Thrombolytic, Membrane Stabilizing, Analgesic activities along with Phytochemical Screening of the Methanolic Extract of Xanthium indicum Koenig Fruits

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ABSTRACT
Objective: This study aimed to assess the thrombolytic, membrane stabilizing, and analgesic activities of the methanolic extract of the fruits of Xanthium indicum Koenig and undertook a phytochemical screening.

Material and Methods: A qualitative analysis method to evaluate phytochemicals was adopted. Analgesic efficacy was assessed in vivo using the acetic acid-induced writhing method in a mouse model. Membrane-stabilizing and thrombolytic tests were done in vitro measuring the percentage of inhibition of hypotonic solution-produced hemolysis and running a clot disruption assay, respectively.

Results: Crude methanolic extract was used for phytochemical screening, confirming the presence of reducing sugars, tannin, saponin, protein, phenol, and diterpenes. In the analgesic activity test, a 500 mg/kg dose of crude extract showed 40% inhibition of writhing, while 200 mg/kg showed a reduction of 22.64%. In the membrane stabilizing activity test, 10 mg/ml extract resulted in the highest inhibition rate of hemolysis with a value of 51.79%, while for acetyl salicylic acid (0.01 mg/ml), this value was 71.35%. In the thrombolytic activity test, 10 mg/ml plant extract showed 27.11% clot lysis, which was the maximum among our tested concentrations; however, 40.08% lysis was achieved by the standard drug streptokinase. In all cases, we found a dose-dependent response. Tannin and flavonoid are known to be responsible for analgesic and thrombolytic response.

Conclusion: As the methanolic extract of the fruits of X. indicum possesses potential pharmacological effects, the plant should be scrutinized comprehensively to detect the bioactive components present and the exact mechanism of their action in view of a drug development program.

Keywords: Xanthium, writhing, membrane stabilizing, thrombolytic agents

ÖZ
Xanthium indicum koenig meyvelerinin metanolik ekstraktının fitokimyasal tarama ve farmakolojik araştırması

Amaç: Bu çalışma, Xanthium indicum Koenig meyvelerinin metanolik özlüğünün trombolitik, membran stabilize edici ve analjezik aktivitelerini fitokimyasal taramaya değerleştirmek için uygulandı.

Gereç ve Yöntem: Bitki kimliği, kimyasal özellikleri, sinyal suyu ve pıhtı bozunumu ile inhibisyonu ile elde edildi.

Bulgular: Metanolik özlü, indirengici şekerler, tanin, saponin, protein, fenol ve diterpenlerin varlığından dolayı fitokimyasal tarama hecho. Analjezik aktivite testinde, 500 mg/kg dozunda hem erkektir %40 oranında kovrim inhibisyonu gösterikten, 200 mg/kg %22.64 oranında görüntüldü. 10 mg/ml ekstir membran stabilize etme aktivitesi testinde %51.79 değerinde en yüksek hemoliz oranını engelledi; aset ilac salicyl asit (0.01 mg/ml) için bu değer %71.35'di. Trombolitik aktivite testinde, 10 mg/ml bitki özlüğü, test edilen konsantrasyonuzun arasında maksimum olan pıht-lizizn %27.11; bununla birlikte standart ilaç streptokinaz ile %40.08邀zümden daha yüksek. Tanin ve flavonoidin analjezik ve trombolitik cevapta rol oynadığı anlaşıldı.

Sonuç: X. indicumun meyvelerinin metanolik ekstrakti potansiyel farmakolojik etkileri sahip olduğundan bitki, ilaç geliştirme programı için kesin etki mekanizmalları ile ilgili bir şekilde incelenmelidir.

Anahtar kelimeler: Xanthium, kovranma, membran stabilizasyonu, trombolitik ajanlar
**Introduction**

The term Xanthium is derived from the Greek Xanthos, meaning yellow, from the ancient name of a certain plant, whose fruit was used to dye the hair (1). *Xanthum indicum* is a coarse annual herb growing about a meter or more tall. *X. indicum* fruits contain glucoide (strumaroside), β-sitosterol, stigmasterol, 3,5-di-O-caffeoylquinic, polyphenols, 1,3,5-tri-O-caffeoylquinic acid, etc. (2).

