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ORIGINAL ARTICLE

Effects of Five Bangladeshi Plant Extracts on *In vitro* Thrombolysis and Cytotoxicity

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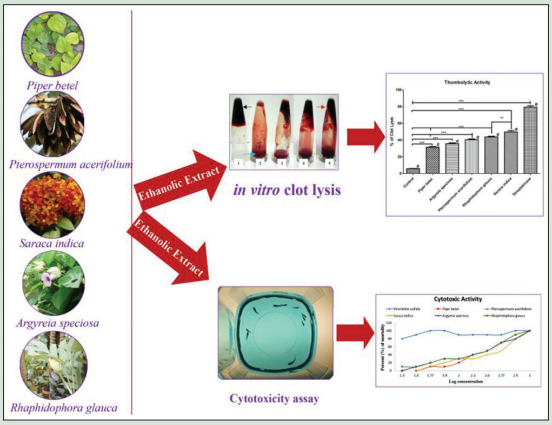
ABSTRACT

Background: *Piper Betle*, *Pterospermum acerifolium*, *Saraca indica*, *Argyreia speciosa*, and *Rhaphidophora glauca* are medicinal plants commonly used as traditional medicine for the treatment of various diseases. To examine, whether organic extracts of these plants possess thrombolytic properties with minimal or no toxicity is our main aim of the study. **Materials and Methods:** *In vitro* thrombolytic model was used to check the clot lysis effects using streptokinase (SK) as a positive control and water as a negative control. Cytotoxicity was screened by brine shrimp lethality bioassay using vincristine sulfate (VS) as positive control. **Results:** Among herbal drugs, different organic extracts of *P. betle*, *P. acerifolium*, *S. indica*, *A. speciosa*, and *R. glauca* showed significant ($P < 0.05$ and $P < 0.0001$) clot lysis activity viz., 31.58% ±0.76%, 40.50% ±0.94%, 49.70% ±1.69%, 35.81% ±0.86%, and 43.80% ±0.91%, respectively, compared to reference drug SK (79.32% ±1.629%). In brine shrimp cytotoxic assay, mortality achieved by the extracts showed lethal concentration 50 (LC_{50}) values 274.64 ± 3.46, 215.60 ± 4.59, 478.40 ± 6.98, 233.37 ± 2.56, and 209.32 ± 1.98 µg/ml, respectively, with reference to VS ($LC_{50,}$ 0.05 ± 0.34). **Conclusion:** In this study, *S. indica*, *R. glauca*, and *P. acerifolium* possessed effective thrombolytic activity. Further studies can be undertaken to identify certain structure of the ingredients in the extracts and to elucidate the precise mechanism of action.

Key words: *Argyreia speciosa*, *Piper betle*, *Pterospermum acerifolium*, *Rhaphidophora glauca*, *Saraca indica*, thrombolysis

SUMMARY

- Five Bangladesh medicinal plants, named Piper betle, *Pterospermum acerifolium*, *Saraca indica*, *Argyreia speciosa*, and *Rhaphidophora glauca* were subjected to comparative antithrombotic and toxicity based analysis. In comparative study, *Saraca indica* showed highest clot lysis (49.70 ± 1.69%) activity among the other plant with lowest toxicity (LC_{50}: 478.40 ± 6.98)



Abbreviations Used: h: Hour; min: Minutes; sec: Second; kg: Kilogram; g: Gram; µg: Microgram; L: Liter; mL: Millilitre; µL: Micro liter; µg/mL: Microgram per Milliliter; mg/kg: Milligram per kilogram; %: Percent; °C: Degree Celsius; *et al.*: et alliori (and others); w/w: Weight by Weight; v/v: Volume by Volume; SEM: Standard Error Mean; LC50: lethal concentration at 50%

