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In vitro Study of Different Partitionates of *Alstonia scholaris* (L.) R.Br. Leaf for Thrombolytic and Membrane Stabilizing Activities

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Abstract

Alstonia scholaris (L.) R.Br. is a medicinal herb belonging to the family Apocyanaceae. Locally it is known as Chatim and abundant with ethno-medicinal properties. The crude ethanolic extract of *A. scholaris* leaves along with its n-hexane, ethyl acetate, dichloromethane and aqueous soluble partitionates were assayed for their probable thrombolytic and membrane stabilizing activities. The activities of the leaf extract were compared to standard drugs, streptokinase for thrombolytic and acetyl salicylic acid for membrane stabilizing activities. In this study, among all extractives the highest thrombolytic activity was exhibited by the crude ethanolic extract (55.33±0.08%) ($p < 0.001$) as compared to Streptokinase (62.07±0.33%) ($p < 0.001$). Alternatively, ethanol soluble materials also significantly inhibited the haemolysis of human erythrocyte membrane both in induced by hypotonic solution (83.47±0.15%) ($p < 0.001$) and by heat (79.82±0.71%) ($p < 0.001$), respectively as compared to standard acetyl salicylic acid (85.09±0.8%) ($p < 0.001$) and (80.98±1.34%) ($p < 0.001$).

Keywords: *Alstonia scholaris*, Apocyanaceae, membrane stabilization activity, thrombolytic activity.

Introduction

Medicinal plants are enriched with many potent compounds that are significant to heal diseases and give pharmacological benefits in biological models. Due to the cost effectiveness and more safety margin medicinal plants are getting attention for modern analysis day by day. Traditionally, different plants are known to have different efficacy for treating various types of diseases. The right plant should be chosen for healing a particular disease and attempts should be made to isolate the bioactive lead molecule (Harvey *et al*., 2015). Plant-derived substances are of great interest owing to their versatile applications. Plants serve human with the valuable components of medicines, seasonings, beverages, cosmetics and dyes (Bhanu *et al.,* 2013).

Thrombosis and inflammation are frequently observed in human body for making very complicated situations. Hence, many drugs have been developed to take care of these problems but side effects still remain as a matter of concern (Rodriguez *et al*., 2012). To obtain safer molecules, comprehensive studies are still going on. Natural products might be very useful resources for finding the desired drug. In developed countries thromboembolic disorders such as pulmonary emboli, deep vein thrombosis, strokes and heart attacks etc. are not common (Moghal *et al*., 2016). Thrombus in the circulatory system followed by vascular blockage may lead to death. Currently used thrombolytic agents that include tissue plasminogen activator, urokinase, streptokinase etc. are used worldwide having risk of hemorrhage, anaphylactic reaction and lack of specificity. So, attempts are still ongoing around the world to develop improved thrombolytic agents (Hilleman *et al.,* 2011).

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Generally, several oxidative damages and related inflammatory actions are accelerated by free radicals generated within the body. The red blood cell (RBC) membrane assimilates to lysosomal membrane, so that the action of drug to stabilize RBC membrane could be anticipated to the membrane stabilizing activity (Islam *et al*., 2015). Compounds with membrane-stabilizing properties can prevent the release of phospholipases that initiate the formation of inflammatory mediators (Shinde *et al*., 1999). The aim of the present study was to successively evaluate the thrombolytic and membrane stabilizing activities of different extractions of leaves of *A. scholaris* in accordance to established methods.

Alstonia scholaris (Linn.) R. Br. (Family Apocyanaceae), is a small evergreen tree with bitter milky juice, distributed throughout the sub-Himalayan belt, West Bengal, Bihar, peninsular India and Southeast Asia (Steve *et al*., 2008). The plant is a medium to large tree, about 40 m high with a rough grey to grayish brown bark, abounding in bitter, white milky latex. Leaves are in whorls of 4-8 in the upper exiles, upper surface is dark green, the lower green-white. The plant has some traditional uses by folks for the antimalarial, antidarrheal, antipyretic, antidysenteric activities (Namaka *et al.,* 2004) and skin disorder (Arora *et al.,* 2015). The milky juice of the plant is purgative and applied on injuries and ulcers to treat pain including rheumatic pains and in cardiac as well as respiratory problems. Bark has antiseptic properties (Arulmozhiet *et al*., 2010; Singh *et al*., 2017). It has been reported as antimicrobial, antiamoebic, antiplasmodial (Khan *et al.,* 2003) potential as well as potency for anaemia and menstrual disorders showing also wound healing activities.

