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Preventive activities against thrombosis and inflammation of *Butea monosperma* (Lam.) leaves methanolic extract *in vitro* model

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ABSTRACT

Aim: To find out the biological activities such as thrombolytic, membrane stabilizing activity, and also quantification of total phenol content *in vitro* model from the leaves extracts of *Butea monosperma*.

Method: The collected leaves were dried and ground. About 250 g of powdered leaves were soaked in 2.5 L (1:2.5 w/v) of 90% methanol for 14 days at room temperature, 25 ± 1°C with irregular shaking. The dark brown color medium was filtered using a filter funnel and hand-held mechanical vacuum pump. The filtrate was dried using rotary evaporator at 120–160 rpm low pressure and temperature of 40°C. The recovered solvent was reused for three successive refluxes carried after every 24 hours of additional soaking. Following the concentration, crude methanol extract (CME) was partitioned into petroleum ether (PESF), carbon tetrachloride (CTSF), chloroform (CSF), and aqueous soluble fractions (AQSF), respectively, by the Kupchan methods. Then all extracts were subjected to evaluate *in vitro* thrombolytic by Daginawala, membrane stabilizing activity by Okoli and Omale, and total phenol content followed by Folin-Ciocalteu methods.

Result: In thrombolytic assay among the extractives, petroleum soluble fraction showed the highest percentage of clot lysis (27.36 ± 0.10%) activity while the standard streptokinase exhibited the lysis of clot by 65.15 ± 0.16%. In case of membrane stabilizing activity, crude methanolic extract profoundly inhibited the lysis of erythrocyte membrane (65.79 ± 0.40%) induced by osmosis, whereas reference standard acetyl salicylic acid resulted in (83.147 ± 0.39%) inhibition of lysis. On the other hand, AQSF showed slightly higher level of membrane stabilizing activity in the heat-induced hemolysis (75.3 ± 0.39). The petroleum soluble fraction was found to have significant level of total phenolic content (249.06 mg of GAE/gm).

Conclusion: This study was conducted to validate the *B. monosperma* leaves used as a folk medicine for the ailment of thrombosis, inflammation such as thrombolytic and membrane stabilizing.

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KEYWORDS

Butea monosperma; thrombolytic; membrane stabilizing activity; total phenolic content

Introduction

Medicinal plants always provide new substances, e.g., antibiotics, alkaloids, cardiac glycosides, quinines, phenols, flavonoids, and saponins, having many biologiactive functions [1]. Thrombosis is an internecine disease which is manifested by the development of a blood clot (thrombus) in the circulatory system because of the inconsistency of homeostatic system of the body. It leads to vascular blockade and while recovering causes deadly outcomes, such as myocardial infarction and even death [2]. Streptokinase (SK) is widely used as fibrinolytic drug and was used in this study as reference standard. Generally, this drug which breaks the thrombus, work by activating the enzyme plasminogen to plasmin that clears the cross-linked fibrin mesh [3]. The possible mechanisms for the membrane stabilizing effect of medicinal plants were not known, but almost all researchers believed

