PHARMACOLOGICAL INVESTIGATION OF THE CHLOROFORM EXTRACTS OF ALSTONIA SCHOLARIS (L.) R.BR

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ABSTRACT

Chloroform extracts of Alstonia scholaris leaves (CLAS) and stem barks (CSAS) have been evaluated for their analgesic, antimicrobial, antioxidant and cytotoxic activity. Acetic acid induced writhing method was used for evaluating analgesic activity. Both the extracts had reduced pain in dose dependent manner, at all the tested doses (200 and 400 mg/kg body weight). Maximum writhing inhibition (75.33 %) was observed at 400 mg/kg dose of CSAS while for CLAS, 400 mg/kg dose exhibited 70.13 % inhibition. The inhibitory effect of indomethacin (45.92 %) was lower than that of the highest dose of CLAS and CSAS. Antimicrobial activity of the extracts was evaluated against various Gram-positive, Gram-negative bacteria and fungi using disk diffusion technique. The average zone of inhibition exhibited by extract was found 10-14 mm and Kanamycin (30 μg/disc) was used as standard. Antioxidant potentiality of the extracts was investigated on DPPH scavenging activity and the IC₅₀ value was found 47.72 μg/ml, 62.03 μg/ml and 45.77 μg/ml for CSAS, CLAS and standard ascorbic acid, respectively. Cytotoxic study was done by brine shrimp lethality bioassay and compared with LC₅₀ (8.90 μg/ml) values of standard vincristine sulphate as a positive control. The cytotoxicity exhibited CLAS and CSAS were promising with LC₅₀ value of 10.21 μg/ml and 9.12 μg/ml, respectively. These results suggest into the plant extracts could be used as a potential therapeutics in many pathological conditions.

Keywords: Acetic acid induced writhing, antimicrobial activity, antioxidant, free radical, brine shrimp lethality bioassay.

INTRODUCTION

Phytochemical aspects of most medicinal plants have been known and used since time memorial. Ethnobotanical advantages conferred by these plant based products have surpassed the chemical counter parts owing to their lesser side effects and more potent therapeutic effect. Natural products continue to play the most significant role in the drug discovery and development process. Hence it is a demanding need of the hour to study the various pharmacologically valuable aspects of these medicinal plants. Alstonia scholaris (Apocynaceae) also called ‘Shahtaparna’ or ‘Devil’s’ tree is a large evergreen tree. It is commonly found in the subtropical regions of South Asia and Africa and is native to Bangladesh. Almost all parts of the plants are used in medicine. Bark of A. scholaris possess spectrum of pharmacological activity ranging from bitter, astringent, thermogenic, febrifugic, digestive, laxative, antipyretic, anthelmintic to galactogogic and cardiotonic properties, therefore used in fevers, malarial fevers, abdominal disorders, dyspepsia, leprosy, skin diseases, asthma, bronchitis, cardiopathy, helminthiasis etc. In folklore medicine, Latex obtained from the exudates of the tree has been in application for ulcers, sores, tumors and in rheumatoid pain; as well as mixed with oil and dropped into ear for earache. Fruits are useful in syphilis and epilepsy and also used a tonic, anti periodic and anthelmintic. Methanolic extracts of roots and flower have exhibited potent antimicrobial activity. Leaves are used in the treatment of beri-beri, congestion of liver, dropsy and ulcers. In the China, the leaves have been historically used in ‘dai’ enthopharmacy to treat chronic respiratory diseases. The leaf extract developed as a commercially available traditional Chinese medicine, used to release tracheitis and cold symptoms. In the quest of searching plants having significant pharmacological and biological activities in Bangladesh, therefore the present study is carried out to investigate the crude chloroform extracts of leaves and stem bark of Alstonia scholaris for its analgesic, antimicrobial, antioxidant and cytotoxic activities.

MATERIALS AND METHODS

Drugs and Chemicals

Acetic acid (Merck, Germany), Indomethacin (Square Pharmaceuticals Ltd.), Tween-80 (BDH Chemicals Ltd), Normal saline solution (0.9 % NaCl) (Beximeo Infusion Ltd.), DPPH (1, 1-diphenyl, 2-picryl hydrazyl) (Sigma Chemical Co., USA), Dimethyl sulfoxide (DMSO) (Merck, Germany) etc. were used for conducting the tests.

