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ANTIOXIDANT, THROMBOLYTIC, ERYTHROCYTE MEMBRANE STABILIZING AND BRINE SHRIMP LETHALITY BIOASSAY OF METHANOLIC LEAVES EXTRACT OF *CLITOREA TERNATEA* (FABACEAE)

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Abstract

The study was designed to conduct methanolic leaf extract (MLE) and fractionated with petroleum ether soluble fraction (PESF), carbon tetrachloride soluble fraction (CTSF), aqueous soluble fraction (AQSF) and dichloromethane soluble fraction (DCMSF) of *Clitorea ternatea (C. ternatea).* Moreover, the extract and fractionated samples were evaluated for biological activities of antioxidant activity, thrombolytic, erythrocyte membrane stabilizing and brine shrimp lethality bioassay. The antioxidant activities of the extractives were determined by 1,1-diphenyl -2- picrylhydrazyl (DPPH) free radical scavenging. Phytochemical screening showed the presence of alkaloid, steroid, flavonoid, saponin and phenol, in the tested sample. The antioxidant activity of CTSF showed a maximum IC_{50} value of 23.7 ± 0.65 µg/ml in comparison to standard Ascorbic acid, 0.36 ± 0.005 µg/ml. The highest amount of total phenolic content was observed 115.9 ± 0.48 GAE/gm in CTSF. In addition, CTSF exhibited 57.20 ± 1.86% clot lysis compared to Streptokinase 66.28 ± 0.90%. However, MLE showed strong level of membrane protection activity against heat induced 57.71 ± 1.12% and hypotonic induced 61.47 ± 0.91% when acetyl salicylic acid (ASA) showed 42.53 ± 0.34% and 70.15 ± 0.16% respectively. The LC_{50} value was observed at 15.3 ± 0.64 µg/ml and 11.88 ± 2.07 µg/ml for CTSF and PESF respectively against Vincristin Sulfate (VS) showing LC_{50} value at 0.75 ± 0.01 µg/ml. Other extractive samples did not exhibit satisfactory cytotoxic activities.The estimated result indicated that the leaves extract of *C. ternatea* possess potential antioxidant, thrombolytic and RBC membrane stabilizing properties.

Keywords: *C. ternatea, antioxidant, thrombolytic, membrane stabilizing, toxicity*

Introduction

Medicinal plants are a great source of raw materials for the manufacturing of different medicines. [1] The curative nature of these agents is for the appearance of different chemical compositions at different concentrations. [2] *C. ternatea* is a member of the family Fabaceae, commonly known as Butterfly pea, Girikarnika. and also called Aparajita in Bangladesh. [3] It has twining fine stems with 0.5-3 m long. The leaves are fluffy, 3-5 cm long and shortly immature underneath, flowers are deep blue in colour and 4-5 cm long. The seeds are olive, black or brown in colour, mottled and 4.5-7 mm long and 3-4 mm wide. Pods are linear, flat, beaked and 6-12 cm long, also 0.7-1.2 mm wide. [4] *C. ternatea* have several pharmacological activities like antidepressant, antipyretic, antistress, sedative, anxiolytic, analgesic, anti-inflammatory [5], anti-microbial activities, tranquilizing property, and antipyretic property. [6] *C. ternatea* have been revealed to possess immune inhibitory and hepato protective anti hyperlipidemic properties. [7] The current study evaluated *in-vitro* thrombolytic, erythrocyte membrane stabilizing, antioxidant and brine shrimp lethality bioassay of *C. ternatea* leaves extract and its various fractions that can provide scientific confirmation for using the plant as herbal remedies. The aim of our present examination was to assess various medicinal values of Bangladeshi origin of *C. ternateae* because as far I can tell no research has been conducted yet with the leaves extract of the plant in Bangladesh. The plant possess numerous traditional values in India, Malaysia and some other countries of South asia, [4, 8] but no significant research data was found for Bangladeshi origin of the plant. Thrombolytic and erythrocyte membrane stabilizing activity conducted first time. In addition, research history did not prove any lethality and serious side effect with traditional use, [9] hence our estimated result also justifies the extended use of the plant. Therefore, our conducted study detect the alternative use of the leaves extract of *C. ternatea* as a possible natural resource with least side effects.

Methods

Plant Sample Collection and processing

The leaf sample of *C. ternatea* was collected from Dhaka in June 2019. The fresh samples were then washed with water and dried in air first and then oven dried also at 45°C and at the final stage grounded by using grinding machine. This powdered sample was then sealed in an air-tight container to prevent the effects of moisture and contaminants. The prepared sample was then stored for further studies.

