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Preliminary *in Vitro* Biological, Phytochemical Screenings and *in Vitro* Antioxidant Activities of *Blumea lacera* (Burm.f.) DC

Amrita Rahman, Mohiuddin Ahmed Bhuiyan and Mohammad Shahriar*
Department of Pharmacy, University of Asia Pacific, Dhaka, Bangladesh

ABSTRACT

In this present study, the leaf extracts of Blumea lacera were subjected to a comparative evaluation of the antioxidant capacities, membrane stabilization, thrombolytic, and cytotoxicity to brine shrimps (Artemia salina). When the DPPH (1,1- Diphenyl-2-picryl hydrazyl) radical scavenging effect was determined spectrophotometrically significant radical scavenging property was observed in ethanol extract with IC_{50} of 19.9 µg/ml. Here, butylated hydroxyl toluene (BHT) and ascorbic acid (ASA) were used as standard antioxidants. The thrombolytic and membrane stabilizing activities were assessed by using human erythrocyte and the results were compared with standard streptokinase (SK) and standard anti-inflammatory drug, acetyl salicylic acid (ASA), respectively. The extracts demonstrated significant toxicity to A. salina with LC_{50} values ranging from 1.85 to 3.16 µg/ml as compared to standard Vincristine sulphate (VS, LC_{50} value 0.927 µg/ml). Preliminary phytochemical investigation suggested the presence of alkaloid, carbohydrate, glycosides, saponins and steroids.

Key words: *Blumea lacera, membrane stabilizing, thrombolytic activity, free radical scavenging, phytochemical screening*

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INTRODUCTION

According to the World Health Organization, 1977 [1] "a medicinal plant" is any plant, which in one or more of its organ contains substances that can be used for the therapeutic purpose or which, are precursors for the synthesis of using drugs. The definition distinguishes those plants whose therapeutic properties and constituents have been established scientifically and plants that are regarded as medicinal but which have not yet been subjected to thorough investigation. The team "herbal drug" determines the part/parts of a plant (leaf, flowers, seeds, roots, barks, stems, etc) used for preparing medicines.

Blumea lacera L., Compositae, is one of the common rabi weeds of India [2]. It is an annual herb, with a strong odour of turpentine. Stem is erect, ash colored, densely glandular, pubescent. Leaves are often incised or lyrate. There are many flower heads in single plant, arranged in axillary cymes or terminal panicle. Pappus is white. Fruits are an achene, oblong and not ribbed. Flowering time January to April [3].

The plant occurs throughout the plains of India from the north-west ascending to 2,000 ft in the Himalayas. It is a common roadside weed in Ceylon and Malaya. It is distributed to the Malay Islands, Australia, China and Tropical Africa. *Blumea* consists of about 80 species [4].

Blumea is described in *Ayurveda* as bitter, astringent, acrid, thermogenic, errhine, anti-inflammatory, styptic, opthalmic, digestive, anthelmintic, liver tonic, expectorant, febrifuge, antipyretic, diuretic, deobstruant, and stimulant [5]. The root kept in the mouth is said to cure disease of the mouth. In the Konkan region of India, the plant is used to drive away fleas and other insects. It is prescribed as an antiscorbutic in West Africa [4].

Study of methonolic extract of leaves of *B. lacera* showed considerable antifungal activity. The highest percentage of inhibition was showed against *A. flavus*, where as lowest percentage of inhibition was shown by *A. niger*. As the concentration of extracts increases, there is increase in percentage of inhibition [6].

Study showed that *Blumea lacera* exhibited broad anti-leukemic activity at magnitudes ranging from moderate to mild and Ixerischinensis is effective at inhibiting the proliferation of K562 cells. *B. lacera* and *Tithonia diversifolia* suppressed the replication of HSV-1 and HSV-2, and had IC_{50} values below 100 µg/ml. The medicinal plants showed no cytotoxic effect at concentrations that inhibited HSV infection. It was, therefore, concluded that the HW extract of tested medicinal plants exhibited anti-leukemic and antiviral activities at different magnitudes of potency [7].