Pain is a complicated disagreeable phenomenon composed of sensory experiences that include time, space, intensity, cognition, emotion, and motivation, originating from damaged tissue or abnormal physiological conditions. Due to the adverse effects of conventional analgesic drugs (e.g. gastric irritation, suffering, and opiate-induced dependency), their use in treatments as analgesic agents may be unsuccessful and sometimes life-threatening. Hence, analgesic treatment without those side effects is being sought all around the globe as a replacement for NSAIDs and opiates. Therefore, plant-based traditional medicine has received a huge interest, given the availability, low cost, small side effects, etc. (3).

On the other hand, myocardial or cerebral infarction, atherothrombotic disease may generate thrombus in arteries. (4,5). Thrombolytic substances like tissue plasminogen activator and urokinase are used to dissolve clots within the blood vessels that have deposited already (6-8). However, all of these drugs have certain limitations, such as a risk of developing fatal disorders, anaphylactic reactions, hemorrhage, lack of specificity, etc. Moreover, nowadays it is found that immunogenicity (due to thrombolytic therapy with streptokinase) may restrict the healing attempts of diseases for some patients (9). Agents isolated from plant sources are likely to be inexpensive and less antigenic. Accurate studies have been designed concerning the search for and development of different plant and microorganism sources that possess anticoagulant, anti-platelet, thrombolytic activity, etc. (10-15).

Inflammation is one of the key outcomes of a number of pathological disorders. Physiologically functional arachidonate metabolites are generated from inflammatory cells alongside a complex mixture of growth factors (16,17). These mediators work collectively to increase vasodilation and blood vessel permeability, which further leads to an elevation of blood flow, plasma protein exudation, and relocation of neutrophils from the outer region of veins and arteries to the inner area of damage tissue. Some pathological data also demonstrate that inflammation is the main initiator for developing atherosclerosis (18-20). The erythrocyte membrane is similar to the lysosomal membrane. As a result, it could be hypothesized that agents for stabilizing the RBC could be used to stabilize the lysosomal membrane (21). Those agents affect the release and action of mediators such as prostaglandins, histamine, leukotrienes, serotonin, etc. (22).

*X. indicum* is included in the list of potent medicinal plants of Bangladesh. Its fruit is used against sores of the lips and mucous membranes. Seeds are used for healing inflammatory swelling, bladder infections, herpes, erysipelas, etc. In this study, we evaluated the thrombolytic, membrane-stabilizing, and analgesic actions of the methanolic extract of fruits from *X. indicum* and undertaken phytochemical screening.

**Material and Methods**

**Material**

Lyophilized alteplase/streptokinase (Genentech Inc., USA), acetyl salicylic acid or aspirin (Sigma Aldrich Corporation, USA), and other chemicals used in the study were bought from the laboratory of the Department of Pharmacy, NSTU, Bangladesh.

**Human subjects and experimental animals**

For the thrombolytic and membrane-stabilizing activity test, blood samples were taken from young healthy persons who did not have a record of using birth-control pills or anticoagulant therapy. Twenty swiss albino mice (weight: 25-30 g, age: 30-35 days) were obtained from JU, Bangladesh (from the animal house of the university). They were kept under controlled temperature (25±2ºC) and RH (60-70%) for 7 days with rodent food and water to adapt before starting the experiment.

**Preparation of the methanolic extract**

Mature fruits of *X. indicum* (Family: Asteraceae) were collected in various areas of Noakhali district, Bangladesh. The plant material was prepared by taxonomist Naimur Rahman of the National Herbarium, Mirpur, Dhaka, and a specimen was kept there for future reference. For preparing the methanolic extracts, 500 gm ground powder material was filled into an opaque colored glass vessel and macerated with methanol (approx. 1800 ml). The container was sealed tightly and stored for 15 days with occasional shaking. Then the substance
macerated in methanol was first passed through a cotton filter and subsequently through filter paper.

