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Canvassing of thrombolytic, cytotoxic, and erythrocyte membrane-stabilizing attributes in *in vitro* screening of *Gynocardia odorata*

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Abstract

Background: In the current study, different plant extracts of *Gynocardia odorata* such as methanol extract (ME), aqueous soluble fraction (AQSF), chloroform soluble fraction (CSF), carbon tetrachloride soluble fraction (CTCSF), and petroleum ether soluble fraction (PESF) were examined for the analysis of thrombolytic, cytotoxic, and erythrocyte membrane-stabilizing activities.

Methods: A well-explicated method was accomplished for plant extractives investigation. The plant extractives were involved in thrombolytic, cytotoxic, and erythrocyte membrane-stabilizing activity evaluation, on the basis of their ability of clot lysis, cytotoxic potentials, and stabilizing erythrocyte membrane under hypotonic solution and heat-induced conditions. Both thrombolytic and erythrocyte membrane-stabilizing activities were performed by using Swiss albino laboratory mice. In addition, plant cytotoxic activity was performed by using the nauplii of brine shrimp as *in vitro* model.

Results: The study of *G. odorata* extracts enumerated basic thrombolytic activity ($19.94 \pm 0.53\%$ to $10.64 \pm 0.46\%$; $p < 0.05$), and basic cytotoxic LC_{50} value (23.09 ± 2.01 μg mL^{-1} to 1.18 ± 0.14 μg mL^{-1}; $p < 0.05$) including statistical analysis confidence limit ranges, chi-square value, and regression equation was entailed. The erythrocyte membrane-stabilizing activity under hypotonic solution-induced hemolysis ($47.41 \pm 0.46\%$ to $18.445 \pm 0.095\%$; $p < 0.05$) and heat-induced hemolysis ($27.95 \pm 0.55\%$ to $17.84 \pm 0.59\%$; $p < 0.05$) was determined. All the statistical calculation was optimized by one-way ANOVA followed by Turkey's *post hoc* test including Dunnett *t* tests.

Conclusion: This study indicates that *G. odorata* could be a natural medication alternative of thrombolytic agents as well as source of potent bioactive compounds.

Keywords: *Gynocardia odorata*; Brine shrimp nauplii; Cytotoxic; Erythrocyte membrane-stabilizing activity

Background

According to the World Health Organization (WHO) estimate, more than 80% of the population of the developing countries rely on conventional plants for initial health care (Mulat et al. 2013). Only in Asia, medicinal plant has a big impact on economy and primary health care. There are approximately 6,500 species used for curative purpose in Asia. Bangladesh has a fertile with reputable inheritance of herbal medicines among the countries in South Asia. In Bangladesh, almost 500 medicinal species are assessed, and 250 species are used for preparation of traditional medicine. In addition, a large amount of pharmaceutical raw materials including medicinal plants and semi-processed plant are used. The majority of these plants have not yet been studied for their pharmacological and toxicological bioactivities (Rajaei et al. 2012; Karki et al. 1999; Uddin et al. 2011). Presently, thrombolytic drugs like urokinase (UK), alteplase, tissue plasminogen activator (t-PA), and streptokinase (SK) are widely used as clinical thrombolytic agents. Nowadays, streptokinase and urokinase are used in developing countries due to their reasonable price, but these drugs are coupled with side

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effects which lead to anaphylactic reaction, hemorrhage, and systemic fibrinolysis. As a result of immunogenicity, multiple treatment of streptokinase is restricted in a given patient (Sherwani et al. 2013; Dewan et al. 2013). Traditional plants are relatively safe for use to treat different types of diseases, including various traditional plants that have thrombolytic, antiplatelet, anticoagulant, and antithrombotic activities which are successfully applied for therapeutic purposes (Das et al. 2013). Present phytochemical research scientists have isolated hundreds of bioactive chemical compounds, for example aconitine, acronycine, compounds from Amaryllidaceae plants, bisindole, camptothecine, cephalotaxus, colchine, ellipticine, emtine, phenanthroquinolizidine, and pyrrolizidine, which are ascertained cytotoxic against tumor cells (Geoffrey et al. 1993).