The aim of present study is to evaluate bioactivities including phytochemical screening, thrombolytic activity, membrane stabilizing activity, cytotoxic activity by using brine shrimp lethality assay and in vitro antioxidant activities of the plant extractives.

MATERIALS AND METHODS

***Plant materials*:** The whole plants of *Blumea lacera* were collected from Mirpur Botanical Garden, Dhaka, Bangladesh, in January 2013. A voucher specimen forthis plant has been maintained in Bangladesh NationalHerbarium, Dhaka, Bangladesh (Accession No. 38224).

The sun dried and powdered plant parts (500 gm) of *Blumea lacera* was successively extracted in a Soxhlet extractor at elevated temperature using 300 ml of distilled methanol (40-60)°C which was followed by ethanol, chloroform, petroleum and n- hexane. All extracts were filtered individually through filter paper and poured on Petri dishes to evaporate the liquid solvents from the extract to get dry extracts. The dry crude extracts were weighed and stored in air-tight container with necessary markings for identification and kept in refrigerator (0-4)°C for future investigation.

***Preliminary phytochemical screening*:** One gram of the methanol extract of *Blumea lacera* was dissolved in 100 ml of methanol and was subjected to preliminary phytochemical screenings for determining nature of phytoconstituents [8, 9].

***Streptokinase (SK)*:** Commercially available lyophilized Altepase (Streptokinase) vial (Beacon pharmaceutical Ltd.) of 15, 00,000 I.U., was collected and 5 ml sterile distilled water was added and mixed properly. This suspension was used as a stock from which 100µl (30,000 I.U) was used for *in vitro* thrombolysis.

***Blood sample*:** Blood (n=6) was drawn from healthy human volunteers without a history of oral contraceptiveor anticoagulant therapy and 1ml of blood was transferred to the previously weighed microcentrifuge tubes and wasallowed to form clots.

***Thrombolytic activity*:** The thrombolytic activity of all extracts was evaluated by the method developed by Daginawala (2006) [10] with slight modification using streptokinase (SK) as the standard.

***Membrane stabilizing activity*:** The erythrocyte membrane resembles to lysosomal membrane and as such, the effect of drugs on the stabilization of erythrocyte could be extrapolated to the stabilization of lysosomal membrane [11]. The membrane stabilizing activity of the extractives was assessed by using hypotonic solution-induced and heat-induced mice erythrocyte haemolysis [12].

To prepare the erythrocyte suspension, whole blood was obtained from healthy human volunteer and was taken in syringes (containing anticoagulant EDTA). The blood was centrifuged and blood cells were washed three times with solution (154 mM NaCl) in 10 mM sodium phosphate buffer (pH 7.4) through centrifugation for 10 min at 3000 g.

Hypotonic solution- induced haemolysis: The test sample consisted of stock erythrocyte (RBC) suspension (0.50 mL) mixed with 5 mL of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing either the extract (1.0 mg/mL) or acetyl salicylic acid (0.1 mg/mL). The control sample consisted of 0.5 mL of RBCs mixed with hypotonic-buffered saline alone. The mixture was incubated for 10 min at room temperature, centrifuged for 10 min at 3000 *g* and the absorbance of the supernatant was measured at 540 nm. The percentage inhibition of either haemolysis or membrane stabilization was calculated using the following equation-

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

Where, OD_1 = optical density of hypotonic-buffered salinesolution alone (control) and OD_2**=** optical density of testsample in hypotonic solution.