A. scholaris were found to contain alkaloids including echitamine, tubotaiwine (stem and root bark), akuammicine (root bark), echitamidine (stem bark), o-akuammidine (leaves, root bark), picrinine (stem bark, leaves, flowers), picralinal, nareline (leaves), strictamine (flowers), (leaves), ditamine (stem bark), echitenine (stem bark), an indole alkaloid (flowers). Moreover, carbohydrates, amino acids, phenols, tannins, cardiac glycosides, saponins, flavonoids, terpinoids, steroids, fixed oils and fats (Vaidyanatha *et al*., 2011) are also found. A new flavanone glycoside: isookanine-7-o-alpha-l-rhamnpyranoside, and a secoiridoid glycoside: alstonoside, has been reported. Presence of α-amyrin, β-amyrin, lupeol acetate, rhazine, yohimbine, linalool, *cis*- and *trans*-linalool oxides, β-terpineol, 2-phenylethyl acetate, terpinen-4-ol and steroids are also recorded phytoconstituents found in this plant (Abhijit, 2011, Werhagen *et al*., 2004). According to Shang *et al*., 2010, Khyade *et al*., 2009, there are reports to locate coumarins, iridoids, leucoanthocyanines in the plant extract. Six pentacyclic triterpenoids as lupeol, betulin, 3-hydroxy-11-ursen-28,13-olide, betulinic acid, oleanolic acid and ursolic acid were structurally identified from this plant (Wang *et al.,* 2016). The flowers of *A. scholaris* are known to contain lupeol and β-amyrin (Sultana *et al.,* 2010).

Materials and Methods

Collection, drying and extraction: The fresh leaves of *A. scholaris* were collected in the month of October (2016) from the plant growing in Botanical Garden, Dhaka and voucher specimen (accession number -73885) has been deposited in Bangladesh National Herbarium (BNH) for future reference.

The leaves of the plants were washed, sundried and ground into coarse powder. About 300 g was soaked in 1500 ml ethanol for 10 days with occasional shaking and stirring. The whole mixture was then filtered through cotton followed by Whatman No. 1 filter paper and was concentrated with a rotary evaporator under reduced pressure at 50°C temperature to afford crude extract (49.34 g). An aliquot (20 g) of the concentrated ethanolic extract was fractionated by modified Kupchan method (VanWagenen *et al.,* 1993) to n-hexane (5.89 g), dichloromethane (4.36 g), ethyl acetate (2.86 g) and aqueous (4.36 g) soluble fractions.

Thrombolytic activity: For thrombolytic assay (Prasad *et al.,* 2007) aliquots (5 ml) of venous blood were drawn from healthy volunteers in ten different

pre weighed sterile vials (1 ml/tube) and incubated at 37°C for 45 minutes. After clot formation, the serum was completely removed without disturbing the clot and each vial having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube – weight of tube alone). To each vial containing pre-weighed clot, each of the extractives (1 mg/100 µl water) was added. Here, 100 µl (equivalent to 30,000 I.U.) of Streptokinase (Altepase, Beacon Pharmaceuticals Limited, Bangladesh) and 100 µl of distilled water were used as positive control and negative control, respectively. Then 100 µl aqueous solutions of different partitionates along with the crude extract was added separately. All the vials were then incubated at 37°C for 90 minutes and observed for clot lysis. After incubation, the released fluid was removed and vials were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis as shown below:

% of thrombolysis = (wt of clot after treatment / wt of clot before treatment) × 100

Membrane stabilizing activity: The membrane stabilizing activity of the crude ethanolic and its soluble partitionates was assessed by evaluating their ability to inhibit hypotonic solution and heat-induced haemolysis of human erythrocytes following the method developed by Sikder *et al*., 2012.

Hypotonic-induced haemolysis: Erythrocyte suspension was prepared by drawing 5 ml of whole blood from healthy human volunteers in a tube containing dipotassium salt of EDTA (2.2 mg/ml of blood). The blood was centrifuged, supernatant was removed and blood cells were washed three times by sodium chloride isotonic solution (154 mM NaCl) in 10 mM sodium phosphate buffer (pH 7.4) using the same volume as supernatant through centrifugation for 10 min at 3000 g. Finally, it was resuspended in the same volume of this isotonic buffer solution. After that solution 0.5 ml of this was mixed with 5 ml of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing either the extract (1 mg/ml) or reference drug, acetyl salicylic acid (0.1 mg/ml). The control sample was consisted of 0.5 ml of RBCs mixed with hypotonic-buffered saline alone. The mixture was incubated for 10 min at room temperature, centrifuged for 10 min at 3000 *g* and the optical density (OD) of the supernatant was measured at 540 nm. The percentage inhibition of hemolysis was calculated using the following equation-

% inhibition of hemolysis = $\{(OD_{control} - OD_{test\ sample})/OD_{control}\} \times 100$

Heat-induced hemolysis: 5 ml of isotonic buffer containing aliquots (1 mg/ml) of the different extractives were taken into two duplicate sets of centrifuge tubes. 5 ml isotonic buffer was used as positive control in different tube. 30 µl erythrocyte suspension was added to each tube and mixed gently for reaction. One pair of the tubes and control samples were incubated at 54ºC for 20 min in a water bath, while the other pair was preserved at 0-5ºC in an ice bath. After the time period, mixtures were centrifuged for 3 min at 1300 *g* and the absorbance of the supernatant was measured at 540 nm. The percentage of inhibition of haemolysis in tests was calculated according to the equation:

% inhibition of haemolysis =

$\{1 - (OD_{heated\ test\ sample} - OD_{unheated\ test\ sample})/(OD_{heated\ control\ sample} - OD_{heated\ test\ sample})\} \times 100$

Statistical analysis: Three replicates (n = 4) of each sample were used for statistical analysis and the values are reported as mean ± standard deviation (SD).

Results and Discussion

Thrombolytic activity: The percentage of clot lysis of different fractionates of the studied plant has been illustrated in table 1.

The highest thrombolytic activity (52.33±0.08%) was noticed by the crude ethanolic extract where the standard Streptokinase showed 62.07±0.33% thrombolysis. Moreover, moderate clot lysis ability was also observed by n-hexane (45.47±0.41%), dichloromethane (49.11±0.3%) and aqueous (50.92±0.74%) fractions. However, weakest

thrombolytic activity was observed by the ethyl acetate fraction 32.35±0.4%.

Table 1. Effect of different extractives of *A. scholaris* on thrombolysis.

Fractions	Mean % of lysis ± STD
EF	55.33 ± 0.08**
NHF	45.47 ± 0.41**
DCMF	49.11 ± 0.3**
EAF	32.35 ± 0.4**
AQF	50.63 ± 0.74**
SK	62.07 ± 0.33**
Blank	15.13 ± 0.54*

*P < 0.005, **P < 0.001; statistically significant as compared to positive control and negative control. (EF: ethanol fraction, NHF: n-hexane fraction, DCMF: dichloromethane fraction, EAF: ethyl acetate fraction, AQF: aqueous fraction)

Membrane stabilizing activity. The percentage of RBC membrane rupture inhibition of different fractionates of the studied plant have been depicted in table 2.

In case of hypotonic solution-induced haemolysis, the highest RBC membrane protecting capacity was evident for the crude ethanolic fraction (83.47±0.15%) as compared to the standard acetyl salicylic acid (85.09±0.8%). Good membrane stabilizing activity was also shown by the ethyl acetate, n hexane and aqueous soluble fraction (above 70%). For heat induced haemolysis, the crude ehanolic extract demonstrated strongest activity (79.82±0.71%), where the standard has 80.98±1.34%. Other fractionates exhibited good stabilization capacity with more than 70% inhibition of RBC membrane rupture.

Table 2. Effect of different extractives of *A. scholaris* on membrane stabilization.

Sample	Mean absorbance ± STD	% inhibition of haemolysis	
		Hypotonic solution-induced	Heat-induced
EF	0.104±0.001708	83.47±0.15**	79.82±0.71
NHF	0.180 ±0.001291	71.03±0.24**	73.92±0.4**
DCMF	0.192±0.001295	68.7±0.82**	76.26±0.57**
EAF	0.121±0.001725	81.45±1.14**	71.05±0.66**
AQF	0.150±0.001291	75.92±0.11**	79.27±1.12
ASA	0.103±0.0017	85.09±0.8**	80.98±1.34**
Control	-	-	-

*P < 0.005, **P < 0.001; statistically significant as compared to positive control ASA (EF: ethanol fraction, NHF: n-hexane fraction, DCMF: dichloromethane fraction, EAF: ethyl acetate fraction, AQF: aqueous fraction)

So from the observed results, it can be proposed that the inhibition of erythrocyte membrane rupture property of *A. scholaris* could be the possible mechanism of action of its anti-inflammatory activity. Most of the extractives showed statistically significant activity ($p < 0.001$) as compared to the standard acetyl salicylic acid (ASA).

Conclusion

The crude ethanolic extract of *A. scholaris* leaves and its different partitionates were evaluated for their thrombolytic and membrane stabilization potential. The plant demonstrated potent thrombolytic and membrane stabilization activities. It may be assumed that these extracts can be considered as good source of thrombolytic and membrane stabilizing agents. Further comprehensive investigations are required to isolate the bioactive compounds and to know their detailed underlying mechanism.

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