Test animals

Healthy Wister rats of either sex weighing about 135-150 g were used for the experiment. They were collected from the Animal Resource Branch of the "International Center for Diarrheal Disease and Research, Bangladesh (ICDDR, B)". They were kept in standard environmental condition (at 24.0 ± 0°C temperature and 55-65 % relative humidity and 12 hour light/12 hour dark cycle) for one week for acclimation after their purchase and fed ICDDR, B formulated rodent food and water. The set of rules followed for animal experiment were approved by the institutional animal ethical committees.

Instruments

The molecular absorption spectra and absorbance at specific wavelengths were recorded with a HACH DR 4000UV visible spectrophotometer equipped with quartz cells of 1-cm light path.

Collection and preparation of plant material

The fresh plant Alstonia scholaris (leaves and stem) were collected from Chauddagram, Comilla, Bangladesh on October, 2011. The plant was identified by the taxonomist of...
Bangladesh National Herbarium, Mirpur, Dhaka, Bangladesh and a voucher specimen was deposited in the herbarium unit (accession number DACB: 37498). The sun dried powdered leaves and stems (250 mg) of A. scholaris were macerated in 500 ml of 99.8 % chloroform (Merck, Germany) separately. After 3 days of occasional shaking and stirring the solutions were filtered using filter cloth and Whatman® filter paper No. 1. The resulting filtrates were then evaporated in water bath maintained at 45°C to dryness and thus a gummy concentrate of greenish colored extract. Finally, about 45.23 g Chloroform extract of leaves of Alstonia scholaris (CLAS) and about 30.22 g Chloroform extract of stem bark of Alstonia scholaris (CSAS) was found.

**In vivo analgesic activity test**

**Acetic acid induced writhing method**
The analgesic activity of the crude Chloroform extracts of CLAS and CSAS were studied using acetic acid induced writhing model in rat.²⁰ At first, thirty six animals were divided into six groups with six mice in each.

- **Group I:** Treated with vehicle (1 % Tween 80 in water, 10 ml kg⁻¹ (p.o.)
- **Group II:** Received Indomethacin (10 mg/kg) body weight (p.o.)
- **Group III** and **Group IV:** Treated with 200 and 400 mg kg⁻¹ body weight (p.o.) of CLAS, respectively.
- **Group V** and **Group VI:** Treated with 200 and 400 mg kg⁻¹ body weight (p.o.) of CSAS, respectively.

The test samples and vehicle were administered orally 30 minutes before intraperitoneal administration of 0.7 % v/v acetic acid but Indomethacin (reference drug) was administered orally 15 minutes before injection of acetic acid. After an interval of 5 minutes, the mice were observed for specific contraction of body referred to as ‘writhing’ for the next 10 minutes. Full writhing was not always accomplished by the animal; this incomplete writhing was considered as half-writhing. Accordingly, two half-writhing were taken as one full writhing. The number of writhes in each treated groups was compared to that of a control group. Samples having analgesic activity will reduce number of writhes of treated mice. The percent inhibition (% analgesic activity) was calculated by

\[
\% \text{ inhibition} = \frac{(A-B)/A}{100}
\]

Where, A = Average number of writhing of control per group;
B = Average number of writhing of test per group

**Antimicrobial assay**

**Microorganisms**
Antimicrobial activity was tested against Bacillus megaterium, B. subtilis, B. cereus, Staphylococcus aureus, Sarcina lutea, Vibrio mimicus, V. parahemolyticus, Pseudomonas aeruginosa, Escherichia coli, Shigella boydii, S. dysenteriae, Salmonella paratyphi, Saccharomyces cerevisae, Candida albicans and Aspergillus niger. These microbial strains were isolated from clinical samples and obtained as pure cultures from the Institute of Nutrition and Food Science (INFS), University of Dhaka, Bangladesh.

