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Evaluation of Antioxidant, Antimicrobial and Cytotoxic Activity of the Bark of *Alstonia scholaris*

Sm Faysal Bellah[1*], Tahasina Jahan Adity[2], Rezaul Karim[1], Billah SMS[3], Alireza SM[4] and Md Obaidullah[5]

[1]*Department of Pharmacy, Manarat International University, Ashulia, Dhaka, Bangladesh*

[2]*Department of Pharmacy, The University of Asia Pacific, Dhaka-1205, Bangladesh*

[3]*Department of Chemistry, Govt. M. M. University College, Jessore-7400, Bangladesh*

[4]*Department of Pharmacy, International Islamic University Chittagong, Chittagong-4203, Bangladesh*

[5]*Pharmacy Discipline, Life Science School, Khulna University, Khulna-9208, Bangladesh*

[*]**Corresponding author:** Sm Faysal Bellah, Department of Pharmacy, Manarat International University, Ashulia, Dhaka-1341, Bangladesh, Tel: 8801913261838; E-mail: faysal_phku@yahoo.com, faysal@manarat.ac.bd

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Abstract

Alstonia scholaris (Family: Apocynaceae) has been investigated for the assessment of the biological activities. The bark of *Alstonia Scholaris* were extracted with pet ether, chloroform, carbon tetrachloride and methanol extract to afford 0.9 g, 0.8 g, 0.7 g, 3 g respectively for the test. We used crude pet ether, chloroform and carbon tetrachloride extract of the plant for the screening of antimicrobial activity against some selected organisms as bacteria and fungi by disc diffusion method. Out of all samples, chloroform and carbon tetrachloride extract showed strongest zone of inhibition and spectrum of activity. *In vitro* antioxidant activity of the extract of *Alstonia scholaris* was estimated by using DPPH free radical scavenging assay method. In DPPH free radical scavenging assay IC50 value of methanolic extract of *Alstonia scholaris* was found to be 39 g/ml which indicates mild to moderate antioxidant activity while Ascorbic acid was the standard drug. In the bioassay of brine shrimp lethality, the methanol extract showed an average of LC50 0.91 µg/ml. This indicated that the cytotoxicity exhibited by methanolic extract was very significant.

Keywords: *Alstonia scholaris;* Antioxidant activity; Antimicrobial activity; Cytotoxic activity

Introduction

As a source of drugs the medicinal plants are used for the management and treatment of various human and livestock health disorders or diseases worldwide from ancient times to the present day. There are a total of 250,000 species of flowering plants referred to as medicinal plants. The World Health Organization (WHO) enlisted about 21,000 medicinal plant species [1]. The global herbal market is about US$ 62 billion per annum. The annual growth of herbal market is about 15 percent and in 2050, it is expected to be about US$ 5 trillion [2]. About 25% of prescribed drugs in the world are of plants source [3]. In developing countries about 80% people believe on traditional plant based medicines for their primary health care purposes [4]. Worldwide several thousands of plants had identified consisting medicinal values and it was used for the treatment of different diseases in various cultures [5]. From the beginning to present majority of people are relying on the traditional medicine for the treatment of their primary health care [6]. The high cost of imported conventional drugs and inaccessibility to western medical care facility, involve that traditional mode of health care is the only form of health care that is reasonably priced and accessible to the people of rural area. On the other hand, western health facilities are obtainable, traditional medicine is viewed as a well-organized and an adequate system from a cultural viewpoint and as a result, traditional medicine usually present side by side in the health care of western region [7].

Alstonia is the genus of evergreen trees or shrubs with white funnel-shaped flowers and milky sap; tropical Africa to southeastern Asia and Polynesia. The plant name was given by the Professor Robert Brown, Department of Botany, Edinburgh University in the year of 1811 and after Charles Alston (1685-1760). It has found diverse common names in different languages e.g. The Bengali name is chattin, Burmese name is lettok, English name is birrba, Hindi name is chatian, Malay name is pulai, pulai linlin, Nepali name is chhataun, chhatiwan, The Trade name is chatiyan wood, pulai, shaitan wood [8,9].

