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In vitro thrombolytic, anthelmintic, anti-oxidant and cytotoxic activity with phytochemical screening of methanolic extract of *Xanthium indicum* leaves

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Abstract

Xanthium indicum is an important medicinal plant traditionally used in Bangladesh as a folkloric treatment. The current study was undertaken to evaluate thrombolytic, anthelmintic, anti-oxidant, cytotoxic properties with phytochemical screening of methanolic extract of *X. indicum* leaves. The analysis of phytochemical screening confirmed the existence of phytosetrols and diterpenes. In thrombolytic assay, a significant clot lysis was observed at four concentrations of plant extract compare to the positive control streptokinase (30,000 IU, 15,000 IU) and negative control normal saline. The extract revealed potent anthelmintic activity at different concentrations. In anti-oxidant activity evaluation by two potential experiments namely total phenolic content determination and free radical scavenging assay by 2, 2-diphenylpicrylhydrazyl (DPPH), the leaves extract possess good anti-oxidant property. In the brine shrimp lethality bioassay, the crude extract showed potent (LC_{50} 1.3 µg/mL) cytotoxic activity compare to the vincristine sulfate as a positive control (LC_{50} 0.8 µg/mL).

Introduction

Plants have been used for mankind as remedies from the very beginning of civilization. Bangladesh possesses a rich flora and expanded genetic resources of medicinal plants. Its wide ranging tropical and growing conditions are very much favorable to the introduction and domestication of new plant species (Sajib et al., 2015). *Xanthium indicum* J. Koenig is a plant belonging to the family of *Asteraceae*, which is commonly known as 'burweed' in English and 'ghagra' in Bengali. A report was demonstrated on this plant, it is used by the Lohit community of Arunachal Pradesh, India for treatment of inflammatory disorders (Namsa et al., 2009) and some recent studies evidenced that the plant possesses antinociceptive activity and hypoglycemic activity (Haque et al., 2013; Hossan et al., 2011) and also traditionally it was used in Bangladesh to manage the blood sugar level in diabetic patients and to relieve pain (Rahmatullah et al., 2011). A current report has validated on anti-bacterial and cytotoxic actions in methanolic extract of this plant (Ullah et al., 2013).

Thus, the present study intended to evaluate the thrombolytic, anthelmintic, anti-oxidant, cytotoxic activity and also to find the existence of phytochemicals in the methanolic crude extract of *X. indicum* leaves.

Materials and Methods

Chemicals

All the chemicals used in this study were of analytical grade, and purchased from Sigma Chemical Co. (St. Louis, MO, USA), and Merck (Darmstadt, Germany). To the commercially available lyophilized S-Kinase™ (Streptokinase) vial (Batch no: VEH 09, Popular Pharmaceutical Ltd., Bangladesh) of 1,500,000 I.U., 5

mL 0.9% sodium chloride was added and mixed properly. This solution was used as a stock from which 100 µL (30,000 I.U) was used for *in vitro* thrombolysis assay.

Collection of plant material and identification

The fresh leaves of *X. indicum* were collected from surrounding area of the Noakhali Science and Technology University in August 2012. It was identified and authenticated by an expert botanist of Bangladesh National Herbarium, Mirpur, Dhaka, where the plant was deposited for future reference.

Preparation of methanol extract

The collected plant leaves were separated from undesirable materials or plants or plant parts. They were air-dried for one week. Then the plant parts were pulverized into a coarse powder with the help of a suitable grinder. About 238 g of powered material was taken in a clean glass container and soaked in 900 mL of 80% methanol (Merck, Germany) and kept for a period of 15 days accompanying occasional shaking and stirring. The whole mixture then filtrated by a piece of clean and white cotton material. Then it was filtered using Whatman No. 42 filter paper. After obtaining clear filtrates, they were then evaporated by using traditional spontaneous natural vaporization method at room temperature. It rendered a gummy concentrate of dark greenish color. The crude extract was stored at 4°C until analysis.