**Determination of the diameters of inhibition zone**
The crude chloroform extracts of leaves and stem bark of Alstonia scholaris were tested in vitro for antimicrobial activity by the standard disc diffusion method²¹,²² against the bacteria. Solutions of known concentration (500 μg/10 μl) of the test samples were made by dissolving measured amount of the samples (50 mg) in 1 ml of solvents. Dried and sterilized filter paper discs (6 mm diameter) were then impregnated with known amounts of the test substances (500 μg/disc) using micropipette and the residual solvents was completely evaporated. Discs containing the test materials were placed on to nutrient agar medium uniformly seeded with the test microorganisms. Standard disc of kanamycin (30 μg/disc) and blank discs (impregnated with solvents followed by evaporation) were used as positive and negative control, respectively. These plates were then kept at low temperature (4°C) for 16 hours to allow maximum diffusion of the test materials and kanamycin. The plates were then incubated at 37°C for 24 hours to allow maximum growth of the organisms. The test material having antimicrobial activity inhibited the growth of the microorganisms and a clear, distinct zone of inhibition was visualized surrounding the discs. The antimicrobial activity of the test agents was determined by measuring the diameter of zone of inhibition expressed in mm. The experiment was carried out in triplicate and the mean value was taken.

**Antioxidant study by DPPH free radical scavenging activity**
The ability of ethanolic extracts of leaves and stem bark of A. scholaris to scavenge 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) free radicals was estimated as previously described by Hasanuzzaman et al.,²³. The percentage inhibition activity was calculated from the following equation.

\[
\text{Percentage of inhibition} = \frac{[A_0 - A_f]/A_0] \times 100
\]

Where \(A_0\) is the absorbance of the control, and \(A_f\) is the absorbance of the extract/ standard. \(K_{50}\) value was calculated from the equation of line obtained by plotting a graph of concentration (μg/ml) versus % inhibition.

**Cytotoxic activity**
The cytotoxicity was conducted using brine shrimp lethality test following the method of Meyer et al²⁴. The brine shrimp eggs were placed in 1 liter of sea water, aerated and hatched for 48 hours at 37°C to become nauplii. After 48 hours, ten brine shrimp nauplii were placed in a small container filled with seawater. CLAS and CSAS, serially diluted with DMSO (Dimethyl sulfoxide), were then added to the container. The mortality of brine shrimp was observed after 24 hours of treatment was given. Vinristine sulphate was used as positive control. The lethal concentration (LC₅₀) of the test samples after 24 hours was determined 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.

**RESULTS**

**Analgesic activity**

**Acetic acid induced writhing method**
The results showed that the pain relief was achieved significant in dose dependent manner, at all test doses (200 and 400 mg/kg body weight) as shown in Table 1. Maximum writhing inhibition (75.33 %) was observed at 400 mg/kg dose of CSAS while for CLAS, 400 mg/kg dose exhibited 70.13 % inhibition. The inhibitory effect of indomethacin (45.92 %) was lower than that of the highest dose of CLAS and CSAS.
**Antimicrobial screening**

The chloroform extracts of *A. scholaris* were screened against twelve human pathogenic bacteria to check antibacterial activities by disc diffusion method. The extracts showed mild to moderate activity against tested pathogenic bacteria which was shown in Table 2. CSAS showed moderate antibacterial activity against tested pathogenic bacteria. CLAS showed good antibacterial activity against Gram (-ve) pathogenic bacteria with an average zone of inhibition of 10-14 mm. This extract was found very active against *Shigella dysenteriae* (14 mm) and *Shigella boydii* (13 mm). No activity was found against *E. coli, B. subtilis, S. paratyphi* for both the extracts. The antifungal activity of CLAS and CSAS against tested fungus was shown in Table 3. Mild activity was found against *Sacharomyces cerevaceae* and no activity was found against *Aspergillus niger* and *Candida albicans*.

**DPPH free radical scavenging activity**

DPPH is most common stable radical commonly used in antioxidant assays. The percentage (%) scavenging of DPPH free radical was found to be concentration dependent i.e. concentration of the extract between 5-100 µg/ml greatly increasing the inhibitory activity (Figure 1). The IC50 of CSAS and CLAS was found 47.72 µg/ml and 62.03 µg/ml, respectively (Table 4). But both extracts had a lower scavenging activity than the standard ascorbic acid (IC50 = 45.77 µg/ml), which was used as standard.

