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Assessment of thrombolytic, membrane stabilizing potential and total phenolic content of *Typha elephantina* Roxb.

Niloy Sen, Latifa Bulbul*, Fahad Hussain and Mohammad Tohidul Amin

Department of Pharmacy, Noakhali Science and Technology University, Bangladesh.

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In this study, the crude methanolic extracts of whole palnt of *Typha elephantina* Roxb. were directed for screening of thrombolytic activity, membrane stabilizing activity, and total phenolic content. Human erythrocytes were taken for the analysis of both thrombolytic and membrane stabilizing activities and here streptokinase (SK) and acetyl salicylic acid (ASA) were used as standard for both tests, respectively. The methanolic extract of whole plant demonstrated high level of thrombolytic activity at the concentration of 4 mg/ml (33.33% of clot lysis) among various concentrations such as 2, 4, 6, 8, 7 and 10 mg/ml. On the other hand, the methanolic extract at different concentrations (1, 3, 5, 7 and 9 mg/ml), dose dependently inhibited the % of haemolysis of red blood cell (RBC) in case of heat induced condition and in case of hyponotic solution induced haemolysis, the methanolic extract just protected the RBC membrane. The total phenolic content (TPC) determination was investigated as a part of antioxidant assay. For the TPC determination, Folin-Ciocalteu method was used and the total phenolic content (126.33±4.33) of methanolic extract of *T. elephantina* was expressed by milligram of gallic acid equivalent to per gram of extract.

Key words: *Typha elephantina* Roxb., thrombolytic activity, membrane stabilizing activity, total phenolic content.

INTRODUCTION

Medicinal plants are defined as the plants which are beneficent for the recuperation of various diseases (Shahriar et al., 2014). In recent time, approximately 30% of the pharmaceuticals are produced from the plants present worldwide (Khan et al., 2010). Atherothrombotic diseases are characterized by the presence of serious impacts of thrombus in blood vessel (Mannan et al., 2011). By adhering to the destructed areas of endothelial surface, thrombocytes exert a key role in the development of atherothrombosis. In thrombosis activated activated platelets form platelets to platelets bonds, also bind to leucocytes and thus bringing them into a

*Corresponding author. E-mail: latifabulbul@gmail.com.

convoluted process of plaque generation and growth. Plasmin, a neutral fibrinolytic agent causes clot lysis by breaking down the fbinogen and fibrin contained in a clot. Additional plasminogen can easily convert to plasmin by streptokinase which forms a 1:1 stochiometric complex with plasminogen (Chowdhury et al., 2011). Moreover, phlorotannin, isolated from marine brown algae, have a singular trait in acceleration of dissolution of intravascular blood clot, via inhibition of antiplasmin (Prasad et al., 2007).

Generally, several oxidative damages and related inflammatory actions are accelerated by free radicals generated within the body. The red blood cell (RBC) membrane assimilates to lysosomal membrane, so that the action of drug to stabilize RBC membrane could be anticipated to the membrane stabilizing activity (Islam et al., 2015). The most frequently used therapeutic agents against oxidation and inflammation are NSAIDS which illicit their action by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membranes (Debnath et al., 2013). As various adverse conditions such as intestinal side effects, mucosal erosion, etc., are associated with NSAIDs, researchers have concentrated to trace medicinal plants in order to find new anti-inflammatory agents with reduced side effects (Islam et al., 2015).

In Bangladesh, most of the people living in far-off hilly regions and also the country is enriched with a potent diversity of medicinal plants disseminate over the forests, crop fields, roadsides gardens and water lands (Shahriar et al., 2014). It is therefore necessary to conduct broad spectrum evaluation of the local flora exploited in traditional medicines for various biological activities and therefore ultimately leading to the new drug development (Chowdhury et al., 2011). In account of this, our concentration has been focused particularly on *Typha elphantina* Roxb. belonging to the family Typhaceae, a bush like small plant which locally known as Hogal. The plant grows plenty in the Sundarban forest as well as in other low lying areas of Sylhet, Chittagong in beels and haors (Khair, 2014). *T. elephantina* Roxb. widely scattered across northern Africa and southern Asia. It is defined as native in many countries all over the world such as Algeria, Egypt, Libya, Uzbekistan, Palestine, Israel, Saudi Arabia, Assam, Bangladesh, India, Bhutan, Nepal, Pakistan, Burma, etc..