Phytochemical screening

The freshly prepared crude methanolic extract was qualitatively tested for the presence of alkaloids, phenols, tannins, flavonoids, saponins, glycosides, reducing sugars, diterpenes, proteins and amino acids, phytosterol and terpenoids. The presence of these chemical constituents were identified by characteristic color changes using standard phytochemical procedures (Howlader et al., 2012; Harborne, 1998).

Thrombolytic activity

In vitro thrombolytic activity of the leaves extract was evaluated by the method (Prasad et al., 2007) with slightly modified (Bhowmick et al., 2014). With ethical considerations, and aseptic precaution, 5 mL of venous blood was drawn from healthy volunteers (n = 10) without a history of cigarette smoking, oral contraceptive or anticoagulant therapy and transferred to different pre weighed sterile micro-centrifuge tube (1 mL/tube). About 200 µL of 2% calcium chloride was then added to each of these tubes, mixed well and incubated at 37°C for 45 min for clotting to occur. After the formation of clot, serum was completely removed from the tubes (carried out without disturbing the clot formed) and each tube having clot was again weighed to determine the weight of the clot (clot weight = weight of clot containing tube – weight of tube alone).

To each micro-centrifuge tube containing pre-weighed clot, 500 µL of different concentrations of the plant extracts (2.5 mg/mL, 5 mg/mL, 10 mg/mL, and 20 mg/mL) were added accordingly. As a positive control, 30,000 IU and 15, 000 IU of reference streptokinase and as a negative non-thrombolytic control, normal saline were separately added to the control tubes numbered. Then all the tubes were incubated again at 37°C for 90 min and observed for clot lysis. After incubation, the obtained fluid was removed from the tubes and they were again weighed to observe the difference in weight after clot disruption. At last, difference obtained in weight was calculated and the result was expressed as percentage of clot lysis following the underneath equation.

% of clot lysis: (weight of released clot/clot wt.) x 100

Anthelmintic activity

The *in vitro* anthelmintic activity was carried out by the method (Ajaiyeoba et al., 2001) with some necessary alterations. Adult earth worm (*Phertima posthuma*) was used to perform the test because of its anatomical and physiological resemblance with intestinal round worm parasite (Vidyarthi, 1967; Lakshmi et al., 2012). The worms were collected from the moist soil of Noakhali Science and Technology University area and washed with normal saline to remove all the fecal matter and waste surrounding their body. Methanolic extract of *X. indicum* leaves was taken at different concentrations (10, 20, 40 and 60 mg/mL) separately. 100 mg of piperazine citrate was dissolved in 10 mL of saline water to prepare a concentration of 10 mg/mL which was used as referred standard. A control group was established with distilled water for the test validation. Earthworms were placed into seven petri dishes in 7 groups, each containing three earthworms where five dishes were used for the five concentrations of methanolic extract of *X. indicum* and one for the reference standard and another for the control group. The paralyzing time was counted only when there was no movement observed except that the worm was shaken vigorously. After ascertaining that the worms moved neither when vigorously shaken nor when dipped in warm water (50°C), the death time was recorded (Islam et al., 2015).

Anti-oxidant activity

The *in vitro* anti-oxidant activity was done in this study by following two methods:

Determination of total phenolic content

The amount of total phenolic content present in plant extract was determined by using Folin-Ciocalteu reagent. As gallic acid was used as standard, the total phenolic contents were expressed as mg/g of gallic acid equivalents (GAE). Concentration of 6.25, 12.5, 25, 50 and 100 mg/mL of gallic acid and concentration of 2 mg/mL of plant extract were also prepared in

methanol. Then 0.5 mL of sample was introduced into test tubes and mixed with 2.5 mL of a 10-fold dilute Folin-Ciocalteu reagent and 2 mL of 7.5% sodium carbonate. The tubes were enclosed with parafilm and allowed to stand for 30 min at room temperature and the absorbance was measured at 760 nm spectrophotometrically (UV-1800, Shimadzu, Japan). Total phenolic content was determined as mg of gallic acid equivalent per gram using the equation obtained from a standard gallic acid calibration curve (Islam et al., 2015).