G. odorata, a medium-sized tree is commonly found in lower hill forest of South Asia, commonly in India, Bangladesh, Nepal, and China. This evergreen tree belongs to the Achariaceae family. People normally used fruit-extracted oils for cooking and lighting purposes. The fruit itself is poisonous before processing (Rai and Rai 1994; Shu 2014). This research aims to study the versatile extracts of *G. odorata* herbal plants of Bangladesh especially on its thrombolytic activity and in stabilizing erythrocyte membrane by using Swiss albino laboratory mice and its cytotoxic activity by using the nauplii of brine shrimp as *in vitro* model.

Methods

Plant collection and identification

The leaves and fruits of *G. odorata* were collected from different areas of Dhaka, Bangladesh. A voucher sample number (DACB-39206) has been issued from the Bangladesh National Herbarium, Mirpur, Dhaka, Bangladesh. The leaves were sun dried for several days and then oven dried for 24 h at considerably low temperature (not more than 40°C) for better grinding. The dried leaves were then crushed to a powder form using a grinding machine in the Phytochemical Research Laboratory, Faculty of Pharmacy, University of Dhaka.

Chemicals and reagents

All chemicals and solvents, i.e., methanol, carbon tetrachloride, n-hexane, chloroform, dimethyl sulfoxide (DMSO), and other reagents, used in these experiments were analytical grade and purchased from Merck (Darmstadt, Germany), and vincristine sulfate (VS) was purchased from Sigma-Aldrich (Steinheim, Germany). Commercially available lyophilized streptokinase (SK) vial (15,000,000 IU) was gifted from Beacon Pharmaceuticals Ltd. (Dhaka, Bangladesh). This suspension was used for *in vitro* thrombolysis as a stock from 100 μL (30,000 IU).

Extraction of the plant material

The desirable sample plants at first were sun dried for few days and then oven dried at 40°C for nearly 24 h for easy grinding. About 500 g of powdered sample plant materials were attenuated in 2.0 L of 95% methanol for 7 days and then filtered through a cotton plug accompanied by Whatman filter paper number 1. Applying a temperature of 40°C to 45°C and reducing the pressure, the extract was concentrated with the assistance of a rotary evaporator. The concentrated methanol extract was partitioned, and the sequent partitioning, i.e., n-hexane (1.2 g), chloroform (800 mg), carbon tetrachloride (1.0 g), and aqueous soluble (1.6 g) fractions, was utilized for the experiment (Anosike et al. 2012).

Animals

In this experiment, adult Swiss albino mice (25 to 30 g) of either sex were used which were grown in the State University of Bangladesh laboratory. They were placed under standard laboratory conditions of 12:12 h light and dark cycle, temperature of 23°C ± 2°C, and maintained relative humidity of 55 ± 5%. During the study, they were fed with standard animal feed and *ad libitum* water. Institutional animal ethical committee (IAEC) approved experimental protocol requirements was supervised by Department of Pharmacy, State University of Bangladesh.

Making of erythrocyte suspension

Whole blood was collected from the mice through the retro-orbital plexus. Anticoagulant ethylenediaminetetraacetic acid (EDTA) was added to preclude clotting. The blood was rinsed three times with 0.9% saline. The bulk of saline was assessed and reconstituted as a 40% (*v*/*v*) suspension with an isotonic buffer solution (pH 7.4) which comprised in 1 L of distilled water with NaH_2PO_4. $2H_2O$ (0.26 g), Na_2HPO_4 (1.15 g), NaCl (9 g), and 10 mM sodium phosphate buffer was centrifuged for 10 min at 3,000 rpm (Kuddus et al. 2012).

Thrombolytic activity

Commercially available lyophilized streptokinase vial (15,000,000 IU) was fused properly with 5 mL phosphate-buffered saline (PBS). This suspension was used as a stock from which appropriate dilutions were made to observe the thrombolytic activity. Five milliliters of blood was withdrawn from each mouse ($n = 10$) through the retro-orbital plexus. Fresh blood was collected in pre-weighed sterile micro-centrifuge tube (1 mL per tube) and incubated at 37°C for 45 min. The observations were taken in triplicate. After clot formation, the serum was completely removed without disturbing the clot, and each tube having the clot was again weighed to determine the clot weight (Clot weight = Weight of clot containing tube – Weight of tube alone). To each micro-centrifuge tube containing the pre-weighed clot, 100-μL aqueous solution of different

plant extracts such as ME, PESF, CTCSF, CSF, and AQSF of the methanolic extract of *G. odorata* were added individually. About 100 μL of SK considered as the positive control and about 100 μL of distilled water considered as the negative control were individually added to the control tubes. At 37°C for 90 min, all the tubes were incubated and observed for clot lysis. After the incubation process, the excess fluid was removed, and the tubes were again weighed to mark the deviation in weight after clot disruption. Deviation obtained in weight taken before and after clot lysis was conveyed as percentage of clot lysis using the following equation: % of clot lysis = (weight of released clot/clot weight) × 100 (Prasad et al. 2007; Rahman et al. 2013; Dhande et al. 2014).