Traditionally the bark is used for dysentery, typhoid, gonorrhea, asthma [10,11], ulcers, toothache, snake bites, cancer, tumour, jaundice, hepatitis, malaria, skin diseases and rheumatic pain [12,13] dog bite [14], fever [15]. It is also used for impotence [16], wounds and earache [17].

The present study is to investigate the antioxidant, antimicrobial and cytotoxic activity of the bark of *Alstonia scholaris*. As the plant have so many traditional use that's why we have focused our concentration for finding our desired results by performing the above test.

Materials and Methods

Plant materials

Plant sample of *Alstonia scholaris* was collected from Jessore, Bangladesh and for the identification of this plant, a sample of this plant was submitted to The National Herbarium, Mirpur, Bangladesh. The required parts of the plants were provoked to sun dried for seven days. For about 24 h of oven dry apply to the plant materials at

considerably low temperature for better grinding. It was then cutting into small pieces for better milling.

Chemicals

All the chemical reagents used in the experiment were of Sigma Chemical Co. Ltd., (St. Louis, MO, USA) and E. Merck (Germany).

Extraction of the Plant material

About 500 g of the powdered material was then placed in a separate round bottomed flask (5 L) and soaked in 2.5 L of methanol. Then kept for a period of 15 days accompanying occasional shaking and stirring. It was then filtered through cotton, then filter with Whatman No.1 filter paper and the filtrate thus obtained was concentrated at 39°C with a Heidolph rotary evaporation. To obtained solid residue the concentrated extract was then air dried. The weight of the methanol soluble crude extract was 15.0 g.

Extraction

Extraction can be done in two ways such as: Cold extraction and Hot extraction. In this investigation only cold extraction is performed.

Cold Extraction

In cold extraction the coarse sample is submerged in a suitable solvent system in an air-flat bottomed container for seven days, with intermittent shaking and stirring. The major portion of the plant material will be dissolved in the solvent during this time.

Solvent-solvent partition of crude extracts modified kupchan partition

5.0 g of the extract was taken and dissolved [18], triturated with 90 ml of methanol plus 10 ml of distilled water. It the mother stock solution, which is then partitioned off sequentially by three different polarity solvent (Table 1).

Partitioning with pet-ether

The mother stock solution was then taken in a separating funnel and extracted with pet-ether. The soluble fractions in pet-ether were collected together and evaporated. The aqueous methanolic fraction was preserved.

Plant	Fraction	Weight	Yield (%)*
Alstonia scholaris	Pet-ether soluble fraction	0.9 g	14%
	Carbon tetrachloride soluble fraction	0.7 g	10%
	Chloroform soluble fraction	0.8 g	16%
	Aqueous soluble fraction	3.0 g	40%

Table 1: Different fractions of methanolic extracts of *Alstonia scholaris* after partitioning. *% Yield is calculated on the 5 g of methanolic crude extract of *Alstonia scholaris*.

Partitioning with carbon tetra chloride

To the mother stock solution left after partitioning with pet-ether, add 12.5 ml of distilled water and mixed well. The mother solution was then taken in a separating funnel and extracted with carbon tetra chloride (CCl_4). These fractions were then collected together and evaporated. The aqueous fraction was also preserved for the next step.

Partitioning with di-chloro methane

To the mother stock solution that left after partitioning with pet-ether and carbon tetra chloride, add 16 ml of distilled water and mixed well. Add 100 ml of the di-chloro methane to it and the funnel was shaken and then kept undisturbed. The organic fractions were collected together and evaporated.

Microorganisms

Both gram positive and gram-negative bacteria and also fungi were taken for antimicrobial test (Table 2).

