactive Compound

The crude extracts from the bark of *A. scholaris* were obtained by percolating with 70 % ethanol and then further partitioning with ethyl acetate.

For the clear separation of bioactive compounds from the ethyl acetate extract was done by thin layer chromatography, different ratios of pet ether and ethyl acetate solvent systems were used. The visualization of separated constituents was examined under UV_{254} and UV_{365} nm, and by spraying with 5 % $FeCl_3$ solution.

Individual constituents from ethyl acetate extract were isolated by column chromatography. A column (diameter 1.84 cm and height 28 cm) was packed using 0.7 g of ethyl acetate extract and 25 g of silica gel. The gradient elution solvents were pet ether and ethyl acetate from the ratio (2.5 : 7.5) to ethyl acetate and methanol (9:1).

Results and Discussions

Pytochemicals constitute one of the most numerous and widely distributed groups of substances in the plant kingdom. Plants produce chemicals known as secondary metabolites that are not directly involved in the process of growth but acts as deterrents to insects and microbial attack. In the present work, phytochemical tests were carried out by test tube methods. It was observed that alkaloids, ∝-amino acids, carbohydrates, flavonoids, glycosides, phenolic compounds, reducing sugars, saponins, steroids, tennins and terpenoids were present and starch was absent in the bark sample. Therefore, the bark of *A.scholaris* is rich in phytochemicals.

Phenolic compounds are a class of antioxidant agents which act as free radical terminators and their bioactivities may be related to their abilities to chelate metals, inhibit lipoxygenase and scavenge free radicals (Karamian and Ghasemlou, 2013). The amount by total phenol was determined with the Folin-Ciocalteu reagent. Gallic acid was used as a standard compound and the total phenols were expressed as μg GAE/mg using the standard curve equation : $y=0.0068x + 0.168$, $R^2 = 0.9993$, where y is absorbance at 760 nm and x is total phenolic content in the extracts of *A.scholaris* expressed in mg/mL. Table 1 and Figure 1 show the variation of mean absorbance with concentration of Gallic acid. Table 2 shows the contents of total phenols that were measured by Folin Ciocalteu reagent in terms of gallic acid equivalent. The total phenol contents were found in 70 % ethanol extract (99.33 μg GAE/mg) and watery extract (61.5 μg GAE/mg).

The efficiency of antioxidant potential of different extracts depends on its ability to scavenge free radicals either by donating hydrogen atom to the oxidizing free radical or decrease the energy of the antioxidant radical that prevents the autooxidation of the antioxidant radical into additional free radicals. Therefore in order to assess the antioxidant potential of extracts of *A. scholaris*, total antioxidant capacity was determined. In the present study, 70 % ethanol extract was observed to have significant (IC_{50}- 27.75 μg/mL) higher antioxidant activity value as compared to watery extract (IC_{50}- 90.56 μg/mL) of *A. scholaris*. The results of radical scavenging activity and IC_{50} values of standard Ascorbic Acid, 70 % ethanol and watery extracts of bark of *A. scholaris* are shown in Table 3 and Figure 2.

The antitumour activity screening of different crude extracts (ethyl acetate, 70 % ethanol, water)of the bark of *A. scholaris* was carried out against *Agrobacterium tumefaciens* by Potato Crown Gall test or Potato Disc Assay method. The results are given in Table 4. Among the results, 70 % ethanol extract inhibited (0.05 g to 0.15 g during 5 days and 7 days) against the formation of tumour cell.

The ethyl acetate crude extract was fractionated by column chromatography. Gradient elution by increasing polarity using pet ether, ethyl acetate and methanol mixture was performed. One of the combined fractions (14-70) having R_f = 0.45 was eluted by the solvent system pet ether:ethyl acetate (2.5:7.5). This combined fraction was found to be one of the phenolic compounds by spraying with 5 % $FeCl_3$. The TLC profiles are shown in Figure 3. This was confirmed by UV and FT IR (Figures 4 and 5, Tables 5 and 6). According to the results, the isolated fraction (compound A_1) is a phenolic compound by alkaline red shift in UV and also supported by FT IR spectrum.