It is cooling and aphrodisiac in nature; used in splenic enlargement, burning sensation, and leprosy. The root-stock has stringent and diuretic properties, also useful in case of dysentery, gonorrhoea and measles and the ripe fruits and soft and woolly floss of male spikes are used as medicated absorbent to wounds and ulcers in emergency cases (Rahman et al., 2014). Several chromatographic and spectroscopic analysis carried out on fruit extract of this plant revealed the presence of four chemical constituents named pentacosane, 1-triacontarol, β-sitosterol, β-sitostery-3-O-β glycopyranoside. These four chemical constituents are supposed to exert various pharmacological activity such as anti-inflammatory, anti-pyretic, anti-tumor activities, etc., (Ruangrungsi et al., 1987). On the other hand, a previous investigation also provides evidence that *T. elephantina* Roxb. possesses analgesic, cytotoxic and anthemintic properties (Bulbul et al., 2013; Rahman et al., 2014). As part of our perpetual investigations on medicinal plants of Bangladesh, the methanolic extract of whole plant of *T. elephantina* Roxb. was studied for the thrombolytic activity, membrane stabilizing activity and also for the antioxidant property in terms of total phenolic content.

METHODOLOGY

Plant collection and preparation of plant materials

The whole plant was collected from NSTU campus, Sonapur, Noakhali, Chittagong during January 2015. Plant sample of *T. elephantina* Roxb. was identified by Mostaq Ahmed, Assistant professor, Department of Botany, Noakhali Government College, Noakhali, Bangladesh, where its voucher specimen (No. 025) was deposited. The plant parts were sun dried for 10 days and then ground by using high capacity grinding machine to produce coarse powder.

Preparation of extract

Powder (600 g) whole plant was soaked in 4.5 L methanol in a desiccator through occasional shaking and stirring. After 15 days, the solvent was removed and filtration was carried out by using sterile cotton and Whatman filter paper no. 1 (Sargent, Welch, USA). Then, rotary evaporation was carried out to concentrate the filtrate and was kept in room temperature in fresh and clean air for obtaining a brownish mass.

Streptokinase

Streptokinase that was commercially available in lyophilized stac vial (incepta pharmaceutical Ltd) of 15,00,000 I.U. and was collected and 5 ml sterile distilled water was added and mixed properly to produce a suspension. It was then used as a stock from which 100 µl (30,000 I.U) was drawn *in vitro* thrombolysis.

Thrombolytic activity

A method developed by Prasad et al. (2006) was used for the assessment of *in vitro* thrombolytic activity of *T. elephantina* extract using streptokinase (SK) as positive control with minor modifications. According to this method, healthy volunteers (n=3) were selected for collecting 5 ml venous blood and then transferred to five distinct pre weighed sterile micro centrifuge tube (1 ml/tube). These five tubes were then incubated for 45 min at 37°C. After clot formation, serum was completely aspirated out from the tubes without hampering the clot formed. Again each tube having clot was weighed to determine the clot weight (clot weight = weight of clot containing tube – weight of tube alone). To each pre weighed clot containing micro centrifuge tube, 100 µl of methanolic extract at several concentrations (2, 4, 6, 8 and 100 mg/ml) suspended overnight were added. As a positive and negative control, 100 µl of streptokinase and 100 µl of sterilized distilled water were separately kept in control tubes, respectively. All tubes were incubated again

for 90 min at 37°C and observed for clot lysis. Finally, the differences in weight taken before and after clot lysis were expressed as percentage of clot lysis following the under beneath equation.

% of clot lysis = (wt. of released clot/clot wt.) × 100

W2 - W3 / W2 × 100

where W2 is the weight of clot after 45 min incubation (g) and W3 is the weight of lysed clot after 90 min incubation (g).

Membrane stabilizing activity

The methanolic extract of *T. elephantina* Roxb. was examined by using hypotonic solution induced and heat induced erythrocyte haemolysis method developed by Omale and Okafor (2008).