Antimicrobial activity

Disc diffusion method was used for the evaluation of antimicrobial activity [19-21]. In this method, antibiotics diffuse from a known source through the nutrient agar gel and generate a concentration gradient. Dried and also sterilized filter paper discs (6 mm diameter) containing the test samples of known amounts of pet ether soluble crude extracts, carbon tetra chloride, chloroform (400 µg/disc) are placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic (Kanamycin, 30 µg/disc) discs and blank discs were used as positive and negative control respectively. For maximum diffusion of the test materials to the surrounding media the plates were kept at low temperature (4°C) for 24 h [22]. After that the plates were then inverted and incubated at 37°C for 24 h for optimum growth of the organisms. The antimicrobial activity of the test agent was then determined by measuring the diameter of zone of inhibition expressed in millimeter [22].

Antioxidant activity

For the estimation of antioxidant activity, the *In vitro* free radical scavenging activity of *Alstonia scholaris* using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) by Brand-Williams method [23]. 2 ml of a methanolic extract solution at different concentration (0.977, 1.953, 3.906, 7.813, 15.625, 31.25, 62.5, 125, 250, 500 µg/mL) were mixed with 3.0 ml of DPPH methanol solution (20 µg/ml). The antioxidant activity was assayed from the bleaching of purple colored methanol solution of DPPH radical by the plant extract as compared to that of tert-butyl-1-hydroxytoluene (BHT) by UV spectrophotometer. The odd electron in DPPH free radical gives a strong absorption maximum at 517 nm. When the odd electron of DPPH radical becomes paired with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H, the color turns purple to yellow as the molar absorptive of the DPPH radical at 517 nm reduces. DPPH radical scavenging activity is described as SC50, which is the concentration of samples to produce 50% reduction of the DPPH.

Cytotoxic activity

The cytotoxic activity was evaluated by using brine shrimp lethality bioassay test [24,25].

Brine shrimp: *Artemia salina* (Brine shrimp) was used for cytotoxic activity. One spoon of cyst was hatched for 48 h in saline water, prepared by dissolving 20 g pure NaCl and 18 g normal edible NaCl into 1 L water. The cyst in turn became living nauplii.

Test microorganisms	Diameter of the zone of inhibition (mm)				
	Methanol (400 µg/disc) mm	Pet Ether (400 µg/disc) mm	Carbon tetrachloride (400 µg/disc) mm	Chloroform (400 µg/disc)mm	Kanamycin (30 µg/disc) mm
Gram Positive Bacteria					
Bacillus cereus	0	0	0	18	26
Bacillus megaterium	0	0	20	22	30
Bacillus subtilis	0	8	23	19	25
Staphylococcus aureus	0	6	14	23	35
Sarcina lutea	0	0	20	16	28
Gram Negative Bacteria					
Pseudomonas aureus	0	0	20	21	31
Escherichia coli	0	0	19	22	25
Salmonella paratyphi	0	5	17	18	25
Salmonella typhi	0	0	21	15	30
Shigella boydii	0	8	20	20	29
Shigella dysenteriae	0	0	18	19	32
Vibrio mimicus	0	0	22	20	29
Vibrio parahemolyticus	0	6	23	23	31
Fungi					
Candida albicans	0	0	22	20	27
Aspergillus niger	0	0	19	22	28
Saccharomyces cerevacae	0	0	15	17	25

Table 2: Antimicrobial activity of plant extracts by various solvent system.

Lethality bioassay: Sample solutions are prepared by dissolving the test materials in pre-calculated amount of Dimethyl sulfoxide (DMSO). For the test, different concentrations (400 µg/ml, 200 µg/ml, 100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml, 3.125 µg/ml, 1.5625 µg/ml, 0.78125 µg/ml, 0.391 µg/ml) of the plant extract prepared and were added to test tubes, each containing 10 shrimps in saline water and finally volume was adjusted by saline water. Control group with 10 shrimps were kept in saline water under the same condition and positive control group, with 10 shrimps in saline water, was treated with a known drug Vincristine Sulfate. All the test tubes were kept in rest for 24 h and then counted for living and dead nauplii. Finally, the percent mortality produced by each concentration was used to determine LC50 (lethal concentration) of the extract.