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that the presence of significant amount of bioactive compounds, such as flavonoids and other phenolic compounds may have been primarily responsible for analgesic and anti-inflammatory activity [4]. In modern healthcare system, people have started looking at the ancient healing systems such as Ayurveda, Siddha, and Unani to treat the various types of ailments due to the serious adverse effects associated with synthetic drugs. Herbal traditional medicines have gained considerable momentum during the past decade and play a paramount role in healthcare systems especially in developing countries [5]. Organic compounds from terrestrial and marine organisms have extensive past and present use in the treatment of many diseases and serve as compounds of interest both in their natural form and as templates for synthetic modification [6]. *Butea monosperma* is a small to medium-sized deciduous tree, 5–15 (maximum 20) m tall which is native to tropical and sub-tropical parts of the Indian Subcontinent and Southeast Asia, ranging across India, Bangladesh, Nepal, Sri Lanka, Myanmar, Thailand, Laos, Cambodia, Vietnam, Malaysia, and western Indonesia. Common names include flame-of-the-forest and bastard teak [7]. Different parts of *B. monosperma* are extensibly used in Ayurveda, Unani, and Homeopathic medicines and have become a cynosure of modern medicine. The plants of this genus are well known for their coloring matters. Commonly, *B. monosperma* is used as tonic, astringent, aphrodisiac and diuretics. It has been reported that the aerial part of this plant contain different important phytochemicals such as triterpene, flavanoids, monospermoside, isospermoside, dihydromonospermoside, chalcones, aurones, isobutyine, and a new bioactive flavone glycoside (5,7-dihydroxy-3,6,4'-trimethoxyflavone-7-O-alpha-L-xylopyranosyl-(1→3)-O-alpha-L-arabinopyranosyl-(1→4)-O-beta-D-galactopyranoside). Roots are useful in treating filariasis, night blindness, helminthiasis, piles, ulcers, and tumors. It is reported to possess anti-fertility, aphrodisiac, and analgesic activities. Flowers are useful in diarrhea, astringent, diuretic, depurative, tonic, leprosy, skin diseases, gout, thirst, and burning sensation. The stem leaves are useful in indigenous medicine for the treatment of dyspepsia, diarrhea, dysentery, ulcer, sore throat, and snake bite. Besides medicinal uses, it is also having the economic use such as leaves are used for making platters, cups, and bowls. Seeds of *B. monosperma* are used in inflammation, skin and eye diseases, bleeding piles, urinary stones, abdominal troubles, intestinal worms and tumor. When seeds are pounded with lemon juice and applied to the skin, they act as a rubefacient [8]. Current research activities have been targeted on phytochemical evaluation of the plants which have traditional, ethno-botanical, and folkloric importance for drug discovery [9]. Due of its availability and traditional history of use, *B. monosperma* leaves presented in Figure 1 were undertaken for the exploration of thrombolytic, membrane stabilizing activity, and total phenolic potential.

Materials and Methods

Drugs and chemicals

SK (1,500,000 I.U. Alteplase) vial was warmly gifted from Beacon Pharmaceuticals Ltd., Bangladesh. Gallic acid (GA), phosphate buffer, sodium hydroxide, hydrochloric acid, sodium carbonate, acetyl salicylic acid (ASA), solvents (methanol, petroleum ether, carbon tetrachloride, chloroform), and also other necessary chemicals and reagents were of analytical grade and collected from local sources.

Collection, identification, drying, and grinding of plant materials

The stem leaves of *B. monosperma* were collected from Suhrawardy Udyan, Dhaka, Bangladesh, in July 2016, and identified by a taxonomist from Bangladesh National Herbarium and then plant specimen was provided with an accession number, DACB-43405. The collected leaves were cleaned properly to remove unwanted dust and dirt followed by dried in a shady place and milled by locally made grinder in order to get a uniform coarse powder. After that milled coarse powder, 210 g was stored in a hermetic glass container till extraction.

Extraction and fractionation of plant materials

The powdered plant material (210 g) was socked in 2,500 µl of 90% methanol and allowed to stand with irregular shaking for 14 days and then the extract was filtered through filter paper. The liquid extract was condensed and dried by a vacuum rotary evaporator (Rotary Vacuum Evaporator,

Figure 1. Photography of the leaves of *B. Monosperma* together with an image of the initial coarse powder (leaves).

Delhi) at 40°C for 50 minutes. After that, concentrated crude methanol extract (CME # 52.2 g) was fractionated using the Kupchan protocol [10], and modified by Van Wagenen method [11]. The fractionated amounts were, petroleum ether soluble fraction (PESF # 18.2 g), carbon tetrachloride fraction (CTSF # 13 g), chloroform soluble fraction (CSF # 11.1 g), and aqueous soluble fraction(AQSF # 9.9 g), respectively.

Phytochemical screenings

All extracts were qualitatively tested by using standard methods established by Sofowora [12]. This method supported us to ensure the qualitative existence of phytoconstituents such as alkaloids, glycosides, steroids, flavonoids, tannins, resins, and phenols in the CME and its fractions.

Estimation of total phenolic content

Total phenolic contents (TPCs) were determined by the modified Folin-Ciocalteu method [13]. An aliquot (2 mg/µl) of the extract and its fractions were mixed with 2 µl Folin-Ciocalteu reagent (previously diluted with water 1:10 v/v) and 2 µl 7.5% of sodium carbonate. For proper mixing, the tubes were vortexed for 30 seconds and allowed to stand for 20 minutes at 25°C for colour development. Absorbance was then measured at 760 nm by UV-spectrophotometer (Shimadzu, USA). Samples of extracts and standard were evaluated at a final concentration of 0.1 mg/µl. TPC were expressed in terms of GA equivalence (GAE; standard curve equation: $y = 0.096x + 0.046$, $R^2 = 0.999$), mg of GA/g of dry extract [14].

Streptokinase solution preparations

Lyophilized SK vials of 1,500,000 I.U. are commercially available on market. One vial was collected and 5 µl sterile distilled water was poured and mixed properly by shaking. This suspension was used as a reference stock from which 100 µl (30,000 I.U.) was used for *in vitro* thrombolytic potential.

In vitro thrombolytic activity

The CME and its fractions were assessed for thrombolytic potential by means of human blood, according to the method previously described by Daginawala et al. [15]. In thrombolytic study, 5 µl of venous blood was collected from healthy male volunteers and transferred into five pre-weighed micro-sterile centrifuge tubes (1 µl/tube) followed by incubation at room temperature for 45 minutes to ensure clot formation. From formed clot, the serum was separated successfully and the tubes were again weighed. CME and each of the extractive suspension (2 mg/1,000 µl of water) was poured in every micro-centrifuge tube containing blood clot and allowed to keep them to stand at room temperature without any disturbance. 100 µl of SK (equivalent to 30,000 I.U.) and 1,000 µl of distilled water were used as positive and negative controls, respectively. After 90 minutes of incubation, the soluble supernatant was decanted carefully from the decantation. Tubes were further weighed to measure the clot lysis. The percentage of clot lysis was determined using the following equation:

$$\text{Clot lysis (\%)} = (\text{weight of clot after treatment}/\text{weight of clot before treatment}) \times 100.$$

Justification of streptokinase use

SK is a thrombolytic medication and enzyme. As a medication it is used to break down the blood clots [16].

In vitro membrane stabilizing activity

The membrane stabilizing activity of the extract was assessed by evaluating their ability to inhibit the breakdown of human red blood cells, provoked osmotically by hypotonic solution or heat by the method developed by Omale and Okafor [17].

Preparation of blood samples for membrane stabilization assays

Two milliliters of blood was withdrawn from healthy male volunteers having hemoglobin within range and free from diseases (using a protocol approved by Ethical Committee of WUB Research Section, World University of Bangladesh). The collected samples were poured into sterile test tubes containing dipotassium salt of ethylenediaminetetraacetic acid (EDTA) (2 mg/µl of blood) under standard conditions of temperature 24 ± 2°C and relative humidity 55 ± 10%. An aliquot of 1.0 µl of blood was transferred into 1.5 µl micro-centrifuge sterile tubes and then centrifuged at 2,500 rpm for 10 minutes; thereafter, the tubes were allowed to stand for 5 minutes. The supernatant was separated; remaining cell suspension was diluted with equal volumes of sterile normal saline (0.9% NaCl) and centrifuged three times, consecutively, to ensure clear supernatant. The clear supernatant was decanted and remaining RBC cells were undertaken for membrane stabilizing activity testing. Finally, cellular components (erythrocyte suspension, 0.5 µl) were mixed with 5 µl hypotonic

solution (50 mM NaCl in 10 mM sodium phosphate buffered saline, pH 7.4) for *in vitro* membrane stabilizing assay.

Justification of dipotassium salt of EDTA use

Dipotassium salt of EDTA is a highly water-soluble salt. It is used here to prevent the coagulation of blood *in vitro*.

Hypotonic solution-Acetyl salicylic acid induced hemolysis

CME and their fractions (2 mg/µl each) and acetyl salicylic acid (0.1 mg/µl) were taken in different sterile centrifuge tubes contained 0.5 µl stock erythrocyte RBC suspension and mixed with 5 µl hypotonic solution by a sonicator. The control sample was prepared without reference standard (ASA). The mixtures were centrifuged for 10 minutes at 3,000 rpm and incubated for 10 minutes at ambient temperature, followed by separation of supernatant by decantation. The absorbance of supernatant was measured at 540 nm using UV/Visible spectrophotometer.

The percentage inhibition of hemolysis was estimated using the equation:

$$\% \text{ inhibition of hemolysis} = 100 \times (OD_1 - OD_2/OD_1)$$

where OD_1 is the optical density of hypotonic-buffered saline solution alone (control) and OD_2 is the optical density of test sample in hypotonic solution.

Heat-induced hemolysis

The heat-induced hemolysis of erythrocytes was conducted according to a method of Okoli *et al.* with little modifications [18]. Two duplicate sets of centrifuge tubes were considered, each containing 2 mg/µl of different extractive solutions and 5 µl of buffered isotonic NaCl solution. One set of centrifuge tubes were prepared by using 5 µl of isotonic buffered solution for heating conditions plus ASA at a concentration of 0.1 mg/µl for positive control and other set regarded as negative control containing only 5 µl of the isotonic buffered solution. Erythrocyte suspension (30 µl) was added to all tubes and mixed gently by inversion. One set of tubes was incubated in a water bath at 54°C for 20 minutes and the other set of tubes were kept on ice at 0–5°C. At the end of the incubation period, the samples were subjected to centrifuge for 10 minutes at 3,000 rpm and the absorbance of the supernatant was measured at 540 nm using UV/Visible spectrophotometer. The percentage inhibition of hemolysis was estimated using the equation:

$$\% \text{ inhibition of hemolysis} = 100 \times [1 - (OD_2 - OD_1/OD_3 - OD_1)]$$

where OD_1 is the test sample unheated, OD_2 is the test sample heated, and OD_3 is the control sample heated.

Justification of ASA used

ASA acts either by inhibiting the action of lysosomal enzymes or by stabilization of lysosomal membranes.

Statistical analysis

The denouements were expressed as mean ± SEM. For statistical and graphical evaluation, SPSS software (version 20) and Microsoft excel 2010 were used. Values of investigations were compared with control or standard which was performed by One-way ANOVA followed by Dunnett's *t*-test.

Results

Phytochemical screening test

The qualitative phytochemical screenings revealed the presence of flavonoids, tannins, steroids, phenols, and other chemical substances (Table 1), which may be the important factors for

Table 1. Phytochemical screening of *B. monosperma* leaves extract and its fractions.

Phytochemicals	CME	PESF	CTSF	CSF	AQSF
Alkaloids	++	–	+	++	++
Carbohydrates	–	–	–	–	–
Saponins	–	–	–	–	–
Tannins	+	+	+	+	–
Resins	+++	+	+++	–	–
Flavonoid	++	+	–	+	–
Steroids	++	++	++	-	–
Phenols	++	++	+	+	+

Based on intensity of the color, where + = present in mild amount, ++ = present in moderate amount, +++ = present in large amount, – = not present. CME: crude methanol extract; PESF: petroleum ether soluble fraction; CTSF: carbon tetrachloride soluble fraction; CSF: chloroform soluble fraction; and AQSF: aqueous soluble fraction.

the anti-inflammation, anti oxidation, and clot lysis potential.

Total phenolic contents determination

In this study, the PESF exhibited the highest phenolic content (249.06 mg of GAE/gm), whereas CTSF showed the lowest amount of phenolic compounds (150.13 mg of GAE/gm) among all the Kupchan fractionates. But the other factions also showed considerable quantities of phenolic compounds (Table 2, Fig. 2). In the phytochemical qualitative screenings, the presence of phenols, flavonoids, tannins, and others chemicals ensure the leaf extracts of *B. monosperma* would be pharmacologically potential.

Thrombolytic activity

The CME and extractives of leaves of *B. monosperma* were evaluated for thrombolytic activity against a positive control SK [19,20] which showed 65.15 ± 0.16% lysis of clot. On the other hand, sterile distilled water, a negative control, exhibited a negligible percent of lysis of clot (8.20 ± 0.16%). The percentages of clot lysis by various fractions were observed in the following order: PSF (27.36 ± 0.23%), AQFS (21.64 ± 0.25%), CTSF (20.74 ± 0.43%), CSF (19.90 ± 0.43%), and CME (12.88 ± 0.33%). Interpreting the results, it may be assumed that (considering >20% moderate); (**$p < 0.01$; *$p < 0.05$) *B. monosperma* leaf extract exhibited moderate thrombolytic activity as presented in Table 3 and Figure 3.

Membrane stabilizing activity

At the concentration of 2 mg/µl, the leaf extract of *B. monosperma* and its fractions significantly inhibited hemolysis of human RBC membrane induced by hypotonic-solution and heat as compared to the standard ASA. The membrane stabilizing activity (Table 3) against heat-induced hemolysis was observed in the following order: ASA (83.55 ± 0.28%), AQSF (75.73 ± 0.48%), CSF (72.33 ± 0.39%), CME (71.70 ± 0.11%), PSF (64.70 ± 0.11%), and CTSF (53.48 ± 0.75%). In hypotonic solution-induced hemolysis, the values were: ASA (83.14 ± 0.39%), CME (65.79 ± 0.47%), PSF (60.67 ± 0.18%), AQSF (57.72 ± 0.40%), CTSF (54.77 ± 0.70%), and CSF (49.24 ± 0.36%). Analyzing the result, it may be assumed that (considering >60% excellent); (**$p < 0.01$; *$p < 0.05$) *B. monosperma* leaf extract exhibited excellent membrane stabilizing activity as presented in Table 3 and Figure 4.

Table 2. Total phenol contents of crude methanolic extract and its partitionates of *B. monosperma*.

Sample/standard	Total phenol contents (mg of GAE/gm)
AQSF	235.94
CTSF	150.13
CME	210.06
PESF	249.06
CSF	272.11

Discussion

The CME of *B. monosperma* leaves and their Kupchan partitionates were under taken to test for the presence of different bioactive compounds. Different phytochemicals such as alkaloids, glycosides, steroids, flavonoids, tannins, resins, phenols, and saponins were tracked out in the tested fractionates. Phytoconstituents characterized in the current study are known to be beneficial in medicinal sciences. The knowledge of this preliminary investigation can be anticipated as decipher in the search of novel and economically valued drug molecules [21,22].

Total phenolic content

The total phenol contents were shown in Table 2. The decision of quantifying TPC in the CME and fractions of AQSF, PSF, CSF, and CTSF were taken after the assurance of the presence of phenols in the preliminary phytochemical investigation. In this study, the PESF exhibited the highest phenolic content (249.06 mg of GAE/gm) where CTSF showed the lowest amount of phenolic compounds (150.13 mg of GAE/gm) among all the Kupchan fractionates. But the other fractions also showed an order of PSF (249.06) > AQSF (235.94) > CME (210.06) with considerable quantity of phenol. Outcome of this study reveals that *B. monosperma* leaf extract contained satisfac-

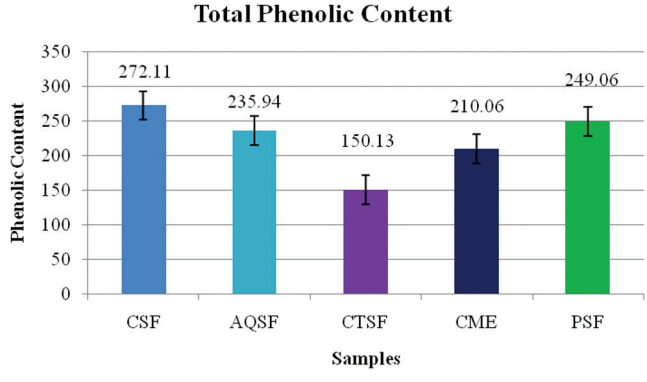

Figure 2. TPC exhibited by different Kupchan fractionates of B. *monosperma*.

Table 3. Membrane stabilizing and thrombolytic activity of the crude methanolic extract and different fractions of *B. monosperma* leaves. Membrane lysis was induced by hypotonic solution (solution having tonicity lower than blood plasma) and by applying heat. Blood was kept at room temperature for the formation of clot.

Sample	Percent inhibition of hemolysis		Percent of clot lysis
	Hypotonic solution-induced	Heat-induced	
CME	*65.79 ± 0.47	**71.70 ± 0.11	12.88 ± 0.33
PSF	*60.67 ± 0.18	*64.70 ± 0.32	*27.36 ± 0.23
CTSF	*54.77 ± 0.70	53.48 ± 0.75	*20.74 ± 0.43
CSF	49.24 ± 0.36	**72.33 ± 0.39	19.90 ± 0.43
AQSF	*57.72 ± 0.40	**75.73 ± 0.48	*21.64 ± 0.25
ASA	**83.14 ± 0.39	**83.55 ± 0.28	–
SK	–	–	**65.15 ± 0.16
Negative control	–	–	8.20 ± 0.16

Values are expressed as mean ± SEM (Standard Error of the Mean), *$p < 0.05$; **$p < 0.01$; significant when compared with the corresponding value of standard and negative control.

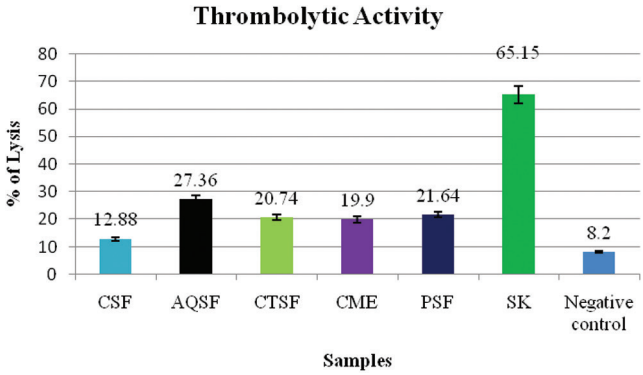

Figure 3. Thrombolytic activity of methanolic extract and its Kupchan fractionates of *B. monosperma.*

tory amount of phenols, from the literature reviewed we found that phenol has ability to interact with proteins and inhibit oxidation and ionic imbalance are among the properties which qualifies to have anti-inflammatory and antioxidant activity.

Thrombolytic activity

Thrombosis process is initiated by activating platelets and also binding to the leucocytes that brings them into a formation and growth of a plaque [23]. Plasmin is a fibrinolytic agent by nature. Plasminogen is easily triggered to plasmin, which could lead to fibrinolysis [24]. SK (a bacterial plasminogen activator) with plasminogen is capable of converting to plasmin [25]. Numerous thrombolytic drugs have been adapted with recombinant technology in order to make those thrombolytic drugs more site specific and effective [26], but some adverse effects like bleeding and embolisms have been resulted from these drugs, which lead to further complications [27]. Our findings revealed that the thrombolytic activities of our test samples (PSF, AQSF, CTSF, CSF, and CME) have moderate potential clot lysis activity.

Membrane stabilizing activity

In our qualitative phytochemical analysis, the presence of significant amount of flavonoids and other phenolic compounds in the leaves extract of *B. monosperma* kept evidence in the study of membrane stabilizing activity (Table 3). From previous studies, we found that at the time of inflammation, lysosomal enzymes and hydrolytic components are secreted from the phagocytes to the extracellular space of

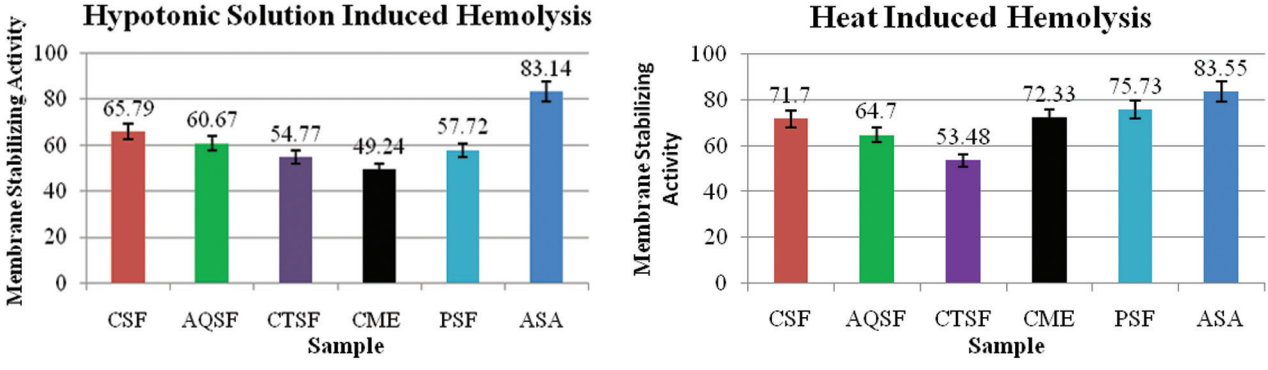

Figure 4. Membrane stabilizing activity of methanolic extract and its different Kupchan fractionates of *B. monosperma* induced by hypotonic solution and heat.

microorganism, which causes injuries to the surrounding organelles and tissues and also provoke many disorders [28]. Generally, Non steroidal anti-inflammatory drugs (NSAIDs), as ASA, acts either by inhibiting the action of lysosomal enzymes or by stabilization of lysosomal membranes. According to researchers, Feirrali expressed that when RBC become exposed to harmful substances such as hypotonic medium, heat, etc., results in the lysis of the membranes, accompanied by the oxidation and the breakdown of hemoglobin [29]. Method of Mounnissamy et al. expressed that the inhibition of hypotonicity and heat-induced RBC membrane lysis was an important parameter of the mechanism of anti-inflammatory activity of the plant extract, because human RBC membranes are considered similar to lysosomal membrane components [30]. So, it is cleared that the possible mechanism of action of the extract and its fractions and standard anti-inflammatory drugs may be connected with binding to the erythrocyte membranes with consequent alteration of surface charges of cells. This could have prevented physical interaction with agents of aggregation or promote dispersion by mutual repulsion of the charges as being involved in the hemolysis of RBCs. Some researchers reported that some chemical components present in the medicinal plants have the same mechanism, which are well known for their anti-inflammatory activity [31]. Both *in vitro* and *in vivo* studies in experimental animals showed that the flavonoids exert stabilizing effects largely on lysosomes [32]. Many researchers believed that tannin and saponins are capable of binding cations and other biomolecules, and are capable of stabilizing the erythrocyte membrane [33]. The *B. monosperma* leaf extracts have tannins, saponin, and lots of flavonoids [34]. So, observing our current study results, it reveals that the CME and its partitionates showed the potent RBC membrane stabilization activity with a good protection against both hypotonic solution and heat-induced lysis. Crude methanolic extract exhibited the highest membrane stabilizing activity both in the hypotonic solution induced hemolysis and in heat induced hemolysis as (65.47 ± 0.47%), (71.70 ± 0.11%), respectively.

Conclusion

This study was conducted to validate the *B. monosperma* leaves used as a folk medicine (for the ailment of thrombosis, inflammation) such as thrombolytic and membrane stabilizing. The outcomes of this research revealed the potential medicinal properties of the leaf extracts of *B. monosperma* against inflammation, formation of thrombus and oxidative degradation of cellular components. Further comprehensive study can be conducted for the chemical characterization of the corresponding chemical compounds responsible for afore mentioned medicinal values

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Conflict of Interest

We don't have any conflict of interest.

Funding

Self funding.

Availability of Data and Materials

All data are placed in the manuscript.

Authors' Contributions

Zubair Khalid Labu developed the necessary methods, analyzed the data, and provided idea to furnish the manuscript. Jalal Uddin and Khurshid Jahan collected the plant part, partitioned and performed the assays. Abu Jafor Biswas studied the literature and performed assay and prepared the primary manuscript. All the authors reviewed and approved the final manuscript.

Ethics Approval and Consent to Participants

The protocol of the study was submitted to the Committee for Ethic and Research of World University of Bangladesh (Grant No. WUBPS # 05512) and was approved. The volunteer blood donors gave their consent for using their blood in our current study.

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