**Phytochemical screening**

Qualitative tests were carried out to evaluate the presence of phytoconstituents in the *X. indicum* extract. By using standard phytochemical procedures, the presence of these constituents in the extract was identified (23).

**Study of analgesic activity**

To evaluate the analgesic activity of *X. indicum*, the acetic acid-induced writhing method was used in a mouse model (24). Reflexive writhing was induced in the mice. At first, 1.0% acetic acid solution was injected intraperitoneally and the animals’ specific contraction (writhing) observed. Comparisons of writhing were made against a standard (Indomethacin), control, and test samples. To prepare the test material at a dose of 250 and 500 mg/kg body weight, 62.5 and 125 mg plant extract, respectively, were triturated with a small amount of DMSO. Proper mixing was done and distilled water was added slowly for adjusting the suspension volume to 2.5 ml. The dose of crude sample extracts was administered orally 30 minutes before acetic acid injection. Distilled water was given as control. Analgesic activity was assessed by counting the number of writhing movements. Experimental mice were selected arbitrarily and divided into four groups (n=5 for each group) designated G-I (control/vehicle only), G-II and G-III for plant extract, and G-IV (standard/indomethacin). Five minutes after injecting acetic acid, a count of the abdominal contractions was done (10 minutes duration for each mouse). The percentage of writhing inhibition was quantified according to the following formula:

\[
\text{Inhibition} = \left( \frac{\text{Mean no. of writhing movements (control)}}{\text{Mean no. of writhing movements (test)}} - 1 \right) \times 100
\]

**Study of thrombolytic activity**

Clot lysis activity (*in vitro*) of *X. indicum* was measured by using the methodology described in Prasad et al. with slight modifications (25). Blood (approx. 7 ml) was taken from healthy people (n=5) and kept in different sterilized microcentrifuge tubes followed by incubation (37°C, 45 minutes). After the formation of clots, serum was discarded totally from the tubes (without disturbing the clots formed already) and the weight of the clots determined (with a Cubis analytical balance: Model: Sartorius, MSE225S-100-DA, Germany). Proper labeling of the all tubes containing clot was done and various concentrations of the extract were added accordingly. We used 10 mg, 20 mg, 30 mg, 40 mg, 50 mg plant extract in 5 ml distilled water, stirring thoroughly, to prepare 2 mg/ml, 4 mg/ml, 6 mg/ml, 8 mg/ml, and 10 mg/ml plant extract, respectively. Distilled water was used as a control, and streptokinase (30,000 IU) was used as a standard. The mixtures were incubated once again (37°C, 90 minutes). Then the fluid was discarded from the tubes and the tubes weighed again to determine the differences. Finally, the weight differences were estimated and results were calculated as clot lysis percentage using the equation below:

\[
\text{Clot lysis (%) = (Weight of released clots /Total weight of the clots) } \times 100
\]

**Study of membrane-stabilizing activities**

We followed the method of Rashid et al. for studying the membrane stabilizing effect of *X. indicum* (24). The blood collected from healthy volunteers (n=5) was rinsed by using isotonic solution (0.9%). In this aspect, NaCl solution (0.16 M) or nonelectrolyte solution (0.3 M) is almost isotonic with human erythrocytes (RBC). 4.505 g NaCl was dissolved in distilled water and mixed properly to prepare 500 ml isotonic solution (154 mM). 1.463 g NaCl was added and mixed properly to prepare 500 ml hypotonic solution (50 mM).

After collecting the blood, we added EDTA to prevent clotting. Ten mM sodium phosphate buffer (pH 7.4) was prepared by centrifuging at 3000 rpm for 10 minutes and used for washing the blood. Washing was done for three times with proper care. The suspension which we collected finally was a stock erythrocyte (RBC) suspension.