Result

Antimicrobial activity

Table 2 showed the antimicrobial activity of *Alstonia scholaris* relative to that of the standard drug kanamycin. The crude pet ether, carbon tetrachloride and chloroform extracts were subjected to antimicrobial screening. The average zone of inhibition formed for carbon tetrachloride is 18-20 mm and 20-22 mm for methanol at 400 µg/disc in agar media. Standard antibiotics disc of Kanamycin at 30 µg/disc was used for comparison. The pet ether extracts showed a little inhibitory action against *Shigella boydii* and *Vibrio parahemolyticus*. The carbon tetrachloride extracts showed strong inhibitory activity against *Vibrio mimicus* and *Vibrio parahemolyticus* having the zone of inhibition 23 mm. The growth of other bacteria was also mildly inhibited. In the same time, the growth of *Vibrio parahemolyticus* was strongly inhibited (23 mm) by chloroform extracts. The extracts showed inhibitory action against *Escherichia coli* having the zone of inhibition 22 mm. In case of the growth of other bacteria and fungi the inhibition was found showing mild to moderate activity.

Antioxidant activity

To determine the antioxidant activity of foods utilizes the stable 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) radical method was used. The odd electron in the DPPH free radical gives a strong absorption maximum

at 517 nm and is purple in color. An electron donated by an antioxidant compound accepted by DPPH, then it is decolorized, which can be quantitatively calculated from the changes in absorbance.

The half maximal inhibitory concentration (IC50) values of *Alstonia scholaris* plant extracts have been furnished in the Table 3.

S. No.	Absorbance of Blank	IC50 value of methanol soluble fraction of *Alstonia scholaris*				IC50 value of ascorbic acid			
		Concentration	Absorbance	%Inhibition	IC50 (µg/ml)	Concentration	Absorbance	%Inhibition	IC50 (µg/ml)
1	0.484	500	0.09	78.001	39	500	0.048	90.082	14
2		250	0.019	77.33		250	0.05	89.669	
3		125	0.123	76.99		125	0.051	89.462	
4		62.5	0.012	76.81		62.5	0.053	89.049	
5		31.25	0.124	76.33		31.25	0.055	88.636	
6		15.625	0.122	75.55		15.625	0.057	88.223	
7		7.813	0.119	74.36		7.813	0.06	87.603	
8		3.906	0.12	74.01		3.906	0.063	86.983	
9		1.953	0.117	73.99		1.953	0.065	86.571	
10		0.977	0.127	73.76		0.977	0.067	86.219	

Table 3: IC50 value of methanol soluble fraction of *Alstonia scholaris* and ascorbic acid.

Highest scavenging was observed with an IC50 value is 39 µg/ml as opposed to the IC50 value of ascorbic acid is 14 µg/ml, a well-known antioxidant (Figures 1 and 2).

Cytotoxic activity

The brine shrimp lethality of the methanol fraction was investigated by the procedure of Meyer's method [24]. Table 4 gives the brine shrimp lethality bioassay results after 24 h exposure to all the samples.

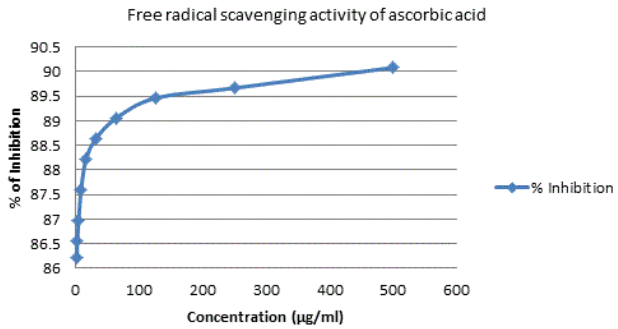

Figure 1: Free radical scavenging activity of ascorbic acid.

