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In-vitro comparative study of cytotoxic and thrombolytic effects of methanolic extract of *Cissus pentagona* and *Thunbergia grandiflora* (Roxb.) leaves

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ABSTRACT

Objective: To compare cytotoxic and thrombolytic activity of crude methanolic extract of *Cissus pentagona* (*C. pentagona*) and *Thunbergia grandiflora* Roxb. (*T. grandiflora*) leaves.
Methods: The screening of cytotoxic activity was done by using brine shrimp lethality bioassay while the thrombolytic activity was evaluated by using the *in vitro* clot lysis model. In brief, venous blood from five healthy volunteers was allowed to form clots which were weighed and treated with the tested plant materials to disrupt the clots. Weight of clot before and after treatment provided a percentage of clot lysis and the results with streptokinase as positive control and water as negative control were compared.
Results: Moderate cytotoxicity was found for both methanol extracts, and it was compared with the standard drug vincristine sulfate in the brine shrimp bioassay. In the present study, the LC_{50} values of the methanol crude extract of *C. pentagona* as well as *T. grandiflora* and vincristine sulfate were 291.33, 243.37 and 12.59 μg/mL, respectively. In thrombolytic study, it was found that *C. pentagona* and *T. grandiflora* showed (24.27 ± 2.61)% and (19.56 ± 2.98)% of clot lysis, respectively. Among the herbs studied, *C. pentagona* showed very significant ($P < 0.001$) percentage of clot lysis than *T. grandiflora*, compared with reference drug streptokinase [(63.54 ± 2.61)%].
Conclusions: The results of the study demonstrated that the leaf of the plants contains preliminary cytotoxic effect on brine shrimp and promising thrombolytic activity *in vitro* when it is tested on human blood. However, further study is needed to evaluate its potential as a thrombolytic agent.

1. Introduction

Cissus pentagona (Vitaceae) Roxb. (*C. pentagona*) is a large woody climber, distributed in Southeast Asian countries such as, Bangladesh, Bhutan, China, India, Indonesia, and Myanmar. In Bangladesh, it occurs in Chittagong (Sitakunda, Hazarikhil), Cox's Bazar (Whykong, Himchari National Park, Chakaria, Sundarban, Shilkhali), Moulvi Bazar (Lowachara)[1]. It is known as "Sona-tola" (in Bangla) and "Hajjarludi" (Chakma), "poipruchala" (Tripura) in local tribes of Chittagong, Bangladesh. The species is applied by Chakma to treat skin disease in the affected areas. Roots are used by Tripura to prepare a paste, which is applied to the affected areas for the treatment of elephantiasis. The roots of this plant in combination with other plants are also used for the treatment of filaria by the tribe in Chittagong Hill Tracts[2-4]. Another medicinal plant, *Thunbergia grandiflora* Roxb. (Acanthaceae) (*T. grandiflora*) is a large climbing or twining shrub, which is found widely all over the world such as India, China, Indo-China, Myanmar and many tropical countries of Africa[5]. It is also found throughout the Bangladesh, especially in forests of Gajipur, Chittagong, Chittagong Hill Tracts, Cox's Bazar, and Tangail[6]. Generally, it is known as black clock vine, and blue trumpet vine. In Bangladesh, it is called as "Kauathuti", "Nallata" and "Nillata" and in local tribes, it is known as "Changra Morich", "Danludi", "Deldipata", "Del Ladi", "Del Ludi", "Jeol

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Ludi", "Jheol Ludi", "Jhiol Ludi", "Lachuney", "Lachoainuyee", "Lakkali", "Lakkani", "Sangara Marish" (Chakma); "Butto Luri", "Lachuia-nui", "La Soain Nuya", "Lawchowanowai", "Luck Chuyee-nu", "Luck Choai Yee" (Marma); "Claicloyong" (Khumi); "Dumangkhong" (Tripura) and "Botualodi" (Tonchonga) [5]. The species is used for the treatment of blood dysentery, cataract, conjunctivitis, diabetes, gout, hydrocele, hysteria, malaria, marasmus, ophthalmia, postpartum pre-eclampsia, rheumatism, spermatorrhoea, stomachache, stomach complaints, elephantiasis, and urinary bladder stone[7-9].

2. Materials and methods

2.1. Plant collection and identification

Leaves of *C. pentagona* and *T. grandiflora* were collected from different parts of the Chittagong region, Bangladesh. The plants were identified by Dr. Shaikh Bokhtear Uddin, taxonomist and associate professor in Department of Botany, University of Chittagong.

2.2. Chemicals and drugs

Lyophilized streptokinase vial (1 500 000 IU) which was imported from Durakinase, Dongkook Phama. Co. Ltd, South Korea and sterile distilled water (5 mL) were added and mixed properly. Mixture of distilled water and lyophilized streptokinase was used as a stock. From this mixture, 100 μL (30 000 IU) was used for determination of cardioprotective activity which means *in vitro* thrombolysis. Two more chemicals were purchased from Sigma-Aldrich (Munich, Germany), which were vincristine sulfate and 99.5% absolute methanol.