**Cytotoxic activity**

In cytotoxic test activity, % mortality increased gradually with the increase in concentration of the test samples of both the extracts (Figure 2). Vincristine sulphate (VS) was used as positive control and the LC50 value was found 8.90 µg/ml. LC50 values obtained from the best-fit line slope were 10.21 µg/ml and 9.12 µg/ml for CLAS and CSAS respectively (Table 5).

**DISCUSSION**

In our present study the analgesic activity of *A. scholaris* extract was assessed by acetic acid induced writhing model. The acetic acid-induced writhing model is extensively used for the determination of analgesic activity because of its sensitivity and response to the molecules at a dose that is not effective in other models25. Acetic acid causes algesia by releasing endogenous substances that excite the pain nerve ending and also because of some other pain mediators like arachidonic acid via cyclooxygenase, and prostaglandin biosynthesis26. It is also seen that the level of lipoxygenase enzyme in peritoneal fluids is also increased by acetic acid27. Substances that inhibit writhings must have significant analgesic activity which may be attributed by the inhibition of prostaglandin synthesis, a peripheral mechanism of pain inhibition28. Several phytochemicals such as flavonoids, tannins, alkaloids etc. have been reported to possess analgesic activity29. The preliminary study on *A. scholaris* revealed that the plant is abundant of a wide spectrum of phytochemicals like tannin, carotene, phytoesterol, resin, gum, iso flavonoids, alkaloids and saponins30,31. These compounds may attribute to the potent analgesic activity of the chloroform extracts of the plant. Bacteria and fungi are responsible for many infectious diseases. But bacteria are becoming resistant to conventional antibiotics32 at an alarming rate. Antibacterial resistance has created desperate need for the search of new antibacterial33,34. The disc diffusion method had shown that the tested chloroform extracts of the steam barks and leaves of *A. scholaris* have moderate antimicrobial activity against the tested organisms. These extracts showed weak potentiality against tested fungus. The DPPH assay is one of the most common and relatively quick methods used for testing radical scavenging activity of various plant extracts35. In our present study, the antioxidant activity was determined using this method. Percentage inhibition of DPPH and IC50 are parameters widely used to measure antioxidant/free radical scavenging power36,37. The results of this study indicated that, the IC50 in chloroform extract of stem barks of *A. scholaris* was significantly lower than that of the leaf extract suggesting that the chloroform extract of leaves had better scavenging activity than the steam bark extract. It was reported that several active compounds such as anthocyanins, saponins, tannins, flavones, and polyphenols etc. are responsible for demonstrating antioxidant activity of plant extracts38. *A. scholaris* is proved to be a potent source of flavonoid, saponins and tannins39,40. Therefore, it may be said that these compounds may play the significant role in revealing the antioxidant property of the plant extracts. The findings of the brine shrimp lethality bioassay method showed that CSAS possesses better cytotoxic activity in comparison to CLAS. Previous studies have proved that several bioactive compounds like glycosides, alkaloids, flavonoids and saponins show cytotoxic activities due to their diverse chemical compounds41. Some of these are present in the plant extract which may be accountable for the cytotoxicity of the plant extracts. However this type of cytotoxicity is non-specific. Thus further studies including animal model should be conducted to test the possible antitumor or anti-carcinogenic activity of the plant extracts.

**Table 1: Effect of chloroform extracts of leaves and stem barks of *A. scholaris* on acetic acid induced writhing in rats**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Dose</th>
<th>No. of writhing</th>
<th>% Writhing inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group-I (Control)</td>
<td>1% Tween 80 in water</td>
<td>10 ml/kg body weight</td>
<td>22.5</td>
<td>45.92</td>
</tr>
<tr>
<td>Group-II (Standard)</td>
<td>Indomethacin</td>
<td>10 mg/kg body weight</td>
<td>12.167</td>
<td>60.35</td>
</tr>
<tr>
<td>Group-III</td>
<td>CLAS</td>
<td>200 mg/kg body weight</td>
<td>8.92</td>
<td>58.5</td>
</tr>
<tr>
<td>Group-IV</td>
<td>CLAS</td>
<td>400 mg/kg body weight</td>
<td>6.72</td>
<td>70.13</td>
</tr>
<tr>
<td>Group-V</td>
<td>CSAS</td>
<td>200 mg/kg body weight</td>
<td>8.45</td>
<td>62.44</td>
</tr>
<tr>
<td>Group-VI</td>
<td>CSAS</td>
<td>400 mg/kg body weight</td>
<td>5.55</td>
<td>75.33</td>
</tr>
</tbody>
</table>