The solution we used in this experiment was hypotonic in nature. The sample consisted of RBC suspension with 5 ml of hypotonic solution in 10 mM Na₂PO₄ buffer containing either various concentrations of extract or standard drug. Before this, we prepared the concentration of 2 mg/ml, 4 mg/ml, 6 mg/ml, 8 mg/ml, 10 mg/ml plant solutions by using 10 mg, 20 mg, 30 mg, 40 mg, and 50 mg plant extract, respectively, in 5 ml distilled water. Distilled water was used as control and acetyl salicylic acid was used as standard in this experiment. The concoctions were incubated at 25°C for 10 minutes, centrifuged...
(3000 rpm, 10 minutes) and the absorbance (OD) was measured at 540 nm wavelength using UV-spectrophotometer (Shimadzu, Japan). Suppression of hemolysis was calculated by using the formula
\[
\text{Inhibition of hemolysis (\%) = } \left(\frac{\text{OD1} - \text{OD2}}{\text{OD1}}\right) \times 100
\]
where, 
\[
\text{OD1} = \text{Optical density of hypotonic-buffered solution (control)} \]
\[
\text{OD2} = \text{Optical density of tested samples in hypotonic solution}
\]

**Statistical Analysis**

One-way ANOVA followed by Dennett's t-tests were performed for analyzing the data of this study. *p \leq 0.05 was considered statistically significant.

**Results**

**Evaluation of phytochemical screening**

Qualitative chemical tests were carried out for identifying the phytochemicals present in *X. indicum* (methanolic extract). The outcomes of the different tests for detecting and ascertaining the chemical components are shown in Table 1.

**Evaluation of analgesic activity**

The effect of *X. indicum* against writhing in animals is summarized in Table 2. The plant extracts reduce writhing in a dose-dependent manner. The reduction is significant when compared with controls. Indomethacin is used as a standard in this experiment.

**Evaluation of membrane stabilizing activity**

In-vitro membrane-stability activity of the methanolic extract of *X. indicum* was assessed for confirming its activity. It is observed that methanolic extract of *X. indicum* has a dose-dependent membrane-stabilizing activity (Figure 1). The percentage of inhibition of RBC hemolysis is 35.54% for the crude methanolic extract of *X. indicum* (conc. 2 mg/ml). This effect increases proportionally with increasing extract concentration.

<p>| Table 1: Phytochemical screening test results of <em>X. indicum</em> fruit extract. |</p>
<table>
<thead>
<tr>
<th>Chemical group</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saponin</td>
<td>-</td>
</tr>
<tr>
<td>Phytosterol</td>
<td>-</td>
</tr>
<tr>
<td>Reducing sugars</td>
<td>+</td>
</tr>
<tr>
<td>Alkaloid</td>
<td>-</td>
</tr>
<tr>
<td>Tannin</td>
<td>+</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>-</td>
</tr>
<tr>
<td>Protein</td>
<td>+</td>
</tr>
<tr>
<td>Phenol</td>
<td>+</td>
</tr>
<tr>
<td>Flavinoid</td>
<td>+</td>
</tr>
<tr>
<td>Diterpenes</td>
<td>+</td>
</tr>
</tbody>
</table>

\((+)=\text{present (-)=absent}\)

<p>| Table 2: Assessment of analgesic activity of methanolic <em>X. indicum</em> fruit extract in mice. |</p>
<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Average number of writhing (after treatment)</th>
<th>Percentage of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-I (control)</td>
<td>11.33 ± 0.58</td>
<td>-</td>
</tr>
<tr>
<td>G-I (250 mg/kg b.w.)</td>
<td>9.83 ± 0.76*</td>
<td>22.64</td>
</tr>
<tr>
<td>G-II (500 mg/kg b.w.)</td>
<td>6.50  ± 0.50*</td>
<td>40.00</td>
</tr>
<tr>
<td>G-IV (Indomethacin, 25 mg/kg)</td>
<td>5.33 ± 0.58*</td>
<td>46.64</td>
</tr>
</tbody>
</table>

\(\text{Values indicated as Mean} \pm \text{STD (n=5)}; \ p* < 0.05 is significant.\)
concentration, and the highest inhibition of 51.79% was found for 10 mg/ml of extract. The standard (acetyl salicylic acid; concentration: 0.1 mg/kg) decreased hemolysis of RBC by 71.35%.

