Phytochemical, Antimicrobial, Cytotoxic, Analgesic and Anti-Inflammatory Properties of *Azadirachta Indica*: A Therapeutic Study

Talha Bin Emran1,*, Mir Muhammad Nasir Uddin2, Atiar Rahman2, Zia Uddin1 and Mohaiminul Islam1

1Department of Pharmacy, BGC Trust University Bangladesh, Chittagong-4000, Bangladesh
2Department of Biochemistry and Molecular Biology, University of Chittagong, Chittagong-4331, Bangladesh

Abstract

In this study the ethanol extract, n-hexane extract and chloroform extract of *Azadirachta indica* were first evaluated for phytochemical study. The phytochemical screening of the three extracts of *Azadirachta indica* exhibited the presence of important secondary metabolites such as flavonoids, terpenoids, steroids and tannins. The extracts showed potential antimicrobial activities against thirteen different strains of microorganisms. Secondly, they were screened *in vitro* for cytotoxicity test by brine shrimp lethality bioassay and results illustrated significant (p<0.05) cytotoxicity against *Artemia salina*. To test the analgesic properties of ethanol extract of *Azadirachta indica*, hot plate and acetic acid induced writhing methods were used. At two different doses (250 and 500 mg/kg body weight), the analgesic tests were performed on Swiss Albino mice. Also, the anti-inflammatory tests were performed by carrageenan induced paw edema method on long Evans rats at the two different doses of 250 and 500 mg/kg body weight using ethanol extract. Our results indicated that *Azadirachta indica* possesses remarkable analgesic and anti-inflammatory activity.

Keywords: *Azadirachta indica*; Phytochemical; Cytotoxicity; Antimicrobial; Analgesic; Anti-inflammatory

Introduction

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources, many based on their use in traditional medicine. It has been noted that the original source of many important pharmaceuticals in current use have been plants used by indigenous people [1]. It has been reported that about 64% of the total global population remains dependent on traditional medicine and medicinal plants for provision of their health-care needs [2]. In order to promote the use of medicinal plants as potential sources of antimicrobial compounds, it is pertinent to thoroughly investigate their composition and activity and thus validate their use [3]. Some phytochemicals produced by plants have antimicrobial activity allowing these plants to be studied and used for the development of new antimicrobial drugs [4]. The effectiveness of phytochemicals in the treatment of various diseases may lie in their antioxidant effects [5]. Secondary plant metabolites are largely unexplored in ‘conventional’ animal production systems. In the past, plant metabolites were generally considered as sources of anti-nutritional factors. Recent bans and restrictions on the use of animal antibiotic growth promoters stimulated interest in bioactive secondary metabolites of plant source as alternative performance enhancers [6].

*Azadirachta indica* (Neem) belonging to Meliaceae family is one of the two species of the genus *Azadirachta*. Although it is native to Bangladesh, it is also available in other tropical and sub-tropical regions such as India, Burma, Sri Lanka, Malaysia and Pakistan [7-9]. Neem is an evergreen and fast-growing tree of reaching an average height of 15-20 meter which might be 35-40 meter in rare cases. Numbers of biologically active compounds have been isolated from the leaf, bark and seeds of this plant. A bitter principle Nimbidin found in Neem bark was found to be antipyretic, non-irritant and effective in skin diseases such as eczema, arsenical dermatitis, burn ulcers, furunculosis, herpes labialis, scabies and seborrheic dermatitis [10,11]. It is also useful for warts and dandruff. Nimbidin and sodium nimbidinate from the bark of Neem were reported to show spermicidal actions. Bark extracts are evident to show very promising diuretic and anti-inflammatory properties. Neem bark has shown anti-bacterial activity against various Gram-positive organisms [12]. The aims of the present investigation were the phytochemical properties of ethanol, n-hexane and chloroform extract of *A. indica*, the antimicrobial activity of three extracts in terms of the diameters of growth of inhibition of supplied test organisms, the cytotoxic effect of the bark extract, analgesic effects of ethanol extract and anti-inflammatory effects of ethanol extract.