Table 1 Absorbance of Gallic Acid Standard Solution at λ_{max} 765 nm

Concentration (μg/mL)	3.125	6.25	12.5	25	50	100
Absorbance	0.188	0.205	0.252	0.352	0.506	0.851

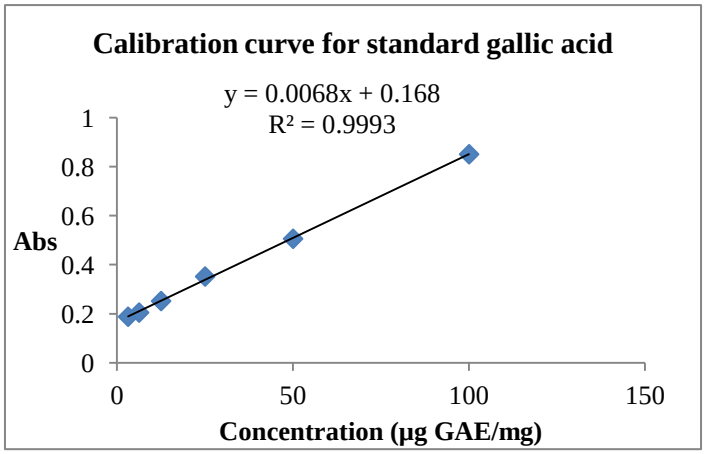


Figure 1 Standard gallic acid calibration curve at λ_{max} 765 nm

Table 2 Total Phenol Contents of 70 % Ethanol and Watery Extracts of the Bark of *Alstonia scholaris* L. (Taung-ma-yo)

No.	Extracts	TPC (μg GAE/mg)
1	Water	61.5
2	70 % Ethanol	99.33

Table 3 % RSA (Radical Scavenging Activity) and IC_{50} Values of Crude Extracts of Bark of *A.scholaris* and Standard Ascorbic Acid

Tested sample	% RSA (mean ±SD) in different concentrations (μg/mL)							IC_{50} (μg/mL)
	3.125	6.25	12.5	25	50	100	200	
70 % Ethanol extract	41.45 ± 6.88	47.50 ± 2.50	47.35 ± 1.04	49.71 ± 1.88	52.36 ± 1.46	70.36 ± 0.21	81.12 ± 3.34	27.75
Watery extract	20.5 ± 2.71	31.42 ± 4.38	30.68 ± 1.25	33.92 ± 0.42	37.32 ± 0.63	52.95 ± 1.46	71.1 ± 0.42	90.56
Standard Ascorbic acid	33.2 ± 0.92	57.66 ± 1.99	88.34 ± 0.88	94.22 ± 1.33	95.87 ± 1.55	96.17 ± 0.22	96.31 ± 0.44	5.27

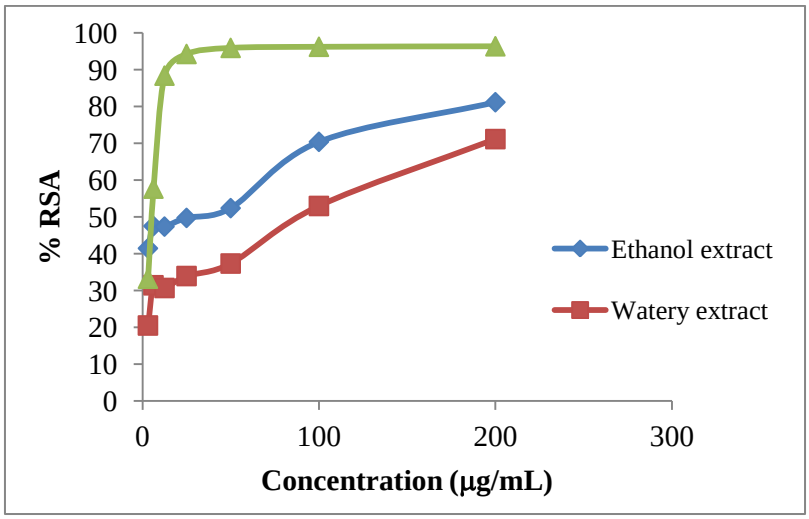


Figure 2 % RSA Vs concentration of crude extracts of bark of *A.scholaris* and standard ascorbic acid and

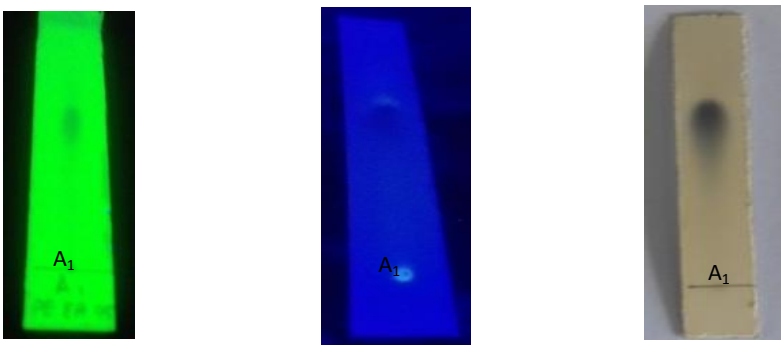
Table 4 Anti-tumour Activity of Crude Extracts from Bark of *Alstonia scholaris* L.