Heat- induced haemolysis: Isotonic buffer containing aliquots (5 ml) of the different extractives were put intotwo duplicate sets of centrifuge tubes. The vehicle, in thesame amount, was added to another tube as control. Erythrocyte suspension (30 µl) was added to each tubeand mixed gently by inversion. One pair of the tubes wasincubated at 54°C for 20 min in a water bath, while theother pair was maintained at (0-5)°C in an ice bath. Thereaction mixture was centrifuged for 3 min at 1300 g andthe absorbance of the supernatant was measured at 540nm. The percentage inhibition or acceleration ofhemolysis in tests and was calculated according to theequation:

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

Where, OD_1 = optical density of unheated test sample,OD_2 = optical density of heated test sample and OD_3 =optical density of heated control sample

***Brine shrimp lethality bioassay*:** Brine shrimp lethality bioassay [13, 14] technique was applied for the determination of general toxic properties of the plant extractives. DMSO solutions of the samples were applied against *Artemia salina* in a 1-day *in vivo* assay. For the experiment1mg of each extracts were

added with 5ml of sea water. Concentration was found 200µg/ml. Then 50µl DMSO was added on these. Sample was prepared. Then the solution was serial diluted to 100, 50, 25, 12.5, 6.25, 3.125, 1.563 µg/ml with sea water. Then 2.5 ml of plant extract solution was added to 2.5 ml seawater containing 10 nauplii. Vincristine sulphate (VS) was used as positive control.

***Free radical scavenging activity*:** The free radical scavenging activity of the whole plants extractives of *Blumea lacera* was evaluated by the published method [15, 16]. DPPH was used to evaluate the free radical scavenging activity (antioxidant potential).

Determination of Total Flavonoids Content: Aluminum chloride colorimetric method was used for Flavonoids determination [17]. 1 ml of the plant extracts/standard of different concentration solution was mixed with 3 ml of methanol, 0.2 ml of aluminum chloride, 0.2 ml of 1 M potassium acetate and 5.6 ml of distilled water. It remained at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm with spectrophotometer against blank. Methanol served as blank. The total content of flavonoid compounds in plant methanol extracts in quercetin equivalents was calculated by the following equation:

$$C = (c \times V)/m$$

Where; C = total content of flavonoid compounds, mg/gm plant extract, in quercetin equivalent, c = the concentration of quercetin established from the calibration curve in mg/ml, V = the volume of extract in ml and m = the weight of crude plant extract in gm.

Determination of Total Antioxidant Capacity: The total antioxidant capacity was evaluated by the phosphomolybdenum method [18]. 0.3 ml of extract and sub-fraction in ethanol, ascorbic acid used as standard (5-200 µg/ml) and blank (ethanol) were combined with 3 ml of reagent mixture separately and incubated at 95°C for 90 minutes. After cooling to room temperature, the absorbance of each sample was measured at 695 nm against the blank. The antioxidant activity is expressed as the number of equivalents of ascorbic acid and was calculated by the following equation:

$$A = (c \times V)/m$$

Where, A = total content of Antioxidant compounds, mg/gm plant extract, in Ascorbic acid Equivalent c = the concentration of Ascorbic acid established from the calibration curve, mg/ml, V = the volume of extract in ml, m = the weight of crude plant extract, gm.

***Cupric Reducing Antioxidant Capacity* (CUPRAC):** The assay was conducted as described previously Apak *et al.*, (2004) [19]. To 0.5 ml of plant extract or standard of different concentrations solution, 1 ml of copper (II) chloride solution (0.01 M prepared from $CuCl_2.2H_2O$), 1 ml of ammonium acetate buffer at pH 7.0 and 1 ml of neocaproin solution (0.0075 M) were mixed. The final volume of the mixture was adjusted to 4.1 ml by adding 0.6 ml of distilled water and the total mixture was incubated for 1 hour at room temperature. Then the absorbance of the solution was measured at 450 nm using a spectrophotometer against blank. Ascorbic acid and BHT was used as a standard.

RESULTS AND DISCUSSION

***Preliminary phytochemical screening*:** In preliminary phytochemical screening, Methanol, ethanol and chloroform extracts of *Blumea lacera* contains alkaloids, alkaloid, carbohydrate, glycosides, saponins and steroids. And the n-hexane extracts contain alkaloids, carbohydrates and glycosides at low concentration. Phenols and tannins are absent in the alcoholic extracts of *blumea lacera* (Table 1).