Materials and Methods

Collection and identification of plants

The plant *A. indica* was collected from Jessore, Bangladesh during the month of November-December. The bark of the plant was collected, sun dried and pilled off. The plant was identified by the experts of Bangladesh National Herbarium, Mirpur, Dhaka (Accession No. 30950).

Extraction of the plant

The collected plant barks were aerated by fan aeration to be partially dried. Then they were heated through Oven to be fully dried at below 40°C for two days. The fully dried barks were then grinded to make them powder by the help of a suitable grinder. The whole powders were extracted by cold extraction with three solvent ethanol, n-hexane and chloroform and kept for a period of three (03) days accompanying extraction at 4°C.
occurring shaking and stirring. The whole mixture then underwent a coarse filtration by a piece of clean, white cotton material. Then these were filtered through Whatman filter paper. The filtrate (ethanol, n-hexane and chloroform extract) obtained was evaporated by Rotary evaporator (Bibby RE-200, Sterlin Ltd., UK) at 5 to 6 rpm and at 68°C temperature. It rendered a gummy concentrate of greenish black color. The gummy concentrate was designated as crude extract. Then the crude extract was dried by freeze drier and preserved at 4°C [13].

**Phytochemical analysis**

All the extracts were subjected to qualitative phytochemical screening to identify the presence of alkaloids, flavonoids, carbohydrates, gums, reducing sugars, saponins, steroids, tannins and terpenoids using the established methods as described by Harborne and Szadz et al. [14,15]. Briefly, Alkaloids, flavonoids and tannins were respectively tested with Wagner reagent, concentrated HCl and 0.1% ferric chloride. Molish reagents & concentrated sulfuric acid for gum, sulfuric acid for steroids, α-naphthol and sulfuric acid for reducing sugar and chloroform and concentrated HCl for terpenoids were used as reagents. Saponin was identified based on the ability to produce suds.

**Antimicrobial assay**

The antimicrobial activity for different extracts was determined by the disc diffusion method [16]. Both Gram-positive and Gram-negative bacterial strains and fungi were used for the test. The bacterial strains used for the investigation are listed below (Table 2). Solutions of known concentration (mg/ml) of the test samples were made by dissolving measured amount of the samples in calculated volume of solvents. Dried and sterilized filter paper discs (6 mm diameter) were then impregnated with known amounts of the test substances using micropipette. Resulting discs were then placed on nutrient agar medium seeded with the microbial strains. A positive control disc impregnated with kanamycin 30 μg/disc) and negative control disc (impregnated with saponin was identified based on the ability to produce suds.

**In vitro Experimental Protocols**

**Hot plate test in mice**

The hot-plate test was carried out with the method described by Lanhers et al. [20] and modified by Mahomed and Ojewole [21]. Mice were divided into four groups of each containing four animals. The mice were placed inside a beaker on the hot plate to record their response to electrical heat induced pain stimulus. Licking of the paws or jumping out of the beaker was taken as an indicator of the animal’s response to heat-induced pain stimulus. The time for licking paws or jumping out of the beaker was taken as reaction time (s). Reaction time was measured once before treatment. The mean of this reaction time constituted initial reaction time before treatment. Each of the test mice was thereafter treated with negative control distilled water, positive control diclofenac sodium (2.5 mg/kg body weight) and ethanol extract at the doses of 250 mg/kg and 500 mg/kg body weight orally. Thirty minutes after treatment, the reaction time of each group was again counted five times individually in one hour interval. Percentage (%) of analgesic score was calculated as:

\[
\text{Percentage} \% \text{ of analgesic effect} = \frac{T_b - T_a}{T_b} \times 100
\]

Where, \(T_b\) = Reaction time (in second) before drug administration

\(T_a\) = Reaction time (in seconds) after drug administration

**Acetic acid induced writhing test in mice**

Acetic acid is administered intra-peritoneally to the experimental animals to create pain sensation. As a positive control, any standard non-steroidal anti-inflammatory drugs (NSAID) can be used. In the present study Ketorolac was used to serve the purpose. The plant extract was administered orally in two different doses (250 and 500 mg/kg body weight) to the Swiss Albino mice after an overnight fast [22]. Test samples and vehicle were administered orally 30 minutes prior to intra-peritoneal administration of 0.7% v/v acetic acid solution (0.1 ml/10 gm). Animals were kept individually under glass jar for observation. Each mouse of all groups were observed individually for counting the number of writhing they made in 10 minutes commencing just 5 minutes after the intra-peritoneal administration of acetic acid solution. Full writhing was not always accomplished by the animal, because sometimes the animals started to give writhing but they did not complete it. 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 group was compared to that of a control group while Ketorolac (10 mg/kg) was used as a reference substance (positive control).

**Anti-inflammatory activity screening**

Carrageenan induced rat paw edema method for anti-inflammatory activity: Anti-inflammatory effect of A. indica extract was carried out by carrageenan induced rat paw edema model

\[
\% \text{mortality} = \frac{\text{(no. of dead nauplii / no. of live nauplii taken)}}{100}
\]
described by Winter et al. [23] with minor modifications [24]. Rats were randomly divided into four groups each consisting of five animals. Group I was given drinking water only (negative control), Group II was given diclofenac as anti-inflammatory agent (positive control), Group III and Group IV were given the test sample at the dose of 250 and 500 mg/kg body weight (test control) respectively. After 30 minutes of oral administration of the test materials, 1% carrageenan was injected to the left hind paw of each animal. The volume of paw edema was measured at 1/2, 1, 2, 3 and 8 hrs using plethysmometer (7150, UGO Basile, Italy) after administration of carrageenan. The right hind paw served as a reference of non-inflamed paw for comparison. The average increase in the percentage of paw volume was calculated and compared against the control group. Percent inhibition was calculated using the formula:

\[
\% \text{ Inhibition of paw edema} = \frac{V_c - V_t}{V_c} \times 100
\]

Where, \( V_c \) and \( V_t \) represent average paw volume of control and treated animal respectively.

### Statistical analysis

The data are expressed as the mean ± SEM (Standard Error Mean) analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's t-test was used as the test of significance. \( p<0.05 \) were considered as the minimum level of significant. All statistical tests were carried out by using statistical software package (SPSS, version 18.0, IBM Corporation, NY, USA).

### Results

#### Acute toxicity

Oral administration of graded doses (250 and 500 mg/kg) of the ethanol extract of \( A. \) indica to rats and mice did not produce any significant changes in behavior, breathing, cutaneous effects, sensory nervous system responses or gastrointestinal effects during the observation period. No mortality was recorded in any group after 24 hrs of administering the extract to the animals.

Preliminary phytochemical screening of the extract of \( A. \) indica revealed the presence of various bioactive components of which alkaloid, cardiac glycosides, steriods, reducing sugars and gums, carbohydrates were the most prominent. The ethanol extract reveals various bioactive components were the most prominent than other extracts and the result of phytochemical test has been summarized in the Table 1. Our results indicate the presence of phytochemicals in the order of ethanol extract>n-hexane extract>chloroform extract. The above data shows the higher yield of phytochemicals in ethanol extract.

### Antimicrobial screening

The ethanol, n-hexane and chloroform extracts (500 µg/disc) of the aerial part of \( A. \) indica were screened against thirteen human pathogenic bacteria and one fungus to check antibacterial activities by disc diffusion method. Chloroform extracts (500 µg/disc) showed antibacterial activity with the average zone of inhibition of 8-10 mm by disc diffusion method (Table 2), among the tested bacteria and fungi, the growth of *Shigella dysentriae* (12 mm) was highly inhibited. In case of ethanol extract the highest activity was also seen against the growth of *Escherichia coli* having the zone of inhibition of 14 mm. Besides this, the ethanol extracts showed good activity against the growth of *Bacillus sereus* (10 mm), *Bacillus subtilis* (10 mm), *Staphylococcus aureus* (11 mm), *Escherichia coli* (14 mm), *Salmonella paratyphi* (10 mm) and *Salmonella typhi* (10 mm), *Shigella dysenteriae* (10 mm), *Pseudomonas aureus* (10 mm) (Table 2).

### Cytotoxicity screening

Following the procedure of Mayer et al. [19] the lethality of the crude ethanol, n-hexane and chloroform extract of \( A. \) indica to brine shrimp was determined on \( A. \) salina after 24 hrs of exposure the samples and the positive control, colchicine. The results of \( A. \) indica on different extracts (concentration of extract 25, 50, 100, 200, 400 and 800 µg/ml respectively) are shown in Table 3. The percentage mortality

<table>
<thead>
<tr>
<th>A. indica extract</th>
<th>Tannins</th>
<th>Flavonoids</th>
<th>Saponins</th>
<th>Gums &amp; Carbohydrate</th>
<th>Steroids</th>
<th>Alkaloids</th>
<th>Reducing sugar</th>
<th>Terpenoids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>n-Hexane</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Chloroform</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>_</td>
</tr>
</tbody>
</table>

Symbol (+) indicates presence and (-) indicates absence of phytochemicals.

<table>
<thead>
<tr>
<th>Test organisms</th>
<th>Ethanol extract (500 µg/disc)</th>
<th>n-hexane extract (500 µg/disc)</th>
<th>Chloroform extract (500 µg/disc)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus sereus</em></td>
<td>10</td>
<td>-</td>
<td>9</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>10</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td><em>Bacillus megaterium</em></td>
<td>8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>11</td>
<td>-</td>
<td>9</td>
</tr>
<tr>
<td><em>Sarcina lutea</em></td>
<td>10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Salmonella paratyphi</em></td>
<td>10</td>
<td>-</td>
<td>6</td>
</tr>
<tr>
<td><em>Salmonella typhi</em></td>
<td>10</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>14</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td><em>Vibrio mimicus</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Vibrio parahaemolyticus</em></td>
<td>6</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td><em>Shigella dysenteriae</em></td>
<td>10</td>
<td>-</td>
<td>8</td>
</tr>
<tr>
<td><em>Shigella boydii</em></td>
<td>8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Pseudomonas aureus</em></td>
<td>10</td>
<td>-</td>
<td>9</td>
</tr>
</tbody>
</table>

Values are expressed as zone of inhibition in millimeter (mm), (n=3).
increased with an increase in concentration. Ethanol, n-hexane extract and colchicine showed 100.0% mortality to brine shrimp at 800 μg/ml. The LC₅₀ obtained from the best-fit line slope were found to be μg/ml, μg/ml and μg/ml for ethanol and n-hexane extract (Table 3).

**Analgesic activity**

**Effect of plant extract on hot-plate test**: The ethanol extract of *A. indica* exhibited statistically significant (p<0.05) analgesic effect in hot plate test of white albino mice. The results presented in table 4 and 5. From these results we could concluded that the meal significantly increased the reaction time of mice in a dose-related manner. The dose dependent effect reached 42.80% and 61.90% at the doses of 250 and 500 mg/kg body weight weight respectively (Tables 4 and 5).

**Acetic acid induced writhing test in mice**: The result of acetic acid induced writhing responses in mice which indicate the analgesic activity of the ethanol extracts of were presented in Table 6. It was found that the extract of *A. indica* and Ketorolac at the assayed doses caused a significant (p<0.05) inhibition on the writhing responses induced by acetic acid when compared to the control (Table 6).

**Anti-inflammatory activity screening**

**Carrageenan induced rat paw edema method for anti-inflammatory activity**: The effective values, calculated for each group are presented in Tables 7 and 8. In control animals, the sub plantar injection of carrageenan produced a local edema that increased progressively to reach a maximal intensity three hour after the injection of the phlