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Highly Anticancer and Moderate Thrombolytic Property of *Accacia rugata* of Mimosaceae Family

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Abstract: Medicinal plants containing potent bioactive compound effective in treating many diseases exert different pharmacological action. This study designed to evaluate the cytotoxic and thrombolytic activity of *Acacia rugata* of Mimosaceae family. The cytotoxic and thrombolytic activity was evaluated by brine-shrimp lethality bioassay and in-vitro clot lysis method. Methanol, petroleum-ether, n-hexane, chloroform and dichloromethane fraction leaves (MEL, PETFL, n-HxFL, CHFL, DCMFL), methanol and n-hexane fraction of fruits (MEF, n-HxFF) and methanol fraction of bark (MEB) were used to evaluate cytotoxicity of the plant. Each extracts showed significant cytotoxic property. The LC_{50} values of MEL, PETFL, n-HxFL, CHFL, DCMFL, MEF, n-HxFF, MEB were observed 1.436, 0.039, 0.974, 0.626, 0.121, 0.176, 0.865, 0.081 µg/ml when compared to standard vincristine (positive control) which LC_{50} value was 0.049 µg/ml. In thrombolytic activity evaluation 20 mg/ml, 10 mg/ml, 5 mg/ml and 2.5 mg/ml dose of MEL showed 40.52 ± 2.91, 35.09 ± 2.71, 31.96 ± 2.02 and 24.91 ± 3.05% clot lysis respectively while 0.9% NaCl solution (negative control) and standard streptokinase (positive control) exhibited 7.41 ± 1.73% and 48.91 ± 3.52% of clot lysis. It can be assumed that different solvent extracts of *A. rugata* have important cytotoxic and thrombolytic activity as compared to standard compounds.

Keywords: Cytotoxic, Thrombolytic, Streptokinase, Vincristine, % of Clot Lysis, *Acacia rugata*

1. Introduction

Medicinal plants, being a healthy source of life have always been considered to be the mean of recuperation for various diseases [1]. According to world Health Organization (WHO), 80% population of developing countries depends on traditional medicine for their primary health care [2]. These secondary metabolites produced by plants can be used as templates to discover newer drugs [3]. Plants derived active compounds are established to be safer, efficient while synthetic drugs are feared to be used in chronic disease [4]. Approximately 20% of medicinal plants that have been used in pharmaceutical studies are useful in treating cancer, invasive aspergillosis and harmful diseases [5]. Since plant possess significant pharmacological activity, low toxicity profile, economic flexibility play role in exploring new disease, investigation for their medicinal properties have been performed [6].

Following cardiovascular disease cancer is a leading cause of mortality and morbidity which results from uncontrolled cell proliferation because of the inhibition of apoptotic process. Standing as a notorious disease of present world cancer responsible for human mortality in large case and approximately half of mortality occur in Asia [7]. Chemotherapy, radiotherapy and chemically derived drugs are the currently available treatments and cause a lot of strain and further damage to the patient t health. Therefore, researchers look forward to using alternative treatments and therapies against cancer [8]. Over the past 30 years Natural products have received increasing attention for their potential as novel cancer preventive and therapeutic agents [9, 10].

Brine shrimp lethality bioassay use to evaluate cytotoxicity because of its simplicity, cost effectiveness and small sample requirements [11].

Thrombosis in portal vein is one kind of venous thrombosis leading to hypertension and reduced blood supply to liver [12]. Cardiovascular diseases associated with thrombus formation are increasing in recent years at an alarming rate [13]. In UK, the rate annual death was reported 25000 because of thrombosis [14]. Thrombolytic therapy recognized as a treatment to alleviate dangerous clots in blood vessels, improve blood flow, and prevent damage to tissues and many organs. In general, Thrombolysis is applied as an emergency treatment which vanish blood clots, the underlying reason of heart attacks and ischemic strokes by feeding the heart and brain with clots [15]. Thrombolysis also may increase the risk of complications in pregnancy or aging, and in people with other conditions. A tiny risk of infection and a slight risk of an allergic reaction to the antithetical dye may arise in patient who sustains thrombosis [16]. Tissue plasminogen activator, streptokinase, urokinase, anti-streptokinase etc. are the commonly known thrombolytic drugs which exert their effect by dissolving the blood clot [17]. Though these thrombolytic drugs are wonderful clot lytics, they still some limitations such as need of large dose, limited fibrin specificity, tendency of bleeding, allergic reactions and resistance to intravenous t-PA6 [18]. Because of these complications scientists are focusing on plants based active compounds to treat thrombus associated disease.