Free radical scavenging activity by DPPH method

The free radical scavenging activity of *X. indicum* leaves extract was measured in terms of hydrogen donating or radical scavenging ability by using the stable radical 1,1 -diphenyl-2-picrylhydrazyl (DPPH) by using the standard method of Brand Willians et al., 1995. 2 mL of methanolic solution of sample (extract/standard) at different concentrations were mixed with 3.0 mL of a DPPH methanol solution (20 mg/mL). The mixture was kept in a dark place at room temperature for 30 min and later absorbance was measured at 517 nm against methanol as blank by UV spectrophotometer. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The anti-oxidant potential was assayed from the bleaching of purple colored methanol solution of DPPH radical by the plant extract as compared to that of butylated hydroxyltoluene (BHT) as positive control by UV spectrophotometer. The graph plotted with inhibition percentage against extract/standard concentration; extract concentration providing 50% inhibition (IC_{50}) was calculated (Sikder et al., 2010).

Cytotoxic Activity

Brine shrimp lethality bioassay (Meyer et al., 1982) is a method used to examine the cytotoxic activities of the extracts. *Artemis salina* leach (brine shrimp eggs) collected from pet shops was used as the test organism. Seawater was taken in the small tank and shrimp eggs were added to one side of the tank and then this side was covered. 48 hours were allowed to hatch the shrimp and to be matured as nauplii. Constant oxygen supply was provided throughout the hatching time. The hatched shrimps were attracted to the lamp through the perforated dam and they were taken for experiment. In this study vincristine sulfate was used as the positive control. Measured amount of the vincristine sulfate was dissolved in DMSO to get an initial concentration of 40 µg/mL from which serial dilutions were made using DMSO to get 20 µg/mL, 10 µg/mL, 5 µg/mL, 2.5 µg/mL, 1.25 µg/mL, 0.625 µg/mL, 0.3125 µg/mL, 0.15625 µg/mL and 0.78125 µg/mL solution from the extracts. Then the positive control solutions were added to the pre-marked vials containing ten living brine shrimp nauplii in 5 mL simulated sea water to get the positive control groups. 100 µL of DMSO was added to each of three pre-marked glass vials containing 5 mL of simulated sea water and 10 shrimp nauplii to use as control groups.

Counting of nauplii

After 24 hours, by using a magnifying glass, the vials were inspected and the number of survived nauplii in each vial was counted. From this data, the percentage of lethality of the brine shrimp nauplii was calculated for each concentration.

Statistical analysis

Descriptive statistics were calculated for all variables by using SPSS software package (version 19.0). All of the values were expressed as mean ± SEM. Data analysis among the groups was compared using one-way ANOVA with Dunnett's post Hoc test. P value of <0.05 was considered as significant.

Results

Phytochemical screening

The preliminary phytochemical screening of crude methanolic leaves extract of *X. indicum* confirmed the only presence of phytosetrols and diterpenes (Table I).

Table I

Phytochemical screening of the methanolic extract of *X. indicum*

Phytochemical	Observation
Alkaloid	-
Carbohydrate	-
Glycoside	-
Saponins	-
Phytosterol	Present
Phenol	-
Tannis	-
Flavonoids	-
Proteins and amino acids	-
Diterpenes	Present