Collection of blood sample

For this study, 2 ml of venous blood was collected from each of the healthy male volunteers of Bangladesh aged between 20 and 23 years having no record of taking oral contraceptive or anticoagulant therapy and free from diseases (using a protocol approved by Institutional Ethics Committee). The collected RBCs were kept in a test tube with an anticoagulant EDTA under standard conditions (temperature 23 ± 2°C and relative humidity 55±10%).

Preparation of erythrocyte suspension

The collected blood containing EDTA was centrifuged for 10 min at 3000 g and being washed for three times with isotonic solution (0.9% NaCl). The volume of saline was measured and reconstituted as a 40% (v/v) suspension with isotonic buffer solution (pH 7.4) which contained 1 L distilled water: $NaH_2PO_4.2H_2O$, 0.26 g; Na_2HPO_4, 1.15 g; NaCl, 9 g (10 mM sodium phosphate buffer). Thus, the suspension finally collected was the stock erythrocyte (RBC) suspension.

Hypotonic solution-induced haemolysis

The test sample consisted of stock erythrocyte (RBC) suspension (0.50 ml) with 5 ml of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffer saline (pH 7.4) containing either the different concentrations of methanolic extract (1, 3, 5, 7 and 9 mg/ml) or acetyl salicylic acid (0.10 mg/ml). Acetyl salicylic acid was used as a reference standard. After the mixtures were subjected to incubation for 10 min at room temperature, followed by subsequent centrifugation for 10 min at 3000 g and the absorbance of the supernatant was measured at 540 nm using UV spectrophotometer (Biswas et al., 2013). The percentage inhibition of either haemolysis or membrane stabilization was calculated using the following equation:

% Inhibition of haemolysis = $100 \times \{(X_1 - X_2) / X_1\}$

where X_1 is the optical density of hypotonic-buffered saline solution alone (control) and X_2 is the optical density of test sample in hypotonic solution.

Heat induced hemolysis

Aliquots (5 ml) of the isotonic buffer, containing different concentrations (1, 3, 5, 7 and 9 mg/ml) of extract of the plant were put into two duplicate sets of centrifuge tubes .The vehicle, in the exact 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. 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 using UV spectrometer (Biswas et al., 2013).

The percentage inhibition or acceleration of hemolysis in tests and was calculated using the following equation:

% Inhibition of haemolysis = $100 \times \{(X_2 - X_1) / (X_3 - X_1\}$

where X_1 is the test sample unheated, X_2 is the test sample heated and X_3 is the control sample heated.

Total phenolic content

Total phenolic content of *T. elephantina* Roxb. was determined with Folin-Ciocaltu reagent using gallic acid as standard. Gallic acid at concentrations of 6.25, 12.5, 25, 50 and 100 mg/ml and concentration of 2 mg/ml of plant extract were also prepared in ethanol.

Then 0.5 ml of extract solution, 2.5 ml of Folin-Ciocalteu reagent (diluted 10 times with water) and 2.0 ml of Na_2CO_3 (7.5% w/v) solution were mixed for analysis. The mixture was incubated for 20 min at room temperature and then the absorbance was measured at 760 nm by UV-spectrophotometer (UV-1800, Shimadzu, Japan). By using the standard curve (Raju et al., 2013) prepared from gallic acid solution with different concentration, the total phenols content of the sample was measured. The phenolic contents of the sample were expressed as mg of gallic acid equivalent (GAE)/g of the extract.

Statistical analysis

Data was expressed as mean ± standard error of mean (SEM).

RESULTS

The present study was an attempt to determine the thrombolytic, membrane stabilizing properties and total phenolic content of methanolic extract of *T. elephantina* Roxb. and the results have been circumscribed in Tables 1, 2 and 3, respectively.

Thrombolytic activity

As a part of searching drugs having capability to facilitate blood clot lysis from natural sources, the extractives of *T. elephantina* was examined for thrombolytic potential and the results are presented in Table 1. 100 µl SK, which was used as positive control (30,000 I.U.) exerted 40.13 ± 0.39% clot lysis activity. On the other hand, as negative control, distilled water showed a negligible percentage of lysis of clot (2.56 ± 0.44%). The plant extract at different concentrations such as 2, 4, 6, 8 and 10 mg/ml demonstrated mild to moderate clot lysis activity where in

Table 1. Effect of different concentrations of the methanolic extract of *Typha elephantina* and the controls on *in vitro* clot lysis.