Acacia rugata (Lam.) is a species belonging to Mimosaceae family and its local name is Banritha. It is a large stragling shrub with more or less hooked prickles. *Acacia rugata* is used as medicinal plant in hill tracts of Bangladesh available in hill tracts of Bangladesh having purgative, anthelminthic, anti-diarrhoeal, emetic and diuretic activities. Seeds are usually used in child birth to facilitate delivery. For leprous patches, prurigo, abscesses, eczema and bubos they are applied externally [19]. A literature overview of *A. rugata* showed that this plant is not studied for its biological activity still. Considering the traditional and local use of *A. rugata*, we designed the study to evaluate cytotoxic and thrombolytic activity by brine-shrimp lethality bioassay and *in-vitro* clot lysis method.

2. Material and Methods

2.1. Crude Plant Parts Collection and Identification

After evaluating the history of traditional use *Acacia rugata* was selected because of its wide use in the tribal communities for different purposes. Leaves, fruits and barks of *Acacia rugata* were collected from Bandarban Hill Districts, Bangladesh in April 2019. Each part of the plant then identified by Dr. Sheikh Bokhtear Uddin, Associate professor, Department of Botany, University of Chittagong. Chittagong, Bangladesh.

2.2. Processing for Powdered Plant Material

The collected plant parts were thoroughly cleaned with tap water and cut into small pieces and dried in the sun. The sun dried materials then processed with grinder to have coarse powder. The dried powdered material then kept air tight pot in a moist free place.

2.3. Test Reagents and Chemicals

All the chemicals and drugs used in these tests were of analytical grade. Vincristine sulphate and Streptokinase were used as standard drugs for brine shrimp cytotoxic activity and thrombolytic activity respectively and are collected from Beximco Pharmaceuticals Ltd, Bangladesh. Tween-80, Methanol, pet-ether, n-hexane, chloroform, dichloromethane, sea salt (NaCl), DMSO all solvents and reagents were analytical grade and obtained from local suppliers.

2.4. Preparation of Extract

Having the dried plant materials 400 mg of leaves powder, 500 mg of fruits powder and 800 gm of barks powder were soaked in 1.8 liters, 2 liters and 3 liters of methanol respectively and these bottles containing the soaked material were kept for 10 days at normal room temperature with 2-3 times shaking in a day. The crude extract of leaves, fruits and barks were filtered using fresh cotton plug and then by using Whatman no. 1 filter paper. The filtrated extract of each part were evaporated to have concentrated extracts using vacuum rotary evaporator. Total percentage yield of each plant part extract was calculated using the following equation [20].

$$\% \text{ of yield of extract} = (W_1/W_2) \times 100$$

Here, W_1 = Weight of extracted material and W_2 = Weight of original plant material used.

The resultant methanol extract of leaves was then partitioned by n-hexane, pet-ether, chloroform and dichloromethane and fruits extract partitioned by n-hexane respectively.

2.5. Assessment of Cytotoxic Activity

The cytotoxic activities of all the extracts of *Acacia rugata* were performed using brine shrimp nauplii following Mayer's method [21-23]. Brine shrimp eggs (Artemia salina Leach) were collected and hatched in a tank containing 1 L of simulated seawater at 37°C and pH 8.4 supplying constant oxygen. The nauplii could hatch and mature for 2 days. 4 mg of MEL, PETFL, n-HxFL, CHFL, DCMFL, MEF, n-HxFF and MEB of *A. rugat*a were taken and dissolved in 200 µl of pure dimethyl sulfoxide (DMSO) in separate vials to get stock solutions and concentration of these prepared solutions was 400 µg/ml. 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781 µg/mL solutions prepared from stock solution for each extract by serial dilution. A vial containing 50 µL DMSO diluted to 5 mL was used as a measurements control.