The comparative study of positive control and negative control (sea water)-giving significant mortality to the shrimp. The lethal concentration (LC50) of the test samples was obtained by a plot of percentage (%) of the shrimps killed against the logarithm of the sample concentration and the best-analysis (Figures 3-5). The average LC50 of methanolic extract of the plant is 0.91 µg/ml and the LC50 of Vincristine sulphate is 0.323 µg/ml.

Discussion

Our result shown that carbon tetrachloride and chloroform extracts have mild to moderate antimicrobial activity against the growth of some bacteria and fungi (Table 2).

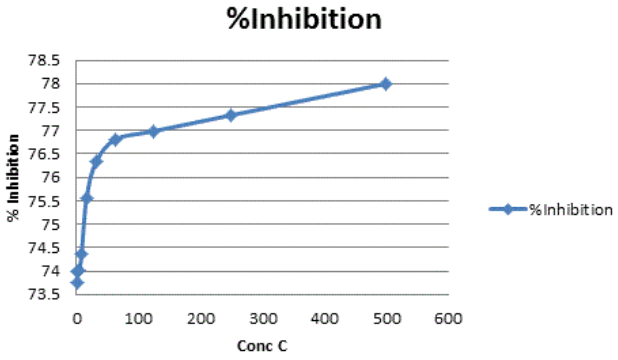

Figure 2: Value Free radical scavenging activity of *Alstonia scholaris*.

In our study with antioxidant test performed by using DPPH, the main characteristic of an antioxidant is its capacity to catch free radicals. The present results suggest that the tested plant extracts have moderate to potent antioxidant activity. Biological systems from a wide variety of sources present highly reactive free radicals and oxygen species. Nucleic acids, proteins, lipids or DNA is oxidize by these free radicals and can initiate degenerative disease. There are many antioxidants such as phenolic acids, polyphenols and flavonoids which scavenge many free radicals such as peroxide, hydroperoxide or lipid

peroxyl and as a result inhibit the oxidative mechanisms that direct to degenerative diseases. The phenolic compounds can play an important role in adsorbing and neutralising free radicals, decomposing peroxides and quenching singlet and triplet oxygen [26]. A number of human neurologic and other disorders such as inflammation, viral infections, autoimmune pathologies, and digestive system disorders including gastrointestinal inflammation and ulcer is produce due to oxidative injury (the fundamental mechanism) [27]. It has been postulated that in diabetes, increased oxidative stress which co-exist with reduction in the antioxidant status: "Oxygen free-radical can prompt peroxidation of lipids, then it is stimulates glycation of protein, enzymes inactivation and structure and function of collagen basement and other membranes alteration play a responsibility in the long term complication of diabetes" [28,29]. Reactive oxygen species are responsible for initiating the multistage carcinogenesis with DNA damage and accumulation of genetic actions in one or few cell lines which leads to progressively dysplastic cellular appearance, uncontrolled cell growth, and finally carcinoma [30].

For this reason, free-radical scavenging antioxidants therapy has potential to prevent delay/stoppage or improve many of these diseases or disorders [31]. Over the earlier period say last two decades, an expanding body of evidence from epidemiological and laboratory studies have confirmed that some edible/non-poisonous plants as a whole, or their identified ingredients with antioxidant properties have extensive protective effects on human carcinogenesis [30,32-34].

Conc. µg/ml	Log C	% Mortality			LC50 µg/ml			Average LC50 µg/ml of Methanol extract	Vincristine Sulfate			
		Methanol extract			Methanol extract							
		Test 1	Test 2	Test 3	Test 1	Test 2	Test 3	Conc µg/ml	Log C	% Mortality	LC50 µg/ml	
400	2.602	100	100	100	0.71	1.1	0.93	0.91	40	1.602	100	0.323
200	2.301	100	90	80					20	1.301	100	
100	2.000	90	80	80					10	1.000	100	
50	1.699	80	80	70					5	0.698	90	
25	1.398	70	70	70					2.5	0.397	80	
12.5	1.097	70	50	60					1.25	0.096	80	
6.25	0.796	60	40	40					0.625	-0.204	60	
3.125	0.495	20	20	20					0.3125	-0.505	50	
1.563	0.194	20	10	0					0.156	-0.806	40	
0.781	0.107	20	20	0					0.078	-1.107	20	

Table 4: Effect of methanol fractions on brine shrimp nauplii.