Cytotoxicity activity

The brine shrimp eggs (*Artemia salina*) were amassed from the Fisheries Department, University of Dhaka, Bangladesh. The simulated seawater (3.8% NaCl) was allowed for 48 h for the brine shrimp eggs to hatch and develop into nauplii. All the plant sample extracts were filled in vials by dissolving them in 100 μL pure DMSO to acquire stock solutions. We consider a 50-μL stock solution with simulated sea water (3.8% NaCl) to reach a series of concentrations approximately 400 μg mL^{-1} each. Standard VS was used as the positive control, and 50-μL pure DMSO diluted to 5 mL was used as the negative control. The matured nauplii were applied to each of all experimental vials and control vial. After 24 h, the vials were scrutinized using a ×3 magnifying glass, and the number of living nauplii in each vial was counted (Meyer et al. 1982; Ali et al. 2013).

Erythrocyte membrane-stabilizing activity

Hypotonic solution-induced hemolysis

The experiment sample comprised of stock of mice erythrocyte (RBC) suspension (0.50 mL) with 5 mL of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate-buffered saline (pH 7.4) containing either the different methanolic extracts (2.0 mg mL^{-1}) or acetyl salicylic acid (0.10 mg mL^{-1}). The acetylsalicylic acid was used as a reference standard. The mixtures were incubated for about 10 min at 25°C and then centrifuged for 10 min at 3,000×*g*, and the absorbance (O.D.) of the supernatant was measured at 540 nm using a Shimadzu UV spectrophotometer (Tokyo, Japan). The percentage inhibition of hemolysis was calculated using the following equation: % inhibition of hemolysis = $100 \times (OD_1 - OD_2)/OD_1$, where OD_1 is the optical density of the hypotonic buffered saline solution alone (control) and OD_2 is the optical density of the test sample in hypotonic solution.

Heat-accelerated hemolysis

Approximately 5 mL of the isotonic buffer containing 1.0 mg mL^{-1} of the different extracts of plants was put into two twin sets of centrifuge tubes. The vehicle with the same quantity was added to another tube as the control. The erythrocyte suspension of mice (30 μL) was added to each tube and mixed gently by upending. One pair of the tubes was incubated at 54°C for 20 min in a water bath. The other pair was placed in an ice bath at 5°C. The whole reaction mixture was centrifuged for 3 min at 1,300 rpm, and the yielding supernatant was measured at 540 nm. The percentage inhibition in the tests was taken and was calculated according to the

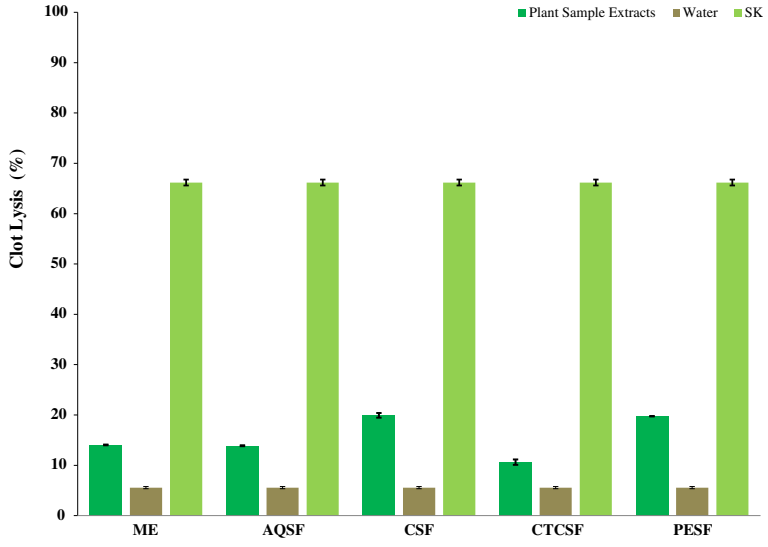

Figure 1 Clot lysis activity of *Gynocardia odorata*. One-way ANOVA on Tukey's *post hoc* test with Dunnett's *t* test ($p < 0.05$).