Sample	Concentrations (mg/ml)	% of clot lysis
Sample 1	2	18.75 ± 0.94
Sample 2	4	33.33 ± 1.06
Sample 3	6	10.79 ± 1.19
Sample 4	8	8.23 ± 1.12
Sample 5	10	7.20 ± 0.41
SK	-	40.13± 0.39
Blank	-	2.56 ± 0.44

Data represented as Mean ± SEM (n=3); SK: Streptokinase; SEM: standard error of mean.

Table 2. Effect of methanolic extract of *Typha elephantina* in hypotonic solution induced haemolysis of erythrocyte membrane.

Treatment	Concentrations (mg/ml)	Optical density of samples in hypotonic solution	% Inhibition of haemolysis
Control	1	3.974± 0.011	-
Sample 1	1	3.075 ±0.055	22.62
Sample 2	3	3.135 ±0.038	21.11
Sample 3	5	3.169 ±0.025	20.25
Sample 4	7	3.216 ±0.014	19.07
Sample 5	9	3.347 ±0.11	15.77
ASA	0.10	1.032 ±1.00	74.01

Data presented as Mean±SEM (n=3); ASA: acetyl salisylic acid; SEM: standard error of mean.

Table 3. Effect of methanolic extract of *Typha elephantina* on heat induced hemolysis of erythrocyte membrane.

Treatment	Concentrations (mg/ml)	OD of sample		% of inhibition of haemolysis
		Heated	Unheated	
Control	1	2.032±0.020	-	-
Sample 1	1	0.868± 0.011	0.603±0.012	18.54
Sample 2	3	0.995± 0.010	0.643±0.024	25.34
Sample 3	5	1.183± 0.064	0.630±0.105	39.00
Sample 4	7	1.382± 0.172	0.741± 0.157	45.26
Sample 5	9	1.382± 0.172	0.741± 0.157	49.65
ASA	0.10	0.672±0.014	0.296± 0.016	75.81

n=3; OD: Optical density; SEM: standard error of mean. Data expressed as Mean ± SEM.

case of 4 mg/ml, the highest clot lysis effect, that is, 33.33 ± 1.06% was achieved. Here also, the % of clot lysis by this plant extract was found to be dose independent and Figure 1 shows the percentage of clot lysis for different concentrations of the methanolic extract, positive control and negative control.

Membrane stabilizing activity

The crude methanolic extract also assayed for membrane stabilizing activity and the results were summarized in Tables 2 and 3 for hypotonic and heat induced haemolysis conditions. In case of hypotonic induced haemolysis, the methanolic extract at all the doses (1, 3, 5, 7, and 9 mg/ml) protected the human erythrocyte membrane. On the other hand, during heat induced haemolysis, all the aforementioned concentrations showed 18.54, 25.34, 39.00, 45.26, and 49.65% inhibition of lysis of RBC membrane, respectively. This inhibition of haemolysis was found to be dose dependent, increasing with increased concentration of the extract and was comparable with that obtained for ASA. On both cases, ASA (0.1 mg/ml) was used as standard and thus