Vincristine sulphate was used as positive control [11]. The formulated test solutions were applied to pre-marked vials with 10 live brine shrimp nauplii in 5 mL of simulated seawater, and incubated for 24 h. After 24 hours the number of survival of nauplii was counted and percentage of mortality was determined using the equation: % mortality=(no. of dead nauplii/ initial no. of live nauplii) x 100. The criterion of toxicity for fractions were established according to Déciga-Campos et al. [24]; LC_{50} values > 1000 µg/mL (non-toxic), 500 ≤ 1000 µg/mL (weak toxicity) and <500 µg/mL (toxic).

2.6. Thrombolytic Activity

The thrombolytic activity of methanol leaves extract of *A. rugata* was evaluated with the method developed by Daginawala [7]. 600 mg crude methanol leaves extract was taken and dissolved in 30 ml of 0.9% NaCl solution to make a stock solution of 20 mg/ml. This stock solution further used to prepare 10 mg/ml, 5 mg/ml, and 2.5 mg/ml solution by serial dilution. 5 ml of phosphate buffer was added to commercially available lyophilized streptokinase (1,500,000 I.U.) vial. So concentration of streptokinase became 30,000 I.U. and used as reference for standard for thrombolytic activity. Venous blood (n=10) was drawn from healthy human volunteers ensuring no history taking oral contraceptive or anticoagulant therapy and citrated with 3.1% sodium citrate solution. Then 500 µl was transferred in different pre weighed sterile micro centrifuge tube and incubated at 37 °C for 45 minutes for clotting to occur. After clot formation the serum was aspirated out and each tube containing clot was again weighed to calculate clot weight. About 500 µl of different extract concentration, 0.9% NaCl (negative control) and streptokinase 30,000 I.U (positive control) was added to clot containing tubes (n=10). These tubes are then incubated for 90 minutes at 37 °C. The released fluid was then removed and tubes were again weighed. The weight difference between before and after clot lysis expressed as % of clot lysis.

$$\text{Clot lysis (\%)} = (\text{wt of released clot/clot wt}) \times 100$$

2.7. Statistical Analysis

% of clot lysis expressed as mean ± SEM. One way ANOVA following Dunnett's multiple comparison was used for statistical analysis. The statistical analysis was carried out in SPSS (version 20.0).

3. Results

3.1. Assessment of Cytotoxic Activity

After 24 hour incubation with test and control samples, LC_{50} value was measured. The LC_{50} value for methanolic leaves extract (MEL), pet ether fraction of leaves (PEFL), n-hexane fraction of leaves (n-HxFL), chloroform fraction of leaves (CHFL), dichloromethane fraction of leaves (DCMFL), methanolic fruits extract (MEF), n-hexane fraction of fruits (n-HxFF), methanol extract of bark (MEB) were found 1.436, 0.039, 0.974, 0.626, 0.121, 0.176, 0.865, 0.081 µg/ml respectively. The standard vincristine (positive control) showed cytotoxicity at 0.049 µg/ml and no mortality was found for the negative control did LC_{50} (µg/ml) less than 500 µg/ml consider as toxic for brine shrimp larvae. Each extract exhibited significant cytotoxic property where PEFL showed more toxicity than vincristine. The LC_{50} after treating the nauplii with standard, plants extracts is shown in *Table 1*.