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INTRODUCTION

Thrombosis is one of the three major causes of cardiovascular morbidity and mortality in the current world.[1] In simple words, thrombosis means localized clotting of the blood, which can take place within the arterial or the venous circulation and contains a significant medical impact.[2] Acute arterial thrombosis would be the proximal reason for many instances of myocardial infarction (stroke) in addition to about 80% of strokes. Venous thromboembolism is also the third leading reason behind cardiovascular-associated death.[3] The pathophysiology of arterial thrombosis is different from that of venous thrombosis as reflected because of the other ways in which these are treated. In broad terms, arterial thrombosis is given drugs that target platelets, and venous thrombosis is treated with drugs that target proteins in the coagulation cascade.[4,5] The accessible antithrombotic medicine is able at reducing arterial thrombosis and venous thrombosis in patients with heart problems. But these synthetic drugs cause bleeding, which limits their use and urge to discover new anticoagulant, antiplatelet and profibrinolytic agents from natural sources.[6,7] Although, due to low cost, urokinase, streptokinase (SK), or tissue plasminogen activators have been used as clinical thrombolytic agent for the treatment of severe or massive deep venous thrombosis, pulmonary embolism, myocardial infarction, and occluded intravenous or dialysis cannulas[8] in India, Bangladesh, and other developing countries in comparison to other thrombolytic drugs but restricted in some patients as a result of immunogenicity, severe anaphylactic, with a history of nervous lesions, gastrointestinal bleeding, or hypertension.[8,9] However, in recent years, herbal medicines

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have attracted more attention due to having combinations of bioactive ingredients which provide the synergistic effects in stroke.[10] Numerous pharmacological studies suggested that these herbal supplements or their corresponding products might dilate cardiocerebral vessels, suppress platelet aggregation, improve microcirculation in brain, protect against ischemic and reperfusion injury, possess neuroprotective properties, or help the tolerance of ischemic tissue to hypoxia.[11,12] Regarding this information, in our present studies, we tried to screen out the thrombolytic efficacy of some Bangladeshi plants and also their comparative studies.

Piper betle L. (*Piperaceae*), Pan in Bengali, leaf possess activity such as antidiabetic, antiulcer, antiplatelet aggregation, antifertility, cardiotonic, antitumor, antimutagenic, respiratory depressant, and anthelmintic.[13] It is used to treat alcoholism, bronchitis, asthma, leprosy, dyspepsia, and antioxidative property.[14] *Pterospermum acerifolium* (L.) Willd (Sterculiaceae) is generally recognized as Kanakchampa, traditionally used for hemostasis, inflammation, ear pain, stomach ache, blood troubles, smallpox, leucorrhea, leprosy, ulcer, and tumors and as an antihyperglycemic agent, laxative, and anthelmintic.[15] *Saraca indica* (*Caesalpiniaceae*), Ashoka in Bengali, is useful as a heart tonic, hypoglycemic agent, in the treatment of abdominal pain, tumors, AIDS, inflammation, and cancer.[16] *Argyreia speciosa* Linn. (Convolvulaceae) is widely distributed species in different parts of India. It has been reported to possess nootropic, aphrodisiac, immunomodulatory, hepatoprotective, antioxidant, anti-inflammatory, antihyperglycemic, antidiarrheal, antimicrobial, antiviral, nematocidal, antiulcer, anticonvulsant, analgesic, and central nervous system depressant activities.[17] The leaves of *A. speciosa* are emollient, vesicant, stimulant, and rubefacient and are traditionally used in the treatment of various skin diseases. The roots are beneficial in anemia, diabetes, obesity, syphilis, tuberculosis, cerebral disorders, and ulcer wound and are also used as aphrodisiac, anti-inflammatory, brain-tonic, cardiotonic, expectorant, digestive, carminative, and appetizer.[18] *Rhaphidophora glauca* (Wall.) Schott (*Araceae*) is found in Alutila, Khagrachari, Bangladesh. Literature showed that it is also available in Himalaya, Nepal, Mawlibhibazar (Syhlet, Bangladesh), etc., *R. glauca* enlisted as "New plant species and record of Bangladesh," which is published by Bangladesh National Herbarium, Ministry of Environment and Forest, Government of Bangladesh.[19]

This study aims to investigate the ethanolic extracts of five Bangladeshi medicinal plants viz. *P. betle*, *P. acerifolium*, *S. indica*, *A. speciosa*, and *R. glauca* for their clot lysis (thrombolytic activity) properties and cytotoxic properties by using *in vitro* models.