Preparation of the extract

The 200 g powdered materials were soaked in 1.5 liters of methanol in an amber-colored glass bottle at room temperature for 14 days with occasional shaking. The whole mixture was then filtered by a piece of clean, white cotton materials. The filtrate then filtered again with Whatman filter paper and total filtrate was concentrated also using a rotary evaporator to get the crude extract. The concentrated extract was partitioned by modified Kupchan method [10] and the resultant partitionates were evaporated to dry to yield PESF, CSF, AQSF and DCMSF. These residues were then stored & used for our current investigations.

Chemicals used in the research

Streptokinase was a gift from Beacon Pharmaceuticals Ltd, Bangladesh. Other experimental reagents and chemicals used here were purchased from local chemical market. All of these reagents were analytical grade.

Qualitative analysis of phytochemicals

The freshly prepared crude extract of *C. ternatea* was tested qualitatively for detecting the presence

of various phytoconstituents by using different standard methods.

1. Test for carbohydrates [11]

Small amount of crude extract was dissolved in 5 ml distilled water and filtered. The filtrate was used to test for the presence of carbohydrates.

a) Molisch's Test: 1.0 ml of filtrate was treated with 2 drops of alcoholic α-naphthol solution in a test tube and the formation of the violet ring at the junction confirms the presence of carbohydrates.

b) Benedict's Test: 1.0 ml of filtrate was treated with Benedict's reagent and then heated gently. Orange red precipitate indicates the presence of reducing sugars.

2. Detection of alkaloids

Small amount of crude extract was dissolved in dilute Hydrochloric acid and filtered.

a) Hager's Test: 1.0 ml of filtrate was treated with Hager's reagent (saturated picric acid solution). Formation of yellow coloured precipitate indicates the presence of alkaloids.

b) Wagner's Test: 1.0 ml of filtrate was treated with Wagner's reagent (Iodine in Potassium Iodide) and the formation of brown/reddish precipitate indicates the presence of alkaloids.

3. Detection of flavonoids

a) Alkaline Reagent Test: Few drops of sodium hydroxide solution was added to the alcoholic solution of the extract and formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.

4. Detection of saponins [12]

a) Foam Test: Small quantity of extract was shaken with little amount of water, if foam produce and persist for 10 minutes, it indicates the presence of saponin.

5. Test for steroids

Few amount of extract was dissolved in small quantity of chloroform, filtered and filtrate was tested for steroid.

a) Liberman- Burchard test: With the filtrate, a few drops of acetic anhydride was added and mixed well. 1.0 ml of concentrated sulphuric acid was added from the side of the test tube and the appearance of green color indicates the presence of steroids.

6. Test for tannins

a) Ferric chloride test: With the small amount of alcoholic extract solution a few drops of 1% neutral ferric chloride solution was added and the formation of blackish blue color indicates the presence of tannins.

7. Detection of phenol [13]

a) Ferric Chloride Test: Small amount of alcoholic extract solution was mixed with 2.0 ml of 2% solution of $FeCl_3$. A blue-green coloration indicates the presence of phenol.

DPPH free radical scavenging assay [14]

The free radical scavenging activity of the plant extract and its fractionate samples on the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was estimated by the method. 0.2 ml of each extractive of each concentration were mixed with 2.0 ml of DPPH solution (0.5 mM). The solution was then kept in a dark place. After 30 minutes of reaction at room temperature in dark place, the absorbance was measured at 517 nm. IC_{50} values (concentration of samples required to scavenge 50% of free radicals)

were calculated from the regression equation, developed by plotting concentration of samples versus percentage inhibition of free radicals. Ascorbic acid was used as positive control.

Total phenolic contents determination [15]

The total phenolic content of the extract was determined by the Folin–Ciocalteu method. Briefly, 200 µl of crude extract 1.0 mg/ml were made up to 3.0 ml with distilled water, mixed thoroughly with 0.5 ml of Folin–Ciocalteu reagent for three minutes, followed by the addition of 2.0 ml of 20% (w/v) sodium carbonate. The mixture could stand in the dark for 60 minutes, and absorbance was measured at 650 nm. The total phenolic content was calculated from the calibration curve, and the results were expressed as mg of gallic acid equivalent per g dry weight.