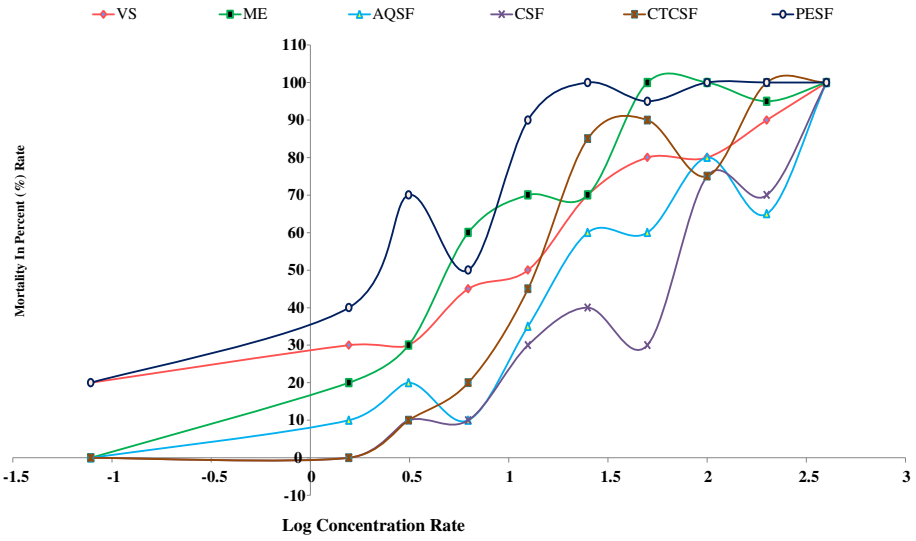

Figure 2 Cytotoxic activity of *Gynocardia odorata*. One-way ANOVA on Tukey's *post hoc* test with Dunnett's *t* test ($p < 0.05$).

following equation: % inhibition of hemolysis = 100 × [1 – (OD_2 – OD_1/OD_3 – OD_1)], where OD_1 is the unheated test sample, OD_2 is the heated test sample, and OD_3 is the heated control sample (Rashid et al. 2011; Sharma et al. 2013).

Statistical analysis

The implication between % clot lysis through SK and plant sample extracts, LC_{50} values through VS, extracts, hypotonic solution-accelerated % inhibition of hemolysis, and heat-accelerated % inhibition of hemolysis was tested using Dunnett's *t* test analysis using the software SPSS (SPSS for Windows, Version 19.0, IBM Corp., Armonk, New York, USA). The data were expressed as mean ± standard deviation. The mean difference between the positive and negative controls was considerably substantial at $p < 0.05$. The LC_{50} value of nauplii was calculated from a linear regression applying 'Biostat-2009' software (AnalystSoft Inc., Vancouver, Canada). All the experiment was performed *in vitro* and expressed as mean ± standard error of the mean.

Results and discussion

Platelets act as a crucial function in atherothrombosis through adhering to the disrupted region of the endothelial surface which further initiates plaque formation and growth. Plasmin, a natural fibrinolytic agent, helps break down fibrinogen and fibrin and initiates the lyses of clot. Typically, SK forms a stoichiometric complex with plasminogen to change plasmin (Chowdhury et al. 2011).

As part of the investigation on the thrombolytic activities of natural sources, the extractives of *G. odorata* were evaluated; the results are depicted in Figure 1. An addition of 100 μl SK, a positive control (30,000 IU) to the clots and subsequent incubation for 90 min at 37°C, showed 66.17 ± 0.59% lysis of clot. Consequently, distilled water was treated as the negative control which exhibited negligible percentages of lyses of clot (5.57 ± 0.23%). The mean difference in clot lysis percentage between the positive and negative controls was found very significant. In this study, *G. odorata* sample extracts CSF and PESF demonstrated the highest thrombolytic activity

Table 1 Cytotoxic activity potential values of *Gynocardia odorata*

Sample	LC_{50} (μg mL^{-1})	Confidence limit ranges (μg mL^{-1})	Chi-square value	Regression equation
VS	0.432 ± 0.02	0.47 to 0.39	25.63	$Y = 0.1008 + 0.0251 \times X$
ME	4.89 ± 0.09	5.06 to 4.70	61.61	$Y = 0.1648 + 0.0178 \times X$
AQSF	23.09 ± 2.01	25.93 to 20.25	87.04	$Y = 0.5682 + 0.0235 \times X$
CSF	43.55 ± 2.50	48.45 to 38.66	152.70	$Y = 0.6943 + 0.0255 \times X$
CTCSF	11.88 ± 0.42	12.70 to 11.06	79.72	$Y = 0.5803 + 0.0181 \times X$
PESF	1.18 ± 0.14	1.45 to 0.91	112.95	$Y = 0.1648 + 0.0178 \times X$