Sample	Tumour formation (5 days)			Tumour formation (7 days)		
	0.05 g	0.1 g	0.15 g	0.05 g	0.1 g	0.15 g
Ethyl acetate extract	+	+	-	+	+	+
70 % Ethanol extract	-	-	-	-	-	-
Watery extract	+	+	+	+	+	+
Control	++			++		

(++) Formation of tumour

(+) Some formation of tumour

(-) No formation of tumour



under UV_{254} nm under UV_{365} nm spraying with 5 % $FeCl_3$

Solvent system – Pet ether : Ethyl acetate (2.5:7.5)

Figure 3 TLC profiles of isolated compound (A_1) from ethyl acetate extract under UV_{254} nm, UV_{365} nm and 5 % $FeCl_3$

Table 5 Assignment of the UV Spectra of the Isolated Compound (A_1)

Medium	λ_{max} (nm)	Electronic Transition	Remark
MeOH	218, 253, 290	$\pi \rightarrow \pi^*$	Alkaline red shift Thus a phenolic compound
MeOH + NaOH	213, 273, 297	$\pi \rightarrow \pi^*$	

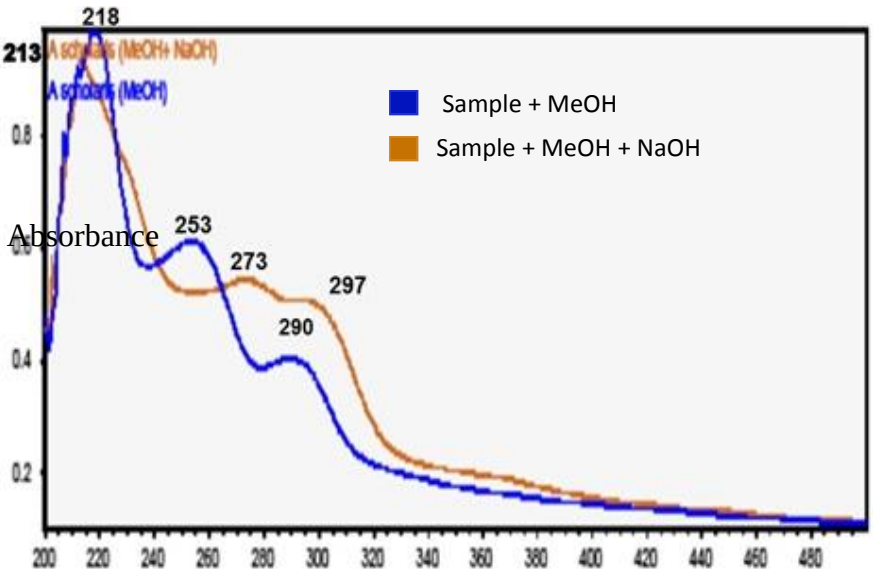


Figure 4 UV spectra for isolated compound from Bark of *Alstonia scholaris* L.

Table 6 FT IR Spectral Data of Isolated Compound (A_1) from Bark of *Alstonia scholaris* L.

No	Wavenumber (cm^{-1})	Vibrational mode	Remark
1	3327	O-H stretching	OH group attached on aromatic ring
2	3079	C-H stretching	Aromatic CH stretching
3	2922, 2852	C-H stretching	CH group in CH_3, CH_2, CH
4	1712	C=O stretching	Conjugated Carbonyl
5	1604, 1494, 1461	C=C stretching	C=C on aromatic ring
6	1186	C-O stretching	C-O group directly attached to aromatic ring

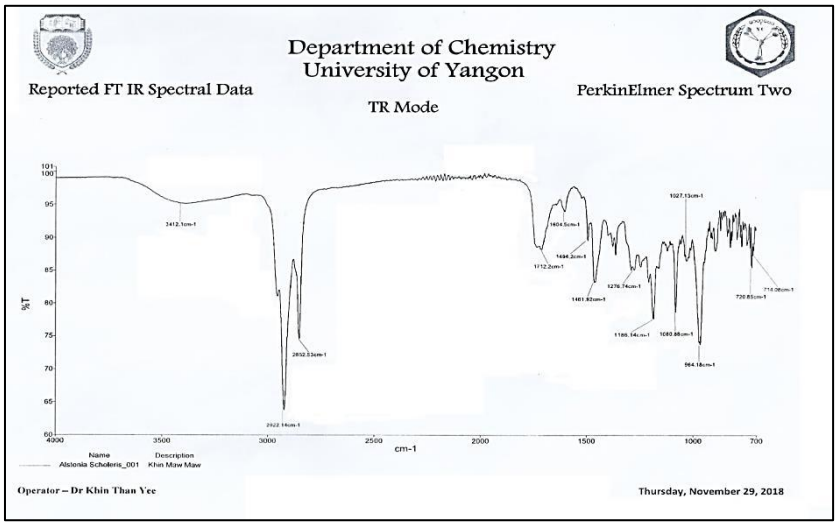


Figure 5 FT IR spectrum of isolated compound obtained from bark of *Alstonia scholaris* L. (Neat)