**Assessment of thrombolytic efficacy**

Thrombolytic efficacy of the crude extract in our study is shown in Figure 2. We found that *X. indicum* possesses a dose-dependent thrombolytic activity. Highest clot lysis (27.11%) was found for the plant extract at a 10 mg/ml concentration. Streptokinase (an enzyme used as standard) induced 71.35% inhibition and negative control (water) provided only 2.55% clot lysis.

**Discussion**

In the phytochemical screening test, we found that *X. indicum* (methanolic extract) contains reducing sugar, tannin, protein, phenol, flavonoid, diterpenes, etc. So, there is a great chance to find bioactive components in this plant extract. Both peripheral and central analgesia can be identified by the test of acetic acid-induced abdominal pain, followed by counting the number of writhing movements of mice. The sensation of pain is triggered by a local reaction starting with the release of arachidonic acid from the tissue phospholipid (26) via cyclooxygenase (COX) producing prostaglandin (PG). Writhing induced by acetic acid injection may be linked with an increment of PGE2 and PGF2α and lipoxygenase products followed by the rise of inflammatory pain by accelerating capillary permeability. This test was effective for achieving locally active analgesia (27,28). Our results showed that methanolic extract of the fruits of *X. indicum* exhibit a significant inhibitory effect on acetic acid-induced writhing. This inhibition is also dose-dependent. The COX inhibitory and antioxidant potential may be decreased either by the synthesis of free arachidonic acid or by inhibiting the enzymatic activities responsible for the production of PG. According to Das et al., flavonoids and tannins might be accountable for the analgesic activity (29). Phytochemical screening in this study also found that methanolic extract of *X. indicum* contains flavonoid and tannins. Perhaps these constituents are responsible for the analgesic activity of *X. indicum*.

Many studies (both *in vitro* and *in vivo*) show that flavonoids exert a stabilizing effect on lysosomes. In addition, tannins, and saponins are also capable of improving the stabilizing effect of erythrocytes because of having a binding capability with cations and other biomolecules (26). Our tested extract also contains flavonoids, saponin, and tannin. So there is a great possibility for *X. indicum* to have a membrane-stabilizing effect due to the presence of these phytoconstituents.

A number of studies have been done to research the anti-thrombolytic effects of natural sources in order to prevent cardiopulmonary disorders and strokes. In order to find better cardio-protective agents from plant sources, we have investigated the thrombolytic activity of *X. indicum* fruits. It was noticed that crude methanolic extract of *X. indicum* showed moderate clot lysis efficacy (27.11%) at a dose of 10 mg/ml. The presence of saponins and tannins in the plant extract may play an important role for this activity by affecting the activation of plasminogen. Plasminogen works as a fibrin-dependent mechanism similar to that of streptokinase (the standard drug). In this case, plasminogen induces extreme synthesis of plasmin, which may disrupt fibrin (the prime component of thrombi) and result in blood clots being easier more easily dissoluble (24,25).

**Conclusion**

Based on our experimental results, we conclude that the methanolic extract of *X. indicum* fruits possesses a high amount of phytoconstituents that might be bioactive. The crude extract possesses remarkable analgesic and membrane-stabilizing activities. In addition, it also showed moderate thrombolytic efficacy. Advanced research explorations are warranted to elucidate the exact bioactive compounds liable for the above activities for understanding the mechanism of such activities for a drug development program.
References

9. Leta GC, Mourao PAS, Tovar AMF. Human venous and arterial glycosaminoglycans have similar affinity for plasma low-density lipoproteins. BBA Mol Basis Dis 2002;1586(3):243-253. [CrossRef]
17. Anosike CA, Obidoa O, Esezonyi IUIS. Membrane stabilization as a mechanism of the anti-inflammatory activity of methanol extract of garden egg (Solanum aethiopicum). DARU J Pharm Sci 2012;20:76. [CrossRef]

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