Thrombolytic activity

The results of effective clot lysis percentage by methanolic extract of *X. indicum* leaves at four concentrations, positive control (streptokinase) and negative control (normal saline) is given in Table II. The percentage of clot lysis was 47.2 ± 1.2% and 24.7 ± 1.1% when addition of 500 µL of streptokinase at concentration of 30,000 IU and 15,000 IU respectively to tubes used as a positive control, while in case of negative control (normal saline) showed percentage of clot lysis very negligible 5.4 ± 1.0%. On the basis of these results, it's shown that four concentrations (2.5 mg/mL, 5 mg/mL, 10 mg/mL and 20 mg/mL) of crude methanolic

extracts induced highly significant ($p<0.001$) clot lysis activity *in vitro* of 17.5 ± 1.3%, 13.2 ± 1.2%, 11.6 ± 1.2%, and 9.1 ± 1.2% respectively comparing with 5.4 ± 1.0%

Table II

Thrombolytic effect of methanolic extract of *X. indicum*

Treatment	Concentration	Percentage of blood clot lysis
Control	Normal saline	5.4 ± 1.0
Streptokinase	30,000 I.U	47.2 ± 1.2[a]
Streptokinase	15,000 I.U	24.7 ± 1.1[a]
X. indicum	2.5 mg/mL	17.5 ± 1.3[a]
X. indicum	5 mg/mL	13.2 ± 1.2[a]
X. indicum	10 mg/mL	11.6 ± 1.2[a]
X. indicum	20 mg/mL	9.1 ± 1.2[a]

Values are presented as mean ± SEM (Standard Error Mean); Probability values calculated as compared to control using one way-ANOVA followed by Dunnet's t-test; [a]indicates that the result is highly significant at $p<0.001$; Number of volunteers each group = 10

clot lysis of normal saline considered as a negative control.

Anthelmintic activity

The gradual increase of sample concentration of methanolic extract of *X. indicum* demonstrates paralysis as well as death of worms in shorter times. At the concentration of 10 mg/mL, 20 mg/mL, 40 mg/mL and 60 mg/mL, the methanolic extract showed paralysis time of 9.7, 6.0, 3.7, 1.3 min and death time of 23.7, 19.3, 9.0 and 4.0 min respectively (Figure 1). These results were compared to that of the standard drug of piperazine citrate at 10 mg/mL concentration showed paralysis time 8.7 min and death time 36.7 min.

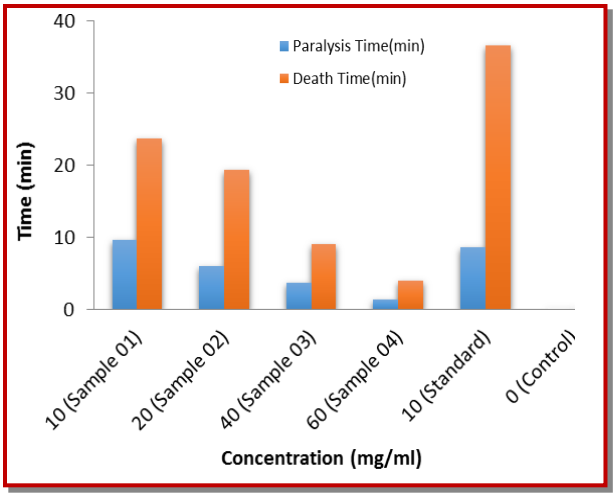

Figure 1: Anthelmintic activity of crude methanolic extract of X. indicum leaves against *Phertima posthuma*

Anti-oxidant activity

Determination of total phenolic content

The results showed that the total phenol content of the extract was found to be 20.1 ± 0.5 mg/g and this suggest that the plant may possess good anti-oxidant activity.

Free radical scavenging activity by DPPH method

The crude methanolic extract showed the poor the free radical scavenging activity compare to reference standard (Figure 2).