Conclusion

From the overall assessment for the present work concerning with the chemical and bioactivity investigation on the *Alstonia scholaris* L. (Taung-ma-yo) bark, the following inferences could be deduced. In the present work on the bark sample, preliminary phytochemical tests revealed the presence of alkaloids, α-amino acids, carbohydrates, flavonoids, glycosides, phenolic compounds, reducing sugars, saponins, steroids, tannins and terpenoids in it. Total phenol content (TPC) of 70 % ethanol crude extract was 99.33 μg GAE/mg and that of water extract was 61.6 μg GAE/mg. Thus, 70 % ethanol extract showed higher phenol contents than watery extract. The antioxidant activity of crude extract in bark sample was screened by DPPH assay method. IC_{50} value of 70 % ethanol extract was 27.75 μg/mL and that of watery extract was 90.56 μg/mL. So, ethanol extract showed more potent antioxidant activity than watery extract. Antitumour activity of crude extracts was investigated by Potato Crown Gall test. Ethyl acetate, 70 % ethanol and watery extracts were used to investigate against tumour cells within 5 days and 7 days. Among three extracts, ethanol extract showed the highest anti-tumour activity. Moreover, isolation of bioactive constituent fractions in bark sample was examined by thin layer chromatographic method. Isolated compound was eluted in the solvent system pet ether : ethyl acetate (2.5:7.5) by column chromatography. This compound was a phenolic which was confirmed by spraying reagent, UV and FT IR.

Acknowledgement

The authors would like to thank the Department of Higher Education (Yangon office), 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 Win Naing, Rector, Dagon University, for his encouragement, Dr Nu Nu Yi and Dr Nay Thwe Kyi, Prorectors, Dagon University, for their invaluable advices and Dr Cho Cho Win, Professor and Head, Dr Hnin Hnin Than, Professor, Department of Chemistry, Dagon University for their helpful advice, valuable suggestions and provision of research facilities at the Department of Chemistry, Dagon University.

References

Anubha, A. and Yashwant, R. (2015). "Review: Phytochemistry, Ethanobotanical and Pharmacological activities of *Alstonia scholaris* R.Br (Apocynaceae)". *International Journal of Advanced Research*, vol. 3 (8), pp. 584 – 590

Baliga, M.S. (2010). "*Alstonia scholaris* Linn R .Br in the Treatment and Prevention of Cancer: Past, Present, and Future". *Integr. Cancer Ther*., vol. 9(3), pp. 261-269

Bhanu, P., Chakraborthy, G.S. and Nandini, M. (2013). "Complete Aspects of *Alstonia schotaris*". *IJPRIF*., vol . 5 (1), pp. 17-26

Kalaria, P., Payal, G., Manodeep,C. and Jagadish, K. (2012). "A Phytopharmacological Review of *Alstonia scholaris* : A Panoramic Herbal Medicine". *IJRAP*., vol. 3 (3), pp. 367-370

Karamian, R. and Ghasemlou, F. (2013). "Screening of Total Phenol and Flavonoid Content,Antioxidant and Antimicrobial Activities of the Methanolic Extracts of Three Silence Species from Iron". *Intl J Agri Crop Sci.*, vol. 5 (3), pp. 305 – 312

Lee, K. G. and Shibamoto. T. (2001). "Antioxidant Property of Aroma Extract Isolated from Clove Buds". *Food Chem*., vol. 74, pp. 443–448

Marini-Bettolo, G.B., Nicoletti, M. and Patamia, M. (1981). "Plant Screening by Chemical and Chromatographic Procedure under Field Conditions". *J. Chromatography*, vol. 213, pp. 113-127

Priestman, J. and Edwards, F.C.G. (1953). *Aids to Qualitative Pharmaceutical Analysis*. London : 1st Edition, Wilkins Co. Baltimore, pp. 98

Thaw Thaw Zin. (2016). *A Phytochemical and Medico–Chemical Investigation of Ginkgo billoba L. (Kabar-Oo) Leaves and Coptis teeta Wall. (Khan. Tauk) Rhizomes.* Ph.D. Dissertation, Department of Chemistry, University of Yangon

Tomoko, Y. (1998). "HPLC Method for Evaluation of the Free Radical Scavenging Activity of Foods by using 1, 1-Diphenyl 2-Picryl Hydrazyl". *Biochem*., vol. 62(6), pp. 1201 – 1204

Vance, C.P., Javanraedi, J., Stushnoff, C., Locke, E. and Vivanco, J. M. (2003). "Antioxidant Activity and Total Phenolic Contents of Iranian Ocimum accessions". *Food Chemistry*. vol. 83, pp. 547-550