LC_{50} values are the mean of the triplicates (mean ± SEM, $p < 0.05$). VS, vincristine sulfate; ME, methanol extract; AQSF, aqueous soluble fraction of the methanol extract; CSF, chloroform soluble fraction; CTCSF, carbon tetrachloride soluble fraction; PESF, petroleum ether soluble fraction.

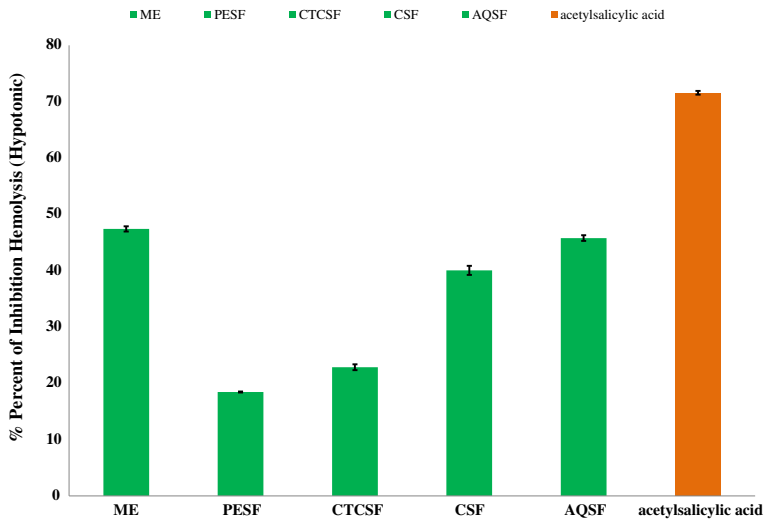

Figure 3 Hypotonic hemolysis of *Gynocardia odorata*. One-way ANOVA on Tukey's *post hoc* test with Dunnett's *t* test ($p < 0.05$).

(19.94 ± 0.53% and 19.75 ± 0.08%, respectively). However, significant thrombolytic activity was also evidenced by ME (14.06 ± 0.06%), AQSF (13.90 ± 0.04%), and CTCSF (10.64 ± 0.46%) which showed moderate thrombolytic activity. The mean difference in clot lyses percentage between the positive and negative controls was very significantly ($p < 0.01$).

Actually, investigation on the toxicity of certain plants allows safety issue for use in further medicinal purpose and focuses on anti-microbial, anti-fungal, and anti-tumor activities (Das et al. 2010). The brine shrimp lethality bioassay is widely used and is quite inexpensive for assaying cytotoxicity of medicinal plants. Bioactive compounds are almost always toxic at higher dose. Thus, *in vivo* lethality in a simple zoological organism can be used as a reliable information for screening and fractionation in the discovery of new bioactive natural products. In the present bioactivity study, all the crude extracts, n-hexane, carbon tetrachloride, chloroform, crude extract, and aqueous soluble fractions of methanolic extract showed positive results, indicating that the test samples are biologically active (Ping et al. 2013). The mortality rates against the concentration are depicted in Figure 2.

Here, the linear regression analysis of LC_{50} and chi-square of all the plant sample extracts with standard VS, as presented in Table 1, is significant ($p < 0.005$). VS was used as the positive control, and LC_{50} was found to be 0.432 ± 0.02 μg mL^{-1} (Table 1) for VS compared with the negative control. VS (positive control) gave significant mortality, and the LC_{50} values of the different extractives were compared to this positive control. The LC_{50} values of the plant sample extracts were significant in PESF (1.18 ± 0.14 μg mL^{-1}) and ME (4.89 ± 0.091μg mL^{-1}). Also, CTCSF (11.88 ± 0.42 μg mL^{-1}), AQSF (23.09 ±

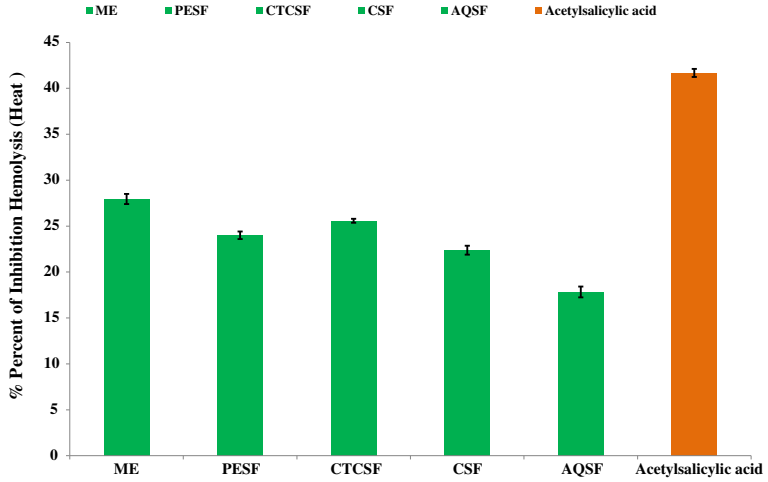

Figure 4 Heat-induced hemolysis of *Gynocardia odorata*. One-way ANOVA on Tukey's *post hoc* test with Dunnett's *t* test ($p < 0.05$).

2.01 μg mL^{-1}), and CSF (43.55 ± 2.50 μg mL^{-1}) showed moderate cytotoxic activity (Table 1).

Membrane-stabilizing attributes were acknowledged for their power to interpose with release of phospholipases that activate the establishment of inflammatory intercessors (Aitadafouri et al. 1996). The erythrocyte membrane matches to the lysosomal membrane, and the consequence of medication on the stabilization of erythrocyte would be inferred to the stabilization of the lysosomal membrane. Therefore, the membrane stabilizes which interferes in the release and the action of intercessors such as serotonin, histamine, leukotrienes, and prostaglandins (Latif et al. 2013). The main purpose of the anti-inflammatory agents is to reduce cyclooxygenase enzymes which convert arachidonic acid to prostaglandins. Different types of plants possess antiinflammatory activities. This study involved hypotonic solution- and heat-induced hemolysis in mice models on the basis of their membrane-stabilizing properties (Saleem et al. 2011; Umukoro et al. 2006).

The different plant sample extracts of *G. odorata* at a concentration of 1.0 mg mL^{-1} significantly saved the lysis of mice erythrocyte membrane induced by a hypotonic solution and at a temperature-induced condition (Figures 3 and 4), which is similar to that using the standard acetyl salicylic acid (0.10 mg mL^{-1}). At a concentration of 1.0 mg mL^{-1}, the reference standard acetylsalicylic acid has a 71.58 ± 0.33% inhibition. The hypotonic solution-induced percent inhibition of hemolysis is highest in ME (47.41 ± 0.46%), AQSF (45.77 ± 0.5%), and chloroform soluble fraction (40.055 ± 0.81). Also, the percent inhibition is relatively lesser in CTCSF (22.84 ± 0.52%) and PESF (18.445 ± 0.095%).

At the heat-induced condition, the lysis of mice erythrocyte membrane is the same as that using the standard acetyl salicylic acid (0.10 mg mL^{-1}). At a concentration of 1.0 mg mL^{-1}, the percent inhibition of the reference standard acetylsalicylic was 41.68 ± 0.46%. The hemolysis percent inhibition of the heat-induced solution is highest in ME (27.95 ± 0.55%), CSF (24.01 ± 0.41%), and AQSF (17.84 ± 0.59%). Also, the percent inhibition is relatively lesser in CTCSF (25.58 ± 0.22%) and PESF (24.01 ± 0.41%).

Conclusions

The different sample plant extracts of *G. odorata* were subjected to different biological investigations, i.e., thrombolytic activity, cytotoxic activity, and erythrocyte membrane-stabilizing activity. This plant is widely used in Bangladesh as a traditional folk medication. This plant has many significant cytotoxic and erythrocyte membrane-stabilizing activities. Also, some different plant sample extracts have abundant amount of thrombolytic activity. Therefore, considering the potential bioactivity, the plant materials can further be studied extensively to find out their undiscovered efficaciousness and to justify their use as traditional folk medication.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
AZB, MNA, and MT designed the experiment and carried out the experiment. FA contributed in composing the article. SRC and MAR supervised the work. All authors read and approved the final manuscript.

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