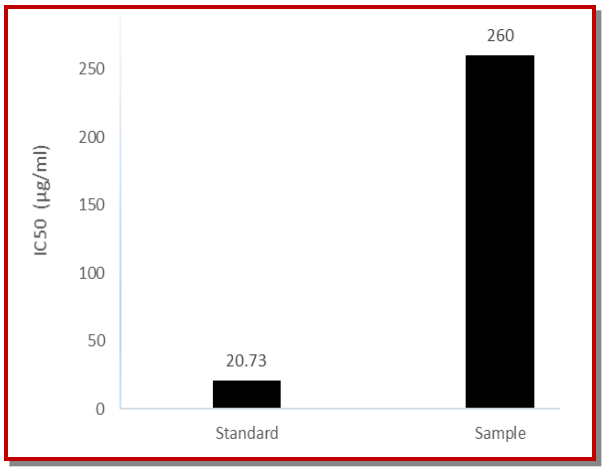

Figure 2: IC_{50} values of the standard BHT and sample methanolic extract of *X. indicum* leaves

Cytotoxic activity

The crude methanolic extract of X. indicum leaves was subjected to cytotoxic activity by the method of brine shrimp lethality bioassay and the obtained data of LC_{50} (lethal concentration of half of the test organism) of vincristine sulfate as a positive control and methanolic extract. The LC_{50} value of methanol extract was found at concentration 1.3 µg/mL whereas LC_{50} of vincristine sulphate showed at concentration of 0.8 µg/mL

Discussion

The phytochemical screening report confirmed that the presence of phytosterol, diterpene as a natural products in the methanolic extract of *X. indicum*. From the previous study, it was reported the presence of phytochemicals in the plant extract having active role in the management of various diseases (Yadav and Agarwal, 2010). This study displays the *in vitro* thrombolytic potential of crude methanolic extract *X. indicum* leaves using human blood. The results show,

slight thrombolytic activity. This is an important finding which may have important implications in cardiovascular health (Hossain et al., 2014) because blood clot formation is considered to be a critical event in which the damaged expanses of the endothelial cell surface or blood vessel are clogged by the deposition of fibrin, platelets and tissue factor (Furie and Furie, 2008). In addition, this finding may indicate the possibility of developing novel thrombolytic agents from the leaves of the plant. It was reported that the presence of phytochemicals like saponin, tannin and alkaloids in the plant extract are the probable reason for demonstrating the thrombolytic activity (Bhowmick et al., 2014; Chowdhury et al., 2011).

In present study, the leaves extract of *X. indicum* possess significant thrombo-lytic activity in spite of absent the responsible phyto-chemicals. It was observed from the study that, the plant extract demonstrated anthelmintic activity. The leaves extract of *X. indicum* exhibited significant dose dependent anthelmintic activity in earthworms in comparison to that of the standard of piperazine. The findings of this test results revealed that, the extract exhibited not only paralysis but also death of earthworms and the calculated time for paralysis and death of earthworms were inversely proportional to the plant extract concentration. Previous studies data reported that, the presence of phenol, tannins, alkaloids, terpenoids may be responsible for exhibit anthelmintic activity (Doughari, 2006; Salhan et al., 2011). In accordance with these studies, although those compound are absent in the leaves extract of *X. indicum* but its exhibit good anthelmintic activity.

For developing new chemotherapeutic agents, the plant species are considered to be an important resources. Hence, the present study was subjected to evaluate the toxicity of the methanolic extract of *X. indicum* by the method of brine shrimp lethality bioassay towards brine shrimps, an important indication of probable cytotoxic properties of the test materials (Meyer et al., 1982). Certain chemical classes from plants such as amonoterpenes, sesquiterpenes, diterpenes, triterpenes, steroids, cucurbitacins, saponins, cardenolides, lignan, quinines, pyrrolizidine and isoquinoline alkaloids are stated to have antitumor and cytotoxic effects (Evans, 1989). Therefore, it can be expected that the presence of phytosterol and diterpenes in the extract as shown by phytochemical screening are responsible for observed cytotoxicity in this study.

Conclusion

The plant extract possess thrombolytic, anti-oxidant, anthelmintic and cytotoxic properties.

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Conflict of Interest

The authors declare that they have on competing interest.

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