MATERIALS AND METHODS

Plant collection and identification

Leaves of *P. betle* (Accession No. 30853) were collected from the local market, leaves of *P. acerifolium* (Accession No. 30316) and *S. indica* (Accession No. 30726) were collected from the local area in Chittagong, whole plants of *A. speciosa* (Accession No. 30286) were collected from Hathazari, Chittagong and leaves of *R. glauca* (Accession No. 31862) were collected from Alutila, Khagrachari. The plants were authenticated as *P. betle, P. acerifolium*, *S. indica*, *A. speciosa*, and *R. glauca* by Dr. Shaikh Bokhtear Uddin, Taxonomist and Associate Professor, Department of Botany, University of Chittagong. The sample specimens of the identified plants have been preserved in the national herbarium with the mentioned accession numbers.

Chemicals and reagents

The test suspension was prepared by adding 5 ml sterile distilled water to the commercially available lyophilized SK vial (Durakinase, Dongkook Phama., Co., Ltd., South Korea) of 1,500,000 I.U. and mixed properly. This suspension was used as a stock from which 100 µl (30,000 I.U) was used for *in vitro* thrombolysis. Absolute ethanol (99.50%) and vincristine sulfate (VS) were purchased from Sigma-Aldrich, Munich, Germany.

Preparation of extracts

For the preparation of extracts, plant materials were air dried perfectly. Plant materials were washed properly. Then, it was chopped into small pieces and air dried for 7 days. After drying, plant powder was made by grinding. The plant materials were soaked into distilled organic solvents for 8 days. Within 10 days, the filtration is repeatedly done for 5 times with the interval of 2 days in a controlled temperature at (25°C ± 0.5°C). For the filtration, cheesecloth and Whatman filter paper number 1 was used and concentrated under reduce pressure at the temperature below 50°C using rotatory evaporator (RE 200, Bibby Sterling Ltd., UK). The concentrated extract was collected in glass petri dishes (90 mm × 15 mm, Pyrex, Germany) and allowed to dry for the complete evaporation of solvent at 37°C. The amount of extract found after extraction and their physical appearances are shown in Table 1. These concentrated extracts were used to investigate the antithrombotic activity of mentioned medicinal plants. A 100 mg each of the extracts was suspended in 10 ml distilled water, and the suspension was shaken vigorously on a vortex mixer. The suspension was kept overnight and decanted to remove the soluble supernatant, which was filtered through a 0.22 µm syringe filter. A 100 µl of this aqueous preparation was added to the microcentrifuge tubes containing the clots to check thrombolytic activity.[9] The same concentration (10 mg/ml) of extracts was prepared for screening the cytotoxic properties.

Antithrombotic effects

Blood specimen

From ten healthy human volunteers, 4 ml of whole blood was drawn without a history of oral contraceptive or anticoagulant therapy using a protocol approved by the Institutional Ethics Committee of Chittagong University. An earlier consent, approval number HET-CU2014/1, was taken from the faculty of medicine, University of Chittagong, for the collection of blood samples from Human volunteers. A 500 µl of blood was transferred to each of the seven previously weighed microcentrifuge tubes to form clots.

Consent of informed donor

A consent form with research project title, name, and details of investigators contacts as well as the purpose of the research were supplied to the volunteer donors. They were also supplied the detail description of the inclusion and exclusion criteria of the donors, whether donors will receive any therapy or not, the volume of blood to be taken, possible discomfort of the puncture sites, and time required for blood sampling. The explanation

Table 1: Different extracts of *Piper betle, Pterospermum acerifolium, Saraca indica, Argyreia speciosa, and Rhaphidophora glauca* and their physical properties

Plant name	Solvent	Powder	Crude extract	Yield (%)	Crude physical appearance
Piper betle	Ethanol	300	30.67	10.22	Green with reddish gummy mass
Pterospermum acerifolium	Ethanol	400	53.34	13.34	Deep green gummy mass
Saraca indica	Ethanol	600	84.97	14.17	Deep green with presence of arbitrary shaped crystals
Argyreia speciosa	Ethanol	600	33.63	5.60	Greenish semisolid
Rhaphidophora glauca	Ethanol	350	29.30	8.37	Greenish black

was made on if future use of the research data beyond the current study is anticipated, whether this is a focus group if so the principal investigator should put a procedure in place in which the researchers caution people about the limit on confidentiality. Access to research information is regarding who would have access to the collected sample, information regarding retention of sample and schedules for their disposal were also detailed. It was indicated to the consent form that the volunteers might refuse to donate blood at any time. Donor whether could withdraw his sample data was disclosed. The sample was restricted for that individual study not for future research projects was presented in the consent form. Possible complications, for example, the possibility of bruising or swelling while giving blood or any other discomforts at the site where blood is drawn and that there might be minimal chance of infection and that these discomforts were brief and transient were also informed. The potential benefits of this study, not directly of the donors, but the society in general or individuals with a similar condition might be benefitted from the results of the study was explained. Confidentiality statement was included in the consent form in the way that confidentiality will be respected and no information that discloses the identity of the participant will be released or published without consent unless required by law of states. The signatures with the date of the donors were also included in the consent form.

Determination of clot lysis

Clot lysis approaches were carried out as reported earlier.[20] A 4 ml venous blood drawn from the healthy volunteers was distributed in ten different preweighed sterile microcentrifuge tubes (0.5 ml/tube) and incubated at 37°C for 45 min. After clot formation, serum was completely removed without disturbing the clot and each tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube – weight of the tube alone). About 100 µl of different extracts of *P. betle*, *P. acerifolium*, *S. indica*, *A. speciosa*, and *R. glauca* were added separately. As a positive and negative control, 100 µl of SK and distilled water, respectively, were added separately. All the tubes were then incubated at 37°C for 90 min and observed for clot lysis. The released fluid was removed and tubes 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 a percentage of clot lysis. The experiment was repeated with the blood samples of the informed donors.

Cytotoxicity assay

Brine shrimp bioassay was carried out to investigate the cytotoxicity of the extracts.[21] to investigate the cytotoxicity of the extracts. The dried extract preparations were re-dissolved in dimethyl sulfoxide to obtain a solution of 10 mg/ml which was subjected to serial dilution to get the concentrations between 20 µg/ml and 800 µg/ml. A 5.0 ml of artificial seawater was added to all the test tubes. Simple zoological organism (*Artemia salina*) was used as a convenient monitor for cytotoxic screening. The eggs of the brine shrimps were collected from the Institute of Marine Science and Fisheries, University of Chittagong, Bangladesh, and hatched in artificial seawater (prepared by using sea salt 38 g/L and adjusted to pH 8.5 using 1N NaOH) under constant aeration for 24 h under the light. The hatched shrimps were allowed to grow by 48 h to get shrimp larvae called nauplii. After 48 h, active nauplii were attracted to one side in a glass petri dish by using a micropipette. The nauplii were then separated from the eggs by allocating them in another glass petri dish containing artificial seawater and used for the assay. Suspension containing twenty nauplii was added to each test tube and was incubated at room temperature (25°C ± 1°C) for 12 h under the light. The tubes were then examined after 24 h and the number of surviving larvae in each tube was counted with the aid of a × 3 magnifying glass. Experiments were conducted along with VS in a set of three tubes per dose. The concentration that would kill 50% of the nauplii lethal concentration 50 (LC_{50}) was determined from a linear regression equation using the software "BioStat-2009."

Statistical analysis

All the data were shown as a mean ± standard deviation and statistical analysis was performed using paired *t*-test analysis and one-way analysis of variance followed by Tukey multiple comparison test with $P < 0.05$ taken as significant (GraphPad Prism 5.0, GraphPad Software, Inc, USA) for thrombolytic assay. LC_{50} values by VS and extracts was tested by the paired *t*-test analysis using the software SPSS, version 19.0 (SPSS for Windows, Version 18.0, IBM Corporation, NY, USA).

Percentage clot lysis = (weight of the clot after lysis by sample and removal of serum/weight of the clot before lysis by sample) ×100.

RESULTS

As described in Table 2, different organic extracts of five medicinally important plants were used for the investigation of antithrombotic effects in human blood sample. In this approach, addition of 100 µl SK (30,000 I.U.) to the clots and subsequent incubation for 90 min at 37°C showed 79.32% ±1.62% clot lysis. On the other hand, distilled water was treated as negative control which showed only 5.86% ±0.30%, a negligible clot lysis. The mean difference in clot lysis percentage between positive and negative control was very significant ($P < 0.0001$ and <0.05, respectively). The most significant ($P < 0.0001$) clot lysis activity was observed in the ethanol extract of *S. indica* (49.70% ±1.69%) among the other extracts. *R. glauca* were showed 43.80% ±0.91% of clot lysis and its $P < 0.0001$. *P. acerifolium* and *A. speciosa* were showed 40.50% ±0.94% and 35.81% ±0.86%, respectively, which was almost similar and also offered significant ($P < 0.0001$ and 0.05, respectively) clot lysis. *P. betle* also showed 31.58% ±0.76% and gave the significant ($P < 0.0001$) clot lysis activity. Again, all the effects of the crude extracts including the positive and negative control were subjected to further comparative based study, where the multiple comparison test was done by Tukey post hoc analysis. From the analysis, it was found that SK showed the most significant effect in comparison to others. In the case of plant extracts, *S. indica* showed the most significant effect in comparison to other plant and negative control group, but moderate significant ($P < 0.01$) in the case of *R. glauca*, Again, *R. glauca* and *P. acerifolium* showed the most significant effect in comparison to *P. betle* and negative control group but not significant when compared to each other. Yet again, *A. speciosa* showed a significant effect in comparison to negative control but not significant to *P. betle* where *P. betle* showed the only significant effect with negative control. The comparative results in a summary are shown in Figure 1.

The regression analysis for brine shrimp bioassay was presented in Table 3. The comparative mortality of brine shrimp and LC_{50} values of different extract are rendered in Figures 2 and 3, respectively. No extract is discovered for being significantly toxic in comparison with positive control.

Table 2: Effect of herbal extracts on *in vitro* clot lysis

Herb/drug	Percentage clot lysis (mean±SD)	*P*
SK (positive control)	79.32±1.62	<0.0001
Water (negative control)	5.86±0.30	<0.0001
Piper betle	31.58±0.76	<0.0001
Pterospermum acerifolium	40.50±0.94	<0.0001
Saraca indica	49.70±1.69	<0.0001
Argyreia speciosa	35.81±0.86	<0.0001
Rhaphidophora glauca	43.80±0.91	<0.0001

Values are mean±SD, (n=10); #P<0.0001, statistical representation of the effective clot lysis percentage by herbal preparations, positive thrombolytic control (SK), and negative control (sterile distilled water) processed by paired *t*-test analysis. SD: Standard deviation; SK: Streptokinase

DISCUSSION

It is well known that the incidence of cardiovascular disease (CVD) remains the leading cause of death, with high morbidity and mortality.[22] However, the management of major risk aspects with conventional drugs is effective in reducing cardiovascular events, and this is supported by extensive evidence from clinical trials.[23] Some are modified further with the use of recombinant technology[24] to make these thrombolytic drugs more site specific and effective. Side effects related to these drugs have been reported that lead to further complications.[25] Recently, there exists growing awareness of the place of dietary factors and herbal supplements for the prevention of CVD and also the chance for their use within the treatment. A lot of this interest centers around the antioxidant vitamins as well as the antioxidant properties of food constituents and herbal materials, but some herbal materials can also improve conventional cardiovascular risk factors or have antithrombotic effects.[26,27] Herbal preparations, if taken in the appropriate dose, can lead to a better option for curing various ailments. In our study of thrombolysis, we have tried five herbal extracts that have been used since ancient times for various diseases. The comparison of positive control with negative control in our thrombolytic assay, it was undoubtedly confirmed that if water was added to the clot, clot dissolution does not occur. When compared with the clot lysis percentage obtained through SK and water, a significant ($P < 0.05$ and $P < 0.0001$) thrombolytic activity was observed after treating the clots with *P. betle*, *P. acerifolium*, *S. indica*, *A. speciosa*, and *R. glauca* extracts. Ethanol extract of *S. indica* showed relatively higher thrombolytic activity among the selected plants. *P. acerifolium* and *R. glauca* were also shown a significant effect but lower than *S. indica*. However, it could be suggested that *S. indica*, *R. glauca*, and *P. acerifolium* can be a promising source of natural drug which has shown a commendable antithrombotic activity.

Table 3: Calculation of lethal concentration 50 values, confidence limits, regression equations, and Chi-square values for different extracts with reference to vincristine sulfate

Sample	LC_{50} (µg/ml)	Range of confidence limit (µg/ml)	Regression equation	χ^2
Vincristine sulfate	0.05±0.34	0.37-0.85	Y=5.93x+78.88	0.36
Piper betle	274.64±3.46	198.39-387.18	Y=61.14x–96.49	2.28
Pterospermum acerifolium	215.60±4.59	138.60-350.68	Y=52.86x–74.03	3.25
Saraca indica	478.40±6.98	345.78-611.01	Y=52.09*x–79.45	0.93
Argyreia speciosa	233.37±2.56	156.74-361.99	Y=51.49x–71.00	1.15
Rhaphidophora glauca	209.32±1.98	137.86-328.18	Y=52.12x–70.40	1.51

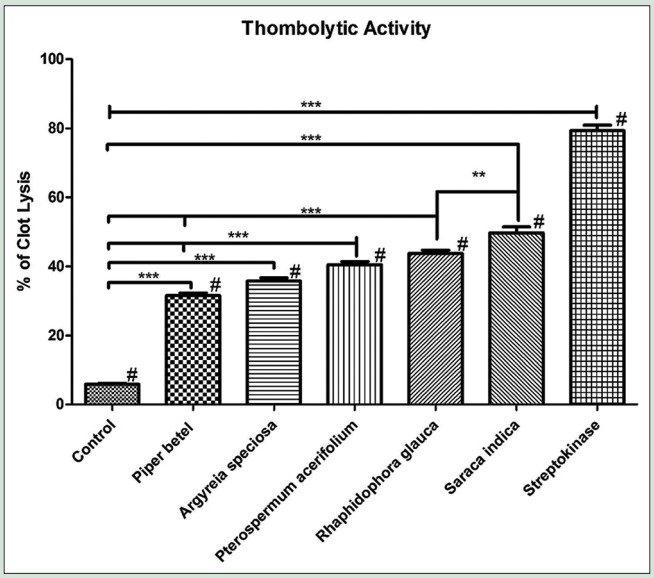

Figure 1: Clot lysis by streptokinase, water, and various organic extracts. Maximum clot lysis (79.32% ±1.62%) was observed in clot treated with streptokinase. Among herbal drugs, the ethanol extract of *Saraca indica* showed highest significant ($P < 0.05$ and $P < 0.0001$) clot lysis activity viz., 49.70% ±1.69%. Sterile distilled water (as a negative control) showed 5.86% ±0.30% clot lysis. Values are mean ± standard deviation, ($n = 10$); #$P < 0.0001$ (two-tailed, processed by paired *t*-test analysis); ***$P < 0.0001$, **$P < 0.01$, resulted by one-way analysis of variance followed by Tukey multiple comparison test

Toxicity of plant materials is a major concern to scientists and medical practitioners.[28,29] Brine shrimp toxicity is a universal bioassay that perceives a broad range of biological activities and a variety of chemical structures. One basic principle is that toxicology is the deal with the pharmacology of a drug with higher doses. If any toxic compound process nontoxic at a lower, dose might elicit a useful, pharmacological, and perturbation on a physiologic system.[30] In all-purpose, the lesser the LC_{50} value, the more toxic the chemical is. The reverse is also true: The larger the LC_{50} value, the lower the toxicity. Again, a great[31] correlation ($r = 0.85$; $P < 0.05$) relating to the LC_{50} with the brine shrimp lethality ensure that the acute oral toxicity assay in mice. The correlation demonstrated that brine shrimp lethality $LC_{50} < 10$ µg/ml (LD_{50} between 100 and 1000 mg/kg) is regarded as being the cutoff value of cytotoxicity. Based on our study, compared to the positive control, no extract was found to have significant toxicity. However, the toxicity of the crude plant extracts is recognized to the phytoconstituents present in them. Moreover, with the age of the plant, intraspecies variation and time of the season, locality, extraction method used and length of storage time presence, and length of storage time usually vary the concentration of bioactive ingredients.[32]

CONCLUSION

Our study found a significant thrombolytic activity for *P. betle*, *P. acerifolium*, *S. indica*, *A. speciosa*, and *R. glauca* plant which may provide a therapeutic approach for the treatment of thrombosis. However, further studies are needed to be undertaken to identify certain structures of the ingredients in the extracts and to elucidate the precise mechanism of action.

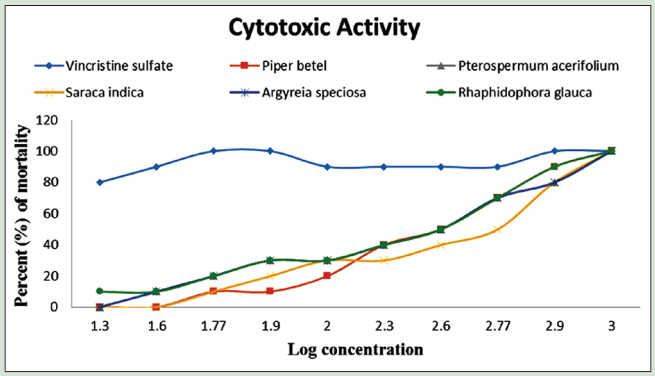

Figure 2: Brine shrimp mortality by vincristine sulfate and different organic extracts. Percent mortality of brine shrimps of five plant extracts and standard cytotoxic agent vincristine sulfate. Data are shown as a mean ± standard deviation of ten shrimps for each concentration. Mortality achieved by the extracts of *Piper betle, Pterospermum acerifolium*, *Saraca indica*, *Argyreia speciosa*, *Phaphidophora glauca* are lower than that by vincristine sulfate. Data were processed with Tukey's *post hoc* test for multiple comparisons, SPSS for Windows, version 18.0, $P < 0.05$ from each other

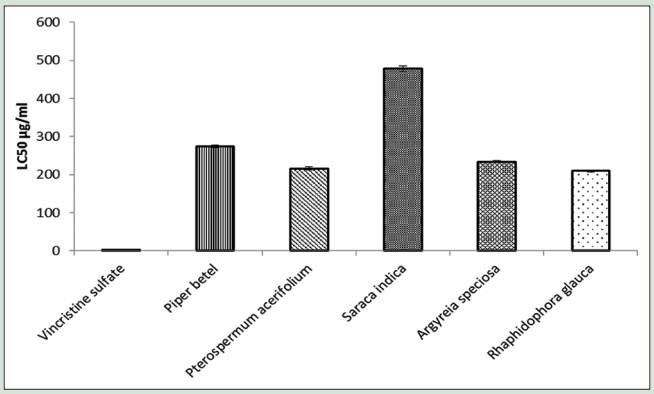

Figure 3: Brine shrimp lethality by vincristine sulfate and different organic extracts. Lethal concentration 50 of vincristine sulfate and different organic extracts for brine shrimp nauplii (*Artemia salina*). Data are shown as a mean ± standard deviation of ten shrimps for each concentration. Mortality achieved by the extracts of *Piper betle. Pterospermum acerifolium*, *Saraca indica*, *Argyreia speciosa*, and *Rhaphidophora glauca* showed lethal concentration 50 values 274.64 ± 3.46, 215.60 ± 4.59, 478.40 ± 6.98, 233.37 ± 2.56, and 209.32 ± 1.98 µg/ml, respectively, with reference to vincristine sulfate (lethal concentration 50, 0.05 ± 0.34). The data were processed with Tukey's *post hoc* test for multiple comparisons, SPSS for Windows, version 18.0, $P < 0.05$ from each other

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Nil.

Conflicts of interest

There are no conflicts of interest.

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