Here, n = 6, CLAS = Chloroform Leaves extract of *A. scholaris*, CSAS = Chloroform Stem bark extract of *A. scholaris*.
Table 2: *In vitro* antibacterial activity of chloroform extracts of *A. scholaris* leaves and steam barks

<table>
<thead>
<tr>
<th>Test organism</th>
<th>Diameter of zone of inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chloroform extract of <em>A. scholaris</em> (CSAS)</td>
</tr>
<tr>
<td><strong>Gram positive bacteria</strong></td>
<td></td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>-</td>
</tr>
<tr>
<td>Bacillus megaterium</td>
<td>10</td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>10</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>10</td>
</tr>
<tr>
<td>Sereina lutea</td>
<td>7</td>
</tr>
<tr>
<td><strong>Gram negative bacteria</strong></td>
<td></td>
</tr>
<tr>
<td>Vibrio mimicus</td>
<td>12</td>
</tr>
<tr>
<td>V. parahaemolyticus</td>
<td>10</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>7</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>-</td>
</tr>
<tr>
<td>Shigella dysenteriae</td>
<td>8</td>
</tr>
<tr>
<td>Shigella boydii</td>
<td>10</td>
</tr>
<tr>
<td>Salmonella paratyphi</td>
<td>-</td>
</tr>
</tbody>
</table>

(-) = No activity

Table 3: *In vitro* antifungal activity of chloroform extracts of *A. scholaris* leaves and steam barks

<table>
<thead>
<tr>
<th>Test organisms</th>
<th>Diameter of zone of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chloroform extract of <em>A. scholaris</em> (bark)</td>
</tr>
<tr>
<td>Aspergillus niger</td>
<td>-</td>
</tr>
<tr>
<td>Saccharomyces cerevaceae</td>
<td>8</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>-</td>
</tr>
</tbody>
</table>

(-) = No activity

Table 4: IC₅₀ of *A. scholaris* extracts and Ascorbic acid

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC₅₀ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid (Standard)</td>
<td>45.77</td>
</tr>
<tr>
<td>CLAS</td>
<td>62.0</td>
</tr>
<tr>
<td>CSAS</td>
<td>47.7</td>
</tr>
</tbody>
</table>

Table 5: Cytotoxic potential of chloroform extracts of leaves and stem barks of *A. scholaris* along with Vincristine sulphate

<table>
<thead>
<tr>
<th>Sample</th>
<th>LC₅₀ (µg/ml)</th>
<th>Regression Equation</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vincristine Sulphate</td>
<td>8.90</td>
<td>y = 36.594x + 22.387</td>
<td>0.993</td>
</tr>
<tr>
<td>CLAS</td>
<td>10.21</td>
<td>y =39.003x + 14.976</td>
<td>0.971</td>
</tr>
<tr>
<td>CSAS</td>
<td>9.12</td>
<td>y =37.524x + 23.635</td>
<td>0.973</td>
</tr>
</tbody>
</table>

Figure 1: DPPH free radical scavenging activity of CLAS and CSAS extracts of *A. scholaris* along with ascorbic acid
CONCLUSION
Our preliminary pharmacological studies on the chloroform extract of *A. scholaris* leaves and stem barks provide in part scientific support for the use of this species in traditional medicine, particularly in various ailments related to pain. The non prescription use of medicinal plants is cited today as an important health problem, in particular their toxicity to the kidney. So, if the plant extract found to show significant antimicrobial activities must take into account acceptable levels of toxicity. Therefore, further pharmacological investigations are required to understand the underlying mechanism of these pharmacological activities.

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