

Studies of Thrombolytic, Antioxidant and Cytotoxic Properties of Two Asteraceous Plants of Bangladesh

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Abstract

Two Bangladeshi medicinal plants *Tridax procumbens* and *Vernonia cinerea* have been investigated for their *in vitro* thrombolytic, membrane stabilizing and cytotoxic properties. The total phenolic content was also determined and expressed in gallic acid equivalent. Among the two plants, the methanol extract of *T. procumbens* exhibited highest thrombolytic activity with clot lysis value of 21.15%. Standard streptokinase and water were used as positive and negative control which demonstrated 66.77% and 2.64% lysis of clot of human blood. The membrane stabilizing activity was assessed by using hypotonic solution and heat-induced methods and was compared with acetyl salicylic acid as standard drug.

Keywords: *Tridax procumbens*, *Vernonia cinerea*, membrane stabilizing, total phenolics

Introduction

Tridax procumbens (Linn.) (Bengali- Tridhara; Family- Asteraceae) is a spreading annual herb grows up to 20 cm in height. This can be found in fields, meadows, croplands, disturbed areas, lawns, and roadsides of tropical or semi-tropical areas and are well known for their medicinal properties among local natives. *T. procumbens* is reputed for its wound healing activities (Dhar *et al.*, 2003). This plant is also traditionally known for its insecticidal and anti-inflammatory activities. In some tribal areas, the leaf juice is used to cure fresh wounds, stop bleeding, as a hair tonic (Suseela *et al.*, 2002). In Nigeria, *T. procumbens* is traditionally used in the treatment of typhoid fever, cough, asthma, epilepsy, diarrhea (Mann *et al.*, 2003) and hypertension (Salahdeen *et al.*, 2004).

Vernonia cinerea (Linn.) (Bengali- Sahadevi; Family- Asteraceae) is an erect annual herb that grows up to 75 cm in height. It is found in tropical or semi-tropical areas as waste land herb (Crellin *et al.*, 1989). Traditionally this herb is widely used in tonsillitis, stomach pain, diarrhea, intermittent fever, eczema, herpes, ringworm and elephantiasis (Elujoba *et al.*, 2005).

Previously less attention has been focused on clot lysing activity of *T. procumbens* and *V. cinerea*. Thus, the aim of this study was to examine the *in vitro* thrombolytic potential of these two medicinal plants of Bangladesh. The present investigation also evaluated the antioxidant, membrane stabilizing and cytotoxic properties of *T. procumbens* and *V. cinerea*. In this paper we report the results of such studies in order to orient future investigations towards new, potent and safe bioactive compounds.

Materials and Methods

Plant materials: The whole plants of *T. procumbens* and *V. cinerea* were collected from the department of Botany, University of Chittagong in the month of February 2011 and voucher specimens for each of the collections have been deposited in Bangladesh National Herbarium (BNH) for future references. The plants were first cleaned, cut into small pieces and air-dried for several days. Then they were dried in oven at 40 °C to facilitate size reduction through grinding.

Extraction: The air-dried and powdered plant parts (300 gm each) were separately soaked in methanol (1.0 L each) for 15 days at room temperature with occasional

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shaking and stirring. They were then filtered through a fresh cotton plug and finally with Whatman No.1 filter paper. The volume of each of the filtrate was reduced using a Buchii Rotary evaporator at low temperature and pressure and subsequent evaporation of solvents afforded extracts of *T. procumbens* (8.5 g) and *V. cinerea* (9.5 g).

Thrombolytic activity: Whole blood was drawn from healthy human volunteers without a history of oral contraceptive or anticoagulant therapy and 1.0 ml of blood was transferred to the previously weighed microcentrifuge tubes and was allowed to clot.

The thrombolytic activity of all extracts was evaluated by the method developed by Dagainwala (2006) using streptokinase (SK) as the standard substance. The extract (100 mg) from each plant was suspended in 10 ml of distilled water and it was kept overnight. Then the soluble supernatant was decanted and filtered through a 0.22-micron syringe filter. For clot lysis venous blood drawn from healthy volunteers was distributed in different pre-weighed sterile microcentrifuge tube (1 ml/tube) and incubated at 37°C for 45 minutes. After clot formation, the serum was completely removed without disturbing the clot and each tube containing the clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube – weight of tube alone).

To each microcentrifuge tube with the pre-weighed clot, 100 µl aqueous solution of different partitionates and crude extract was added separately. Then, 100 µl of streptokinase (SK) and 100 mg of distilled water were separately added to the control tube as positive and negative controls respectively. All the tubes were then incubated at 37 °C for 90 minutes and observed for clot lysis. After incubation, 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 percentage of clot lysis as shown below:

$$\% \text{ of clot lysis} = (\text{wt of released clot} / \text{clot wt}) \times 100$$

Streptokinase (SK): Commercially available lyophilized Altepaste (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 100 µl (30,000 I.U) was used for *in vitro* thrombolysis.

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 (Omale, 2008). The membrane stabilizing activity of the extractives was assessed by using hypotonic solution-induced and heat-induced mice erythrocyte haemolysis (Shinde *et al.*, 1999). To prepare the erythrocyte suspension, whole blood was obtained from mice and was taken in syringes (containing anticoagulant EDTA) through cardiac puncture. 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-

$\% \text{ inhibition of haemolysis} = 100 \times (\text{OD}_1 - \text{OD}_2 / \text{OD}_1)$, where, OD_1 = optical density of hypotonic-buffered saline solution alone (control) and OD_2 = optical density of test sample in hypotonic solution.