Table 1. *Screening of cytotoxic activity of the different fractions of A. rugata by using brine shrimp lethality bioassay.*

Sample	Equation	R^2	LC_{50} (µg/ml)
Vincristine	y = 23.365 + 48.863	0.935	0.049
MEL	y = 15.907 + 27.157	0.856	1.436
PEFL	y = 22.350 + 49.119	0.931	0.039
n-HxFL	y = 18.323 + 32.142	0.947	0.974
CHFL	y = 12.886 + 41.924	0.912	0.626
DCMFL	y = 15.101 + 48.161	0.848	0.121
MEF	y = 20.538 + 46.379	0.938	0.176
n-HxFL	y = 26.175 + 27.346	0.914	0.865
MEB	y = 23.155 + 48.114	0.952	0.081

3.2. Thrombolytic Activity

In *in vitro* clot lysis method, after 90 minutes incubation at 37°C, minute clot lysis was observed when 500 µl of normal saline (negative control) added to clot tube (7.41±1.73%). Streptokinase (30,000, I.U) showed 48.91±3.52% clot lysis which is significantly ((i.e., $p<0.001$) differ from negative control. Percent of clot lysis after treating the blood clot with streptokinase 30,000, I.U (positive control), control and plant extracts is shown in *Table 2*. 20 mg/ml, 10 mg/ml, 5 mg/ml, and 2.5mg/ml of methanol leaves extract of *A. rugata* showed significant ($p<0.001$) clot lysis by 40.52±2.91%, 35.09±2.71%, 31.96±2.02%, 24.91±3.05% respectively. Here the methanol extract showed dose dependent significant thrombolytic activity.

Table 2. *Effect of methanolic leaves extract of A. rugata on blood clot lysis.*

Group	Concentration	% of clot lysis
NaCl solution	0.9%	07.41 ± 1.73
Streptokinase	30,000 I.U.	48.91 ± 3.52***
MEL	20 mg/ml	40.52 ± 2.91***
MEL	10 mg/ml	35.09 ± 2.71***
MEL	5 mg/ml	31.96 ± 2.02***
MEL	2.5 mg/ml	24.91 ± 3.05***

Values are represented as mean ± SEM (n=10). *$p<0.05$ compared with control done by one way ANOVA followed by Dunnett's't'-test.

4. Discussion

A. rugata has wide range of medicinal properties. This study revealed the cytotoxic and thrombolytic attributes of different fractions of *A. rugata* in vitro.

Medicinal plants are regarded as a vital source of phytoconstituents [25]. Moreover, as a result of the biodiversity plants possess different active principles and they produce different pharmacological activities on human body

[26]. From the ancient time plants had been used for the treatment of many diseases and nowadays drug discovery from plants can be achieved following phytopharmacological investigation, which renewed the attention towards herbal medicines [27]. Though anticancer drugs should not exert anticancer property to the normal cells but they show toxicity towards rapidly growing cells [13]. Cytotoxicity studies are considered as a key factor for the identification of possible cytotoxicity of numerous substances, for instance, chemicals, plant extracts, and biologically active compound. That is why plant cytotoxicity studies have been regarded as a salient feature for research scientist [21]. From previous data, it has been cleared that, several species of the *Acacia* have possessed cytotoxic activity [28, 29]. The cytotoxic potential of *A. rugata* is highly potent and further investigation may lead to isolate the compound responsible for this crucial effect.

Failure of hemostasis results in the development of thrombus in the circulatory system which cause vascular blockage leading to serious consequences in atherothrombotic disease like myocardial infarction which at times leading to death [30]. Thrombolytic agents like reteplase alteplase, urokinase, streptokinase etc. are serine proteases which convert plasminogen into plasmin that further break down fibrinogen and fibrin and dissolve the clot. Research works have been undertaken to discover antithrombotic agents of plant sources to combat and prevention of coronary heart disease and strokes [31]. *A. rugata* was evaluated for thrombolytic action and found with having moderate thrombolytic activity and such finding may have important implications in cardiovascular health.

5. Conclusion

Cancer is growing in an alarming rate in both developed and developing countries. Alternative treatments with naturally occurring plant derived anticancer agents are increasingly in demand. From this study it can be reached with a satisfactory conclusion that *A. rugata* has highly effective cytotoxic property and may lead to have bioactive compounds for anticancer drugs. Moreover, the plant possesses moderate dose dependent anti-thrombosis activity which may be future thrombolytic drugs source. This finding may be further explored for the discovery of anticancer lead compound.

Conflict of Interest

The authors do not have any conflict of interest. All the blood donors were informed about the blood withdrawal process and ethical rules were maintained before working with human blood.

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