Table 1: Analysis of phytochemicals in the different extracts of *Blumea lacera*

Name of Tests	Name of Extracts				
	Methanol	Ethanol	Chloroform	Pet-ether	n-Hexane
Alkaloids	+	+	+	+	+
Carbohydrates	+	+	+	+	+
Flavonoids	+	+	+	-	-
Glycosides	+	+	+	+	+
Phenols	_	_	_	_	_
Saponins	+	+	+	+	_
Steroids	+	+	+	+	_
Tannins	_	_	-	-	-

(+) = Presence &(-)= Absent

***Thrombolytic activity*:** As a part of discovery of cardio-protective drugs from natural sources the extractives of *Blumea lacera* were assessed for thrombolytic activity and the results are presented in Table 2. Addition of 100µl SK, a positive control (30,000 I.U.), to the clots and subsequent incubation for 90 minutes at 37°C, showed 56.82% lysis of clot. At the same time, distilled water was treated as negative control which exhibited negligible lysis of clot (3.28%). In this study, the methanol soluble fraction exhibited highest thrombolytic activity (61.75%). However, significant thrombolytic activity was demonstrated by the ethanol extract and chloroform extract of *Blumea lacera* (26.35% & 49.43%).

Table 2: Thrombolytic activity of different fractions of *Blumea lacera*

Name of samples	% of clotlysis
Standard (SK)	56.82
Control	3.28
Methanol	61.75
Ethanol	26.35
Chloroform	49.43
Petroleum ether	17.39
n-Hexane	18.23

SK= Streptokinase

***Membrane stabilizing activity*:** The different extractives of *Blumea lacera* at 1.0 mg/ml concentration significantly protected the lysis of human erythrocyte membrane by heat-induced haemolysis, as compared to the standard acetyl salicylic acid (0.10 mg/mL) (Table 3). In heat-induced conditions, the methanol extract of *B. lacera* inhibited 65.55% haemolysis of RBCs, as compared to 79.41% inhibited by acetyl salicylic acid (0.10 mg/mL). The ethanol and chloroform soluble extractives also revealed significant inhibition of haemolysis of RBCs. And in hypotonic solution induced conditions, the ethanol extract of the plant inhibited 79.20% of RBCs, as compared to 89.41% inhibited by standard (Table 4).

Table 3: Effect of extractives of *Blumea lacera* on heat- induced haemolysis

Name of the samples	Concentration(mg/ml)	Haemolysis inhibition (%)
Control	Only isotonic solution	
Standard (acetyl salicylic acid)	0.1	79.41±0 .052
Methanol	1	65.55± 0.028
Ethanol	1	74.20± 0.087
Chloroform	1	72.06± 0.019

Table 4: Effect of extractives of *Blumea lacera* on hypotonic solution of erythrocyte membrane

Name of the samples	Concentration(mg/ml)	Haemolysis inhibition(%)
Control	Only hypotonic solution	
Standard (acetyl salicylic acid)	0.1	89.41±0 .072
Methanol	1	62.88± 0.034
Ethanol	1	79.20± 0.057
Chloroform	1	68.06± 0.019

The present investigation suggests that the membrane stabilizing activity of *Blumea lacera* may be playing a significant role in its anti-inflammatory activity.

***Brine shrimp lethality bioassay*:** In the brine shrimp lethality bioassaythe lethal concentration (LC_{50}) of the test samples after 24hours was obtained by a plot of percentage of the shrimps died against the logarithm of the sample concentration (toxicant concentration) and the best-fit line was obtained from the curve data by means of regression analysis.

Vincristine sulphate (VS) was used as positive control, LC_{50} was found 0.927µg/ml. Compared with negative control, Vincristine sulphate (positive control) gave significant mortality and the LC_{50} values of different extractives were compared with negative control.