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

% 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

Total phenolics analysis: Total phenolic content of *T. procumbens* and *V. cinerea* extracts were measured by employing the method (Skerget *et al.*, 2005) involving Folin-Ciocalteu reagent as an oxidizing agent and gallic acid as a standard. 0.5 ml of extract solution (2 mg/ml) in water, 2.5 ml of Folin-Ciocalteu reagent (diluted 10 times with water) and 2.0 ml of sodium carbonate (7.5 % w/v) solution were added. After 20 minutes of incubation at room temperature the absorbance was measured at 760 nm using a UV-visible spectrophotometer. Total phenolics were quantified by calibration curve obtained from measuring the known concentrations of gallic acid (0-100 µg/ml) and were expressed as mg of GAE (gallic acid equivalent) / gm of the dried extract.

Brine shrimp lethality bioassay: Brine shrimp lethality bioassay (Meyer *et al.*, 1982 and McLaughlin *et al.*, 1998) 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 experiment, 4 mg of each of the pet - ether, carbon tetrachloride and chloroform soluble fractions were dissolved in DMSO and solutions of varying concentrations (400, 200, 100, 50, 25, 12.50, 6.25, 3.125, 1.563, 0.781 µg/ml) were obtained by serial dilution technique using DMSO. Vincristine sulphate was used as positive control

Results and Discussion

As a part of discovery of cardio protective drugs from natural resources, methanol extracts of *T. procumbens* and *V. cinerea* were assessed for thrombolytic activity and the

results are presented in Table 1. Addition of 100 µl SK, a positive control (30,000 I.U.), to the clots and subsequent incubation for 90 minutes at 37°C, showed 66.76% lysis of clot. On the other hand, distilled water was treated as negative control which exhibited a negligible percentage of lysis of clot 2.64%. The mean difference in clot lysis percentage between positive and negative control was found statistically very significant. In this study, methanol extract of *T. procumbens* exhibited highest thrombolytic activity (21.15%). However, significant thrombolytic activity was demonstrated by the methanol extracts of *V. cinerea* (17.461%).

The membrane stabilizing activity of methanol extracts of *T. procumbens* and *V. cinerea* was also determined. All the extractives at 1.0 mg/mL significantly protected the lysis of human erythrocyte membrane induced by hypotonic solution and heat induced haemolysis, as compared to the standard acetyl salicylic acid (0.10 mg/mL) (Table 2). In hypotonic solution and heat induced conditions, the methanol extract of *V. cinerea* inhibited 62.43% and 53.13% haemolysis of RBCs, respectively as compared to 71.9% and 48.105% inhibited by acetyl salicylic acid, respectively (0.10 mg/mL). The methanol extract of *T. procumbens* also significantly inhibited the haemolysis RBCs in both hypotonic solution and heat induced conditions.

The total phenolic content varied for different plant extracts ranging from 26.51 mg to 69.84 mg of GAE/ gm of dried extract (Table 3). The highest total phenolics were found in methanol extract of *T. procumbens* (69.84 mg of GAE/ gm of dried extract).

In cytotoxicity screening, the lethality of the methanol extracts of both plants was evaluated against *A. salina*. Table 3 shows the results of the brine shrimp lethality testing after 24 hours of exposure to the samples and the

Table 1. Thrombolytic activity (in terms of % of clot lysis) of *T. procumbens* and *V. cinerea*.

Sample	W ₁	W ₂	W ₃	% of lysis
<i>T. procumbens</i>	0.88 ± 0.009	1.21 ± 0.005	1.15 ± 0.005	21.15 ± 0.60
<i>V. cinerea</i>	0.86 ± 0.006	1.22 ± 0.003	1.21 ± 0.075	17.46 ± 2.36
SK	0.88 ± 0.001	1.52 ± 0.063	1.10 ± 0.017	66.77 ± 0.66
Blank	0.88 ± 0.014	1.451 ± 0.021	1.43 ± 0.021	2.64 ± 0.22

W₁ = weight of micro centrifuge tube alone; W₂ = weight of clot containing tube; W₃ = weight of clot containing tube after clot disruption; SK =Streptokinase

Table 2. Effect of extractives of *T. procumbens* and *V. cinerea* on hypotonic solution and heat-induced haemolysis of erythrocyte membrane.

Sample	Concentration (mg/mL)	Haemolysis inhibition (%)	
		Heat induced	Hypotonic solution induced
<i>T. procumbens</i>	1.0	38.54 ± 0.74	26.38 ± 0.47
<i>V. cinerea</i>	1.0	53.13 ± 0.94	62.43 ± 0.84
Acetyl salicylic acid	0.1	48.10 ± 0.76	71.9 ± 0.99

Table 3. The total phenolic content and cytotoxic activity of of *T. procumbens* and *V. cinerea*.

Sample	Total Phenolic Content (mg of GAE/ gm of dried extract)	Cytotoxic activity (LC ₅₀ µg/ml)
VS	-	0.45 ± 0.04
<i>T. procumbens</i>	69.84 ± 1.38	31.68 ± 0.38
<i>V. cinerea</i>	26.51 ± 0.57	-

The average values of three calculations are presented as mean ± S.D. (standard deviation)

positive control, vincristine sulphate (VS). The methanol extract of *T. procumbens* showed potent cytotoxic activity having LC₅₀ of 31.68 µg/ml as compared to 0.45 µg/ml for vincristine sulphate. The test samples of *V. cinerea* demonstrated zero mortality rates as no nauplii died after 24 hours of observation. So, no median lethal concentration (LC₅₀, the concentration at which 50% mortality of brine shrimp nauplii occurred) was observed.

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