2.3. Preparation of extract

Plant materials of *C. pentagona* and *T. grandiflora* were dried and ground into powder (40-80 mesh, 500 g) through Moulinex Blender AK-241. Then the powder was soaked in 2 L of methanol at room temperature [(23.0 ± 0.5) ℃] for a week. By using Whatman filter paper No. 1 and cheesecloth, filtrate was obtained. Filtrated solution was concentrated under reduced pressure by using a rotary evaporator. Less than 50 ℃ was maintained for this filtration process. Glass Petri dishes were used to keep the extract. Each of the extracts (100 mg) was suspended in 10 mL distilled water. By using a vortex mixer, the suspension was shaken vigorously. Suspension of extract and distilled water was kept overnight and gradually poured through a 0.22 μm syringe filter for the filtration. In this way, soluble supernatant was removed. Then 100 μL of this filtrated aqueous preparation was added to microcentrifuge tubes which contained the clots to check the *ex-vivo* cardioprotective (thrombolytic) activity. The same concentration (10 mg/mL) of both plant extracts was prepared for the *ex-vivo* screening of cytotoxic effects.

2.4. Cytotoxicity screening

Evaluation of cytotoxic effect of methanolic extracts was performed by brine shrimp lethality bioassay, which is mostly used for bioactive compound screening[10,11]. A simple zoological organism (*Artemia salina*) was used for the experiment. At first, eggs of brine shrimps were collected from an aquarium shop (Dhaka, Bangladesh). Then brine shrimps were hatched in artificial seawater (3.8% NaCl solution) for 48 h and developed from brine shrimp eggs to larval shrimp (nauplii). Meyer's method was used for this cytotoxic assessment of brine shrimp nauplii. Tested samples (extracts) were prepared by dissolving them in dimethyl sulfoxide which was not more than than 50 μL in 5 mL solution. Seawater (3.8% NaCl solution) was added so that concentrations of 10, 50, 100, 150, 200 and 300 μg/mL were attained. After that, a vial with 5 mL dimethyl sulfoxide was taken and used as control. Vincristine sulphate (standard drug) was used as positive control. Mature shrimps were placed in each of the experimental vials. Then vials were inspected after 24 h by using magnifying glass and the number of surviving nauplii in each vial was counted. Those data represented the percentage of lethality of brine shrimp nauplii, from which each concentration can be evaluated by using the formula below:

$$\% \text{ of mortality} = \frac{Nt}{No} \times 100\%$$

Where, Nt is the number of dead nauplii after 24-hour incubation; No is the total number of nauplii transferred, *i.e.*, 10.

The LC_{50} was determined from the log concentrations versus percentage of mortality curve.

2.5. Thrombolytic activity

2.5.1. Blood sample

About 2 mL of blood was drawn from five healthy humans. They had no history of taking any types of contraceptive or anticoagulant therapy. A total of 500 μL of blood was transferred to each of the three previously weighed microcentrifuge tubes to form clots.

2.5.2. Clot lysis

At first, three different sterile microcentrifuge tubes (0.5 mL/tube) were taken and weighed. Then 2 mL venous blood from human volunteers was added in pre-weighed sterile microcentrifuge tubes. The tubes were incubated at 37 ℃ for 45 min. In this process, serum was totally eliminated after the formation of clots without disturbing the clots. Each tube containg clot was again weighed to know the weight of clot. For the determination of clot weight, weight of tube alone were excluded from the weight of clot and tube. For each microcentrifuge tube containing pre-weighed clot, 100 μL of methanol extracts of both plants (*C. pentagona* and *T. grandiflora*) were added separately. About 100 μL of streptokinase was used as a positive control and 100 μL of distilled water was used as a negative control. At last, all the tubes were incubated at 37 ℃ for 90 min. In

this way, clot lysis was observed. After incubation, released fluid was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference in weight before and after clot lysis was expressed as percentage of clot lysis[12]. Evaluation of thrombolytic effects of both methanolic extracts were performed by the formula below:

% of clot lysis = (Weight of released clot/clot weight) × 100

The experiment was repeated 5 times with the blood samples from the 5 healthy volunteers.

2.6. Statistical analysis

Statistical significance between % of clot lysis by streptokinase and plant extracts was evaluated by paired *t*-test analysis. The test was performed by using the software SPSS version 20.0 (SPSS for Windows, IBM Corporation, New York, USA). Expression of data was expressed as mean ± SD. The mean difference between positive and negative controls was considered significant at $P < 0.05$ and $P < 0.001$.