The LC_{50} values of methanol, ethanol, chloroform, n-hexane and pet-ether were found to be 3.16, 2.08, 1.99, 2.04 and 1.85 respectively (Table 5).

Table 5: LC_{50} values of the five extracts of *Blumea lacera* and standard

Test samples	LC_{50}
Vincristine sulphate (standard)	0.927
Methanol	3.16
Ethanol	2.08
Chloroform	1.99
n-hexane	2.04
Pet- ether	1.85

***Free radical scavenging activity*:** All the extractives of *Blumea lacera* were subjected to free radical scavenging activity using DPPH by usingascorbic acid (ASA) and *tert*-butyl-1-hydroxytoluene (BHT) as reference standards (Table 6). IC_{50} indicate the potency of scavenging activity.In this investigationStandard ascorbic acid and BHT were found to have an IC_{50} of 10.23µg/ml, 9.33µg/ml respectively. In comparisons to standard methanol, ethanol, chloroform, n-hexane and pet-ether extract showed IC_{50} of 29.51, 19.9, 37.15, 316.22 and 158.4µg/ml respectively (Table 6).

Table 6: IC50 values of different extracts of *Blumea lacera* in DPPH scavenging assay

Name of standard and samples	IC_{50}
Ascorbic acid	10.23
BHT	9.33
Methanol	29.51
Ethanol	19.9
Chloroform	37.15
n-hexane	316.22
Pet-ether	158.4

Total Flavonoid Content: Aluminium chloride colorimetric method was used to determine the total flavonoid contents of the different extracts of *Blumea lacera*. Total flavonoids contents was calculated using the standard curve of quercetin (y= 0.0019x + 0.0219; R^2= 0.99218) and was expressed as quercetin equivalents (QE) per gram of the plant extract. Methanol extract of *Blumea lacera* was found to contain the highest amount of flavonoids (Table 7). Flavonoids content of the extracts were found to decrease in the following order: Methanol extract> Petroleum ether extract> n-Hexane extract> Chloroform extract> Ethanol extract (Table 7).

Table 7: Total flavonoid contents of the different extracts of *Blumea lacera*

Name of Extracts	Total Flavonoid Content (mg/gm; Quercetin Equivalent)
Methanol	11.21 ± 9.7
Ethanol	7.15 ± 7.4
Chloroform	8.73 ± 6.7
Petroleum ether	10.02 ± 8.1
n-Hexane	9.42 ± 7.3

Values are the mean of duplicate experiments and represented as mean ± SD.

Total Antioxidant Capacity: Total antioxidant capacity of the different extracts of *Blumea lacera* was evaluated by the phosphomolybdenum method and was expressed as ascorbic acid equivalents (AAE) per gram of plant extract. Total antioxidant capacity of the test samples was calculated using the standard curve of ascorbic acid (y= 0.0022x+ 0.0032; R^2= 0.9996). n-hexane extract of was found to posses the highest amount of antioxidant (Table 8).Total antioxidant capacity of the extracts was found to decrease in the following order: n-hexane extract> chloroform extract> ethanol extract> methanol extract> petroleum ether extract (Table 8).

Table 8: Total antioxidant capacity of the different extracts of *Blumea lacera*

Name of Extracts	Total Anti-oxidant capacity (mg/gm), Ascorbic Acid Equivalent
Methanol	4.18±2.35
Ethanol	4.42±2.96
Chloroform	5.32±2.92
Petroleum ether	3.21±1.86
n- hexane	6.65±4.62

Values are the mean of duplicate experiments and represented as mean ± SD

Cupric Reducing Antioxidant Capacity (CUPRAC):Reduction of Cu2+ ion to Cu+ was found to rise with increasing concentrations of the different extracts. The standard ascorbic acid and BHT showed highest reducing capacity. Amongthe extracts methanol and ethanol extracts showed maximum reducing capacity that is comparable to ascorbic acid (AA) and butylated hydroxyl toluene (BHT) shown in Figure 1.

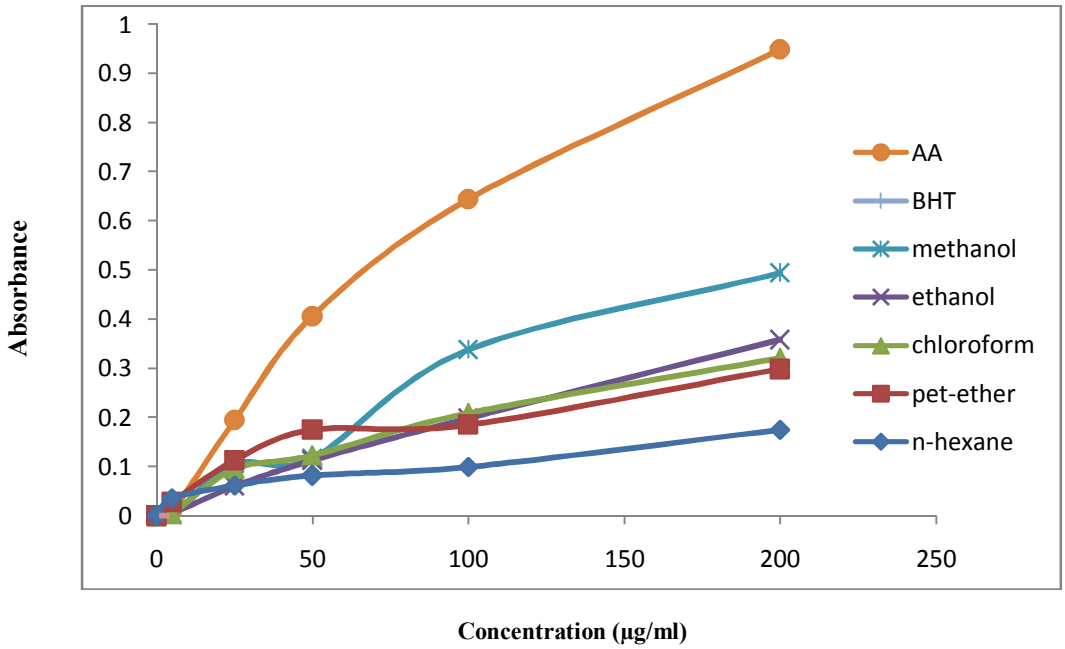

Figure 1: Comparative Cupric Reducing Antioxidant Capacity (CUPRAC) of different extracts of *B. lacera* with ascorbic acid (AA) and butylated hydroxyl toluene (BHT)

CONCLUSION

It can be concluded that the methanol, ethanol and chloroform extracts of the *Blumea lacera* had significant thrombolytic and membrane stabilizing activities. The Phytochemical screening revealed the presence of alkaloids, carbohydrates, flavonoids, glycosides, saponins and steroids. Antioxidant activities assay by DPPH free radical scavenging test, methanol, ethanol and chloroform extract of *Blumea lacera* displayed antioxidant function at low concentration (30 µg/ml). In percent mortality of brine shrimp nauplii produced by the extracts of the plant indicates the presence of cytotoxic principles in these extracts.In total antioxidant capacity test, n-hexane extract of was found to posses the highest (6.65mg/gm) total antioxidant capacity. The total antioxidant capacity of the extracts was found to decrease in the following order: n-hexane extract> chloroform extract> ethanol extract> methanol extract> petroleum ether extract. Methanol extract of *Blumea lacera* was found to contain the highest (11.21 mg/gm) amount of flavonoids. Flavonoids content of the extracts were found to decrease in the following order: Methanol extract> Petroleum ether extract> n-Hexane extract> Chloroform extract> Ethanol extract. In cupric reducing activity test of antioxidant, the methanol and ethanol extracts showed maximum reducing capacity that is comparable to standard (ascorbic acid and BHT).

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