Thrombolytic assay [16]

The thrombolytic activity was evaluated by the method developed by (Prasad et al. 2007). In this method whole blood was drawn from healthy human volunteers and 1.0 ml of blood was transferred to the sterile Eppendorf tubes that were previously weighed and then the tubes were kept forming clots. After clot formation, the serum was removed completely very carefully without disturbing the clot. To each Eppendorf tube containing pre-weighed clot, 100 µl aqueous solutions of different extracts along with the crude extracts were added separately. As a positive control, 100 µl of streptokinase (SK) and as a negative non thrombolytic control, 100 µl of distilled water were separately added to the Eppendorf tubes. The steps were done carefully without disturbing the clots. The tubes were then incubated at 37°C for 90 minutes and observed for clot lysis. The released fluid was removed, and Eppendorf tubes were again weighed to measure the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis. Percentage of clot lysis for evaluation of thrombolytic activity was measured.

Erythrocyte membrane stabilizing activity [17]

The membrane stabilizing activity was determined with human erythrocytes by following the method developed by Omale and Okafor (2008). In this method the extractive samples were examined by using a hypotonic solution and heat induced RBC haemolysis. To prepare the erythrocyte suspension, 5.0 ml of whole blood was withdrawn from healthy human volunteer by hypodermal syringes (containing anticoagulant 3.1% Na-citrate) and was then centrifuged and blood cells were washed with solution (154 mM NaCl) in 10 mM sodium phosphate buffer (pH 7.4) three times through centrifugation for about 10 minutes at 3000 rpm.

Brine Shrimp Lethality Bioassay [18]

Cytotoxicity of the extract and its fractionate samples were evaluated three times by brine shrimp lethality bioassay following the standard method. Brine shrimp eggs were hatched in saline solution to be matured as nauplii. 20.0 mg of each of the extractive was dissolved in 1.0 ml of DMSO and then diluted to several concentrations (5.0 µg/ml, 25.0 µg/ml, 50.0 µg/ml, 100 µg/ml, 250 µg/ml, 500 µg/ml, 1000 µg/ml and 2500 µg/ml respectively). Ten living nauplii were taken carefully in each test tube containing samples of different concentrations along with salt water. Observation was done after 24 hours where the number of nauplii died was counted from which % of mortality was calculated. VS was used as positive control whereas DMSO used as a negative control. The % of mortality was calculated. Finally, LC_{50} value of each sample was derived using a regression equation obtained by

plotting the logarithm of concentration against the % of mortality.

Statistical analysis

For the bioassays, three replicates of each sample were taken for statistical analysis and values are reported as (mean ± S.D, n=3).

Results

Preliminary phytochemical analysis of *C. ternatea*

Phytochemical analysis of *C. ternatea* was carried out in MLE, where it was clearly observed that steroid, phenol, flavonoid, alkaloid, saponin, was present (Table 1). Moreover, previous research also reported a number of secondary metabolites like flavonoids, glycosides, anthocyanins, triterpenoids from *C. ternatea* extract. [19]

DPPH Free Radical Scavenging Assay

The antioxidant activity was determined through the DPPH free radical scavenging assay. Whereas, CTSF and AQSF possessed strong scavenging activity with IC_{50} value of 23.7 ± 0.65 µg/ml and 30.69 ± 0.49 µg/ml respectively compared to the standard ascorbic acid with IC_{50} value of 0.36 ± 0.005 µg/ml. However MLE and PESF was found to be weak scavengers of free radical activity as IC_{50} value was observed 103.18 ± 1.87 µg/ml and 177.17 ± 0.56 µg/ml respectively (Table 2).

Total Phenolic Contents Determination

The antioxidant potential was determined by total phenolic contents where estimated values were found to be ranging from 75.28 to 116.54 mg equivalent to GAE/g of extract (Table 2). CTSF showed the highest level of phenolic content 115.9 ± 0.48 GAE/ g of extract.

Thrombolytic assay

The extract and its fractionate samples were evaluated for thrombolytic activity where 100 µl of SK (30,000 I.U.) was added to the clots as a positive control following 66.28 ± 0.90% clot lysis. Negative control revealed a negligible percentage 2.66 ± 0.33% of clot lysis. The other extractives showed thrombolytic activity of MLE 30.79 ± 1.86%, PESF 31.51 ± 0.94%, CTSF 57.20 ± 1.86%, AQSF 51.55 ± 1.10% and DCMSF 36.81 ± 1.6% of clot lysis respectively. The CTSF showed a maximum percentage of clot lysis 57.20 ± 1.86% (Table 3).