In Brine shrimp lethality bioassay indicates cytotoxicity & a large range of pharmacological activities, such as pesticidal and antitumor activities obtained from *Alstonia scholaris* bark extract [24,25]. This study found that the crude methanolic extract exhibited lower potency than vincristine sulphate. Therefore, further isolation of the highly active fractions may lead to the discovery of new cytotoxic compounds. Besides cytotoxic activity, these fractions should also be evaluated for the pesticide activity.

An alkaloid called alstonine was present in *Alstonia scholaris* plant which has antitumor activity in lymphoma and Ehrlich ascites carcinoma cells. Presence of alkaloid indicates the plants have anticancer, antitumour and cytotoxic activity. Bisindole and villalstonine are also found in this plant which showed marked activity against human cancer cell lines, large cell carcinoma cell line and human lung adenocarcinoma [35].

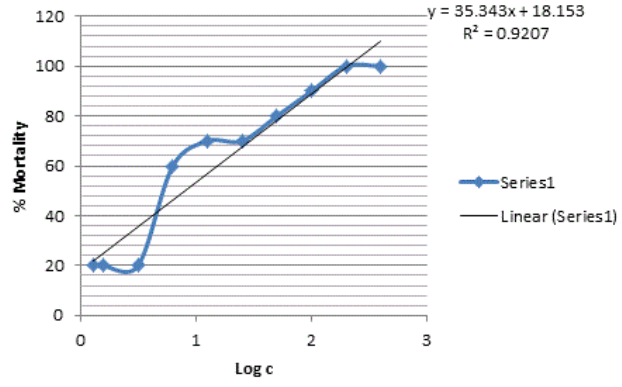

Figure 3: Effect of methanol extract on brine shrimp nauplii. Graph mortality vs. Log C for test 1.

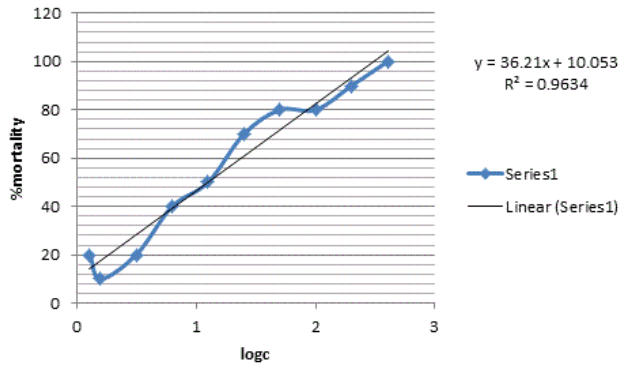

Figure 4: Effect of methanol extract on brine shrimp nauplii. Graph mortality vs. Log C for test 2.

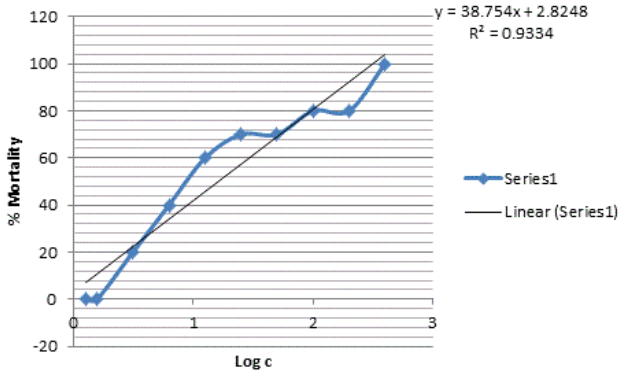

Figure 5: Effect of methanol extract on brine shrimp nauplii. Graph mortality vs. Log C for test 3.

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