3. Results

3.1. Brine shrimp lethality bioassay

Following the procedure of Meyer, the lethality of methanolic extracts of *C. pentagona* and *T. grandiflora* leaves were determined on *Artemia salina* after sample exposure for 24 h. Determination of the cytotoxic effect of the extracts was conducted by making comparison between negative control (dimethyl sulfoxide only) and positive control (vincristine sulphate). Table 1 represents the percentage of mortality of brine shrimp caused by the plant extracts at six different concentrations (10 to 300 μg/mL) of the extracts. It was precise that the percentage of lethality was directly proportional to the concentrations of extracts. LC_{50} values of methanol extracts of *C. pentagona* and *T. grandiflora* obtained in the present experiment were 291.33 and 243.37 μg/mL, respectively. From the comparison between *C. pentagona* and *T. grandiflora*, *T. grandiflora* exhibited stronger cytotoxic effects than *C. pentagona*. In case of vincristine sulphate (positive control), the LC_{50} value was 12.59 μg/mL. However, no mortality was obtained from the negative control.

Table 1

Mortality of brine shrimp caused by both extracts (*C. pentagona* and *T. grandiflora*) at six different concentrations. %.

Concentration (μg/mL)	Log C	CP	TG	Vincristine sulphate
10	1.000	10.00	10.00	40.00
50	1.699	20.00	20.00	80.00
100	2.000	20.00	20.00	100.00
200	2.301	40.00	40.00	100.00
300	2.477	50.00	70.00	100.00

CP: *C. pentagona*; TG: *T. grandiflora*. Log C: Log concentrations.

3.2. Thrombolytic activity

At first, 100 μL of streptokinase (positive control) was added to the clots and kept in incubator at 37 ˚C for 90 min, which showed significant lysis of clot [(63.54 ± 2.61)%]. When distilled water (negative control) was used to treat clots, it showed negligible clot lysis [(4.21 ± 0.73)%]. A significant value (probability value of $P < 0.05$) was obtained by calculating the mean difference in the clot lysis (%) between streptokinase (positive control) and distilled water (negative control). When *C. pentagona* and *T. grandiflora* extracts were used to treat clots, the lysis of clot was (24.27 ± 2.61)% and (19.56 ± 2.98)%, respectively. A statistically significant value (probability value of $P < 0.001$) was obtained from the mean percentage of clot lysis of *C. pentagona* and *T. grandiflora*. *C. pentagona* exhibited a relatively higher percentage of clot lysis than *T. grandiflora*. However, both plant extracts showed statistically significant values when compared with streptokinase (positive control) and distilled water (negative control) (probability value of $P < 0.001$).

4. Discussion

Toxicity profile of plant materials is mainly an important criterion to experts and medical practitioners[13-15], and cytotoxic brine shrimp lethality (LC_{50}, 24 h) test was conducted in this experiment to know about the toxicity of the plant extracts. Derived equation from Parra[16], showed a great correlation ($r = 0.85$; $P < 0.05$) between the LC_{50} of brine shrimp lethality test and the severe oral toxicity assay in mice. Based on derivation of the correlation, cutoff value for cytotoxicity is determined by LC_{50} which should be less than 10 μg/mL (LD_{50} between 100 and 1 000 mg/kg)[16,17]. In this experiment, the modest cytotoxicity was found for both extracts, compared with the standard drug vincristine sulfate.

In the case of thrombolytic studies, both methanolic plant extracts as well as positive control (streptokinase) truly demonstrated the effect on clot lysis. By comparing the clot lysis percentage obtained through streptokinase and distilled water, a promisingly significant ($P < 0.05$) thrombolytic effect was seen after the clots were treated with both *C. pentagona* and *T. grandiflora* extracts. It is established from the previous experiment that there are some bacterial pollutants of plants that have plasminogen receptors which are specific for plasminogen. Certain plasminogen on cell surface is rapidly activated to plasmin that could lead to fibrinolysis[18]. Bacterial plasminogen activator which also acts as cofactor molecules, such as staphylokinase, and streptokinase, can cause formation of exosite and increase the substrate activity towards the enzyme. Staphylokinase activates plasminogen to be in a position to break down clots, and also damages the extracellular molecules secreted by cells and fibrin particles that keep cells organized[19-21]. From the above experiment, it would be interesting to examine both of the mechanisms correlated to clot lytic effects showed by *C. pentagona* and *T. grandiflora* extracts. However, these activities might be due to the presence of biologically active or inhibitory compounds or synergism by the existence of some compounds. A different type of constituents, such as saponins, polyphenols, alkaloids, and flavonoids, may be present in the extracts, so further vast investigations are required to determine the specific cytotoxic and

thrombolytic effects of the leaf extracts. Both of the leaves extract of *C. pentagona* and *T. grandiflora* contain moderate cytotoxic and thrombolytic activities *in vitro*. However, *in vitro* clot disbanding property and active components of *C. pentagona* for clot lysis are yet to be investigated. Additional investigations are required to be performed because phytochemicals derived from this plant could be incorporated as a thrombolytic agent for the improvement of the patients suffering from cardiac diseases.

Conflict of interest statement

We declare that we have no conflict of interest.

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