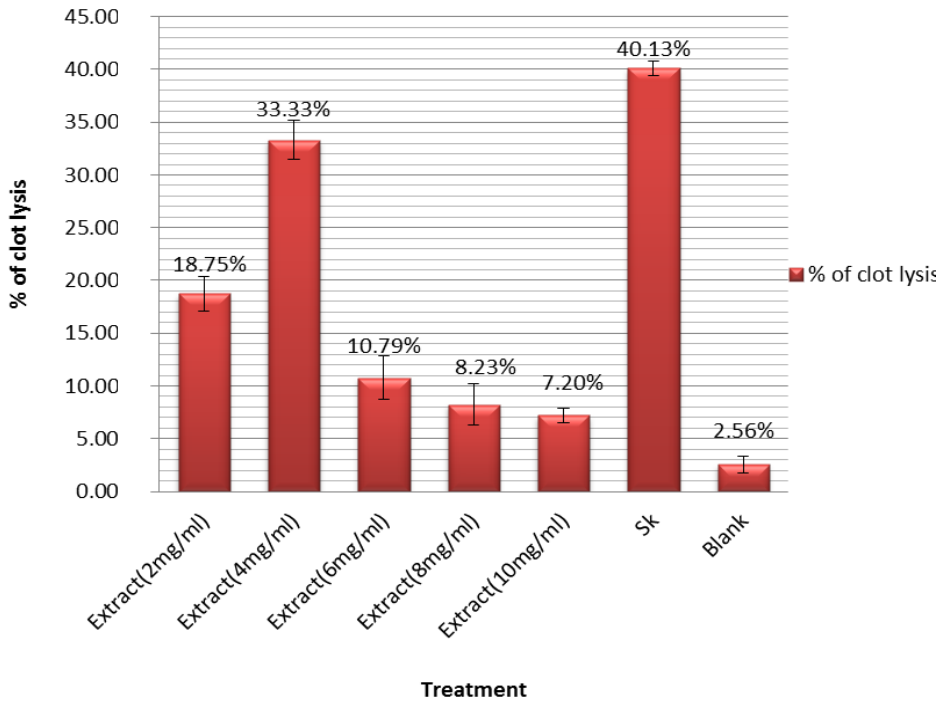

Figure 1. Thrombolytic activity of *Typha elephantina* at different concentrations.

Table 4. Total phenolic content determination of methanolic extract of *Typha elephantina* Roxb.

Sample	S/N	Absorbance of the sample	Absorbance	Total phenolic content (mg of GAE/g) of extract
Methanol extract	1	0.805	0.840±0.025	126.33±4.33
	2	0.827		
	3	0.889		

Data presented as Mean± SEM (n=3); SEM: Standard error of mean.

protected the RBC membrane by inhibiting lysis by 74.01 and 75.81%, respectively. Figure 2 exhibited the total phenomenon regarding membrane stabilizing activity in case of both hypnotic and heat induced haemolysis.

Total phenolic content

Table 4 shows the total phenolic content of the *T. elephantina* extract. The result is reported as gallic acid equivalents and it was 126.33 ± 4.33 mg of GAE/g of extract. Thus, the result manifested that plant extract has good antioxidant activity.

DISCUSSION

Various researchers have conducted several studies to find out the herbs and natural foods having thrombolytic effect and there is also evidence that, coronary events and stroke can be prevented by consuming such foods (Khan et al., 2010). The clots already formed in the blood can be dissolved by various thrombolytic agent; but these drugs are not free from adverse reactions and can be responsible for serious life threatening consequences (Mannan et al., 2011). An extensively used thrombolytic agent known as streptokinase plays seminal role in converting additional plasminogen to plasmin. But adverse complications such as bleeding and embolism are related with this agent and lead the researchers to conduct research work in order to discover novel sources of herbs and natural foods having thrombolytic effect with minimal side effects (Bhowmick et al., 2014). As a part of this research work, we tried to search out whether the methanolic extract of whole plant possesses clot lysis potentiality or not. The comparison study between positive and negative control clearly revealed that clot lysis did not take place when water was added to the clot.