Erythrocyte Membrane Stabilizing Activity

The extractive samples at a concentration of 1.0 mg/ml, prevent the haemolysis of RBC induced by heat and hypotonic solution as compared to the standard ASA (0.10 mg/ml). MLE showed maximum 61.47 ± 0.91% and 51.71 ± 1.12% haemolysis of RBC for hypotonic and heat induced as compared to the standard 70.15 ± 0.17% and 42.53 ± 0.34% for hypotonic and heat induced respectively (Table 4).

Brine shrimp lethality bioassay

In determining brine shrimp lethality bioassay, the lowest LC_{50} values represent the more toxic effects. [20] The cytotoxic evaluation showed no significant lethality. Only CTSF and PESF showed the LC_{50} value of 15.3 ± 0.64 µg/ml and 11.88 ± 2.07 µg/ml respectively comparing to the standard VS with LC_{50} value of 0.75 ± 0.01 µg/ml (Table 5).

Discussions

For the variation of medicinal properties, herbal medicines always considered as a prime source of health care for the global population. Preliminary phytochemical research of leaf extract of *C. ternatea* showed the existence of phytoconstituents like, steroid, phenol, flavonoid, alkaloid, and saponin. Where it has been reported that leaf extract exhibited different phytoconstituents like alkaloids, reducing sugars, flavonoids, steroids, and glycosides [8]. The

presence of these agents confirmed different pharmacological activities.

The leaf extract of *C. ternatea* possessed strong scavenging activity by the DPPH free radical scavenging assay. CTSF showed the highest IC_{50} value of 23.7 ± 0.65 µg/ml, which indicates that the antioxidant activity might be declared for being safe. [21]

Formation of thrombus inside the blood vessels causes blockage of blood flow that leads to myocardial infarction, hypertension, stroke and so many vascular complexities. Tissue plasminogen activator, streptokinase, etc. are currently used for treating thrombosis and medicinal plants possess antithrombotic activity also. Our conducted research exhibited that *C. ternatea* is potential for thrombolytic activity [22] where frequently employed method showed that, CTSF and AQSF exhibited 57.20 ± 1.86% and 51.55 ± 1.10% of clot lysis which is an evidence that the leaves extract is potential for thrombolytic activity. To find out cardio protective drugs from medicinal plants, thrombolytic activity was conducted.

As RBC membrane resembles to lysosomal membrane, the effect of erythrocyte membrane stabilizing agents on the stabilization of red blood cells could be generalized to the stabilization of lysosomal membrane. [23] Our recent study conclude that the MLE of *C. ternatea* possesses potential RBC membrane stabilizing properties with 61.47 ± 0.91% and 51.71 ± 1.12% for hypotonic and heat induced respectively. So the crude extract can prevent haemolysis induced by hypotonic and heat induced.

Our conducted research did not reveal any cytotoxicity where evaluation was conducted by brine shrimp lethality test. Reports mentioned that C. ternatea and its components having tumor suppressing property, interfere with progression of cell cycle and also enhance immune activity. [24]

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Table 1. Phytochemical analysis of MLE of *C. ternatea*

S/N	Phytoconstituents	Test methods	Result
01	Carbohydrate	Molisch's test	-ve
		Benedict's test	-ve
02	Alkaloids	Hager's test	+ve
		Wagner's test	+ve
03	Flavonoids	Alkaline Reagent test	+ve
04	Saponin	Foam test	+ve
05	Tannins	Ferric Chloride test	-ve
06	Steroids	Liberman-Burchard test	+ve
07	Phenols	Ferric Chloride test	+ve

+ Symbol indicates presence and – indicates absence with respect to extractive solvents

Table 2. IC_{50} value of DPPH radical scavenging activity and Total phenolic content of *C. ternatea* expressed as (Mean ± S. D, n=3)

S/N	Sample	DPPH IC_{50} (µg/ml)	TPC (GAE/ g of extract)
01	CTSF	23.7 ± 0.65	115.9 ± 0.48
02	AQSF	30.69 ± 0.49	89.15 ± 0.64
03	MLE	103.18 ± 1.87	75.69 ± 0.32
04	PESF	177.17 ± 0.56	86.99 ± 0.54

Table 3. *In vitro* thrombolytic activity of *C. ternatea.* Data are presented as (Mean ± S. D, n=3)

S/N	Fraction	% of clot lysis
01	MLE	30.79 ± 1.86
02	PESF	31.51 ± 0.94
03	CTSF	57.20 ± 1.86
04	AQSF	51.55 ± 1.10
05	DCMSF	36.81 ± 1.64
06	Blank	2.66 ± 0.33
07	SK	66.28 ± 0.90

Table 4. Erythrocyte Membrane stabilizing activity of *C. ternatea*. Data are presented as (Mean ± S.D, n=3)

S/N	Fraction	% Inhibition Haemolysis	
		Hypotonic Induced Haemolysis	Heat Induced Haemolysis
01	MLE	61.47 ± 0.91	51.71 ± 1.12
02	PESF	43.25 ± 1.40	41.42 ± 1.55
03	CTSF	32.42 ± 1.07	21.62 ± 1.38
04	AQSF	33.38 ± 1.60	24.25 ±1.79
05	DCMSF	55.89 ± 1.49	32.60 ± 1.52
06	ASA	70.152 ± 0.17	42.53 ± 0.34

Table 5. LC_{50} values of Brine shrimp lethality bioassay of *C. ternatea* expressed as (Mean ±S.D, n=3)

S/N	Sample	LC_{50} (µg/ml)
01	Vincristine Sulfate	0.75 ± 0.01
02	CTSF	15.3 ± 0.64
03	PESF	11.88 ± 2.07

Figure 1. Graphical representation of Thrombolytic activity of the extractive samples.

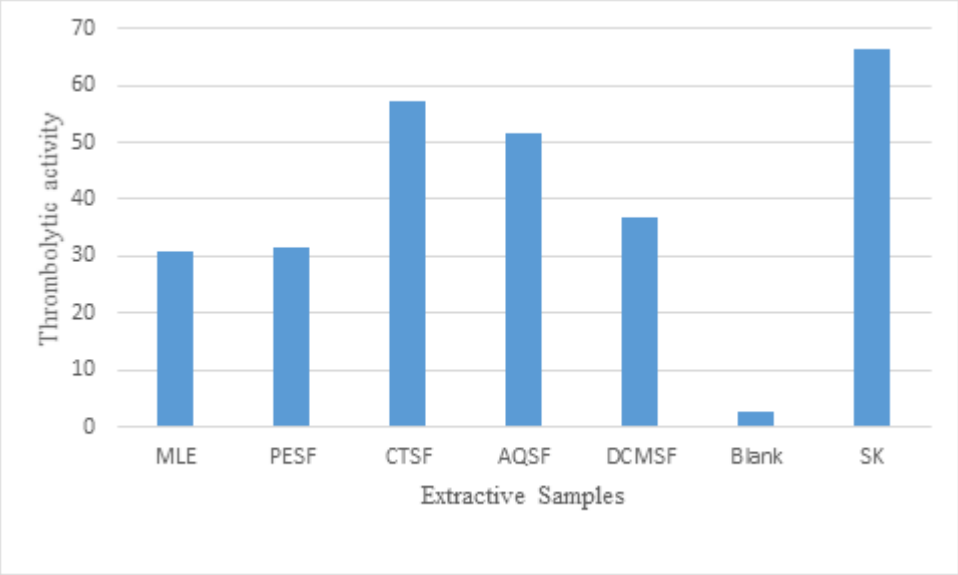

Figure 2. Graphical representation of IC_{50} values of the extractive samples.

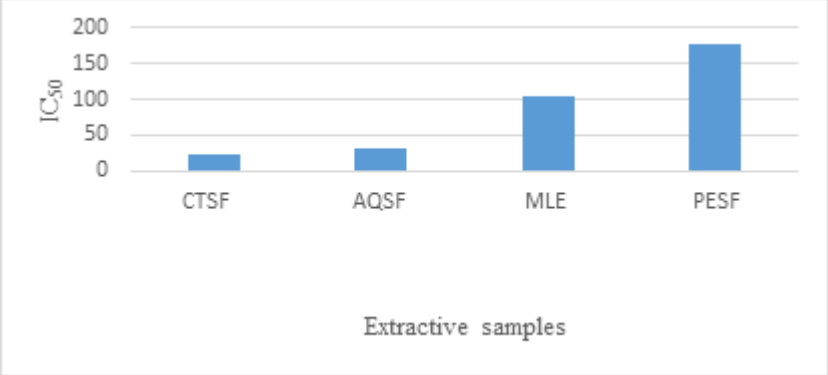

Figure 3. Graphical representation of LC_{50} values of the extractive samples.