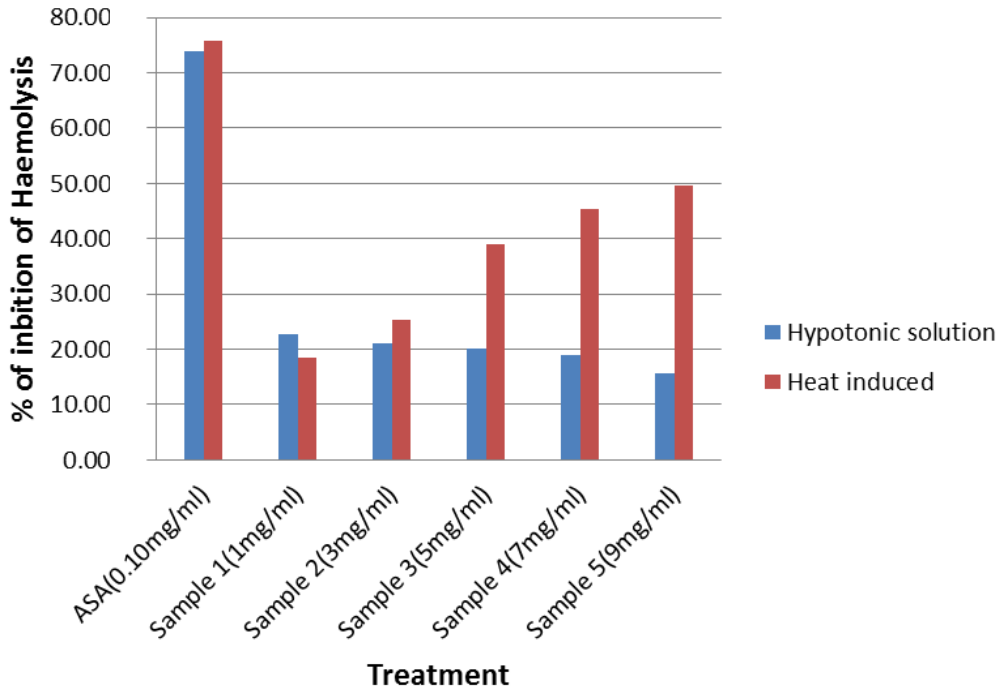

Figure 2. Effect of different concentration of *Typha elephantina* on hypnotic and heat induced haemolysis.

This prominent result encouraged us to compare five different concentrations of the test sample in same way with negative control and thus showed antithrombotic effect in a dose independent manner. It was also observed from the results that the sample in concentration 4 mg/ml exhibited the highest clot lysis activity (33.33±1.06%) among the five concentrations. In an investigation carried out by Umesh et al. (2014) on *T. angustifolia* L. leaves extract, another species of Typhaceae family also provide evidence about the presence of thrombolytic properties in this family species. Since phytochemical analysis revealed the presence of flavonoids, tannin, phenols, saponin, alkaloid in the crude extract of *T. elephantina* Roxb (Rahman et al., 2014) and it could be prognosticated that mainly tannin, saponin and alkaloid phytochemicals may confirm its clot lysis activity.

In this study, the membrane stabilizing activity of this plant at different concentrations was also investigated. Various lysozomal enzymes and hydrolytic components are excreted by phagocytes during inflammation period. These chemical agents account for damages to surrounding organelles and tissues (Dewan et al., 2013). As RBCs membranes are injured when exposed to various detrimental substances such as hypotonic medium, heat, etc., that is why hypotonic solution and heat induced haemolysis of erythrocyte membrane was chosen as an assessment for membrane stabilizing activity (Bhowmick et al., 2014). Earlier investigation has asserted that various herbal preparations rich in flavonoids and other phenolic compounds are capable of stabilizing the erythrocyte membrane and exert anti-inflammatory activity (Sadique et al., 1989). Their anti-inflammatory activities are namely due to their inhibitory effect on enzymes related to the production of the inflammatory mediators and metabolism of arachidonic acid (Metowogo et al., 2008). The results of this study displayed that, methanolic extract of *T. elephantina* Roxb. at different concentrations protected the RBC membrane against lysis induced by hypotonic solution and heat. As articulated earlier about the presence of phytochemicals such as flavonoids and other phenolic compounds in this plant, these phytochemicals may be liable for their membrane stabilizing activity by either preventing the release of phospholipase or by inhibiting cyclo-oxygenases which are denoted as the crucial catalytic elements in inflammatory pathway.

On the other hand, surplus production of reactive oxygen species play significant role to the development of hazardous tissue damage with variety of pathological process like ischaemia, inflammation, atherosclerosis and thrombosis (Diaz et al., 1997). The lack of balance between peroxidants and antioxidants play crucial role for the development of atherosclerosis (Khan et al., 1998). An investigation conducted in previous time has revealed that plant flavonoids instigate potent anti-inflammatory and anti-oxidant properties (Middleton et al., 1992). This paper also delivered us about the amount of total phenolic content as a part of the antioxidant test.

Conclusions

These findings conclude that, the methanolic extract of *T. elephantina* Roxb. have potentiality for thrombolytic and membrane stabilizing activities. The total phenolic content (126.33 ± 4.33 mg of GAE/g of extract) is also in close conformity to the presence of antioxidant property in this plant. Therefore, the plant demands further systemic, chemical and biological investigations to determine and isolate the active principles.

Conflict of Interests

The authors have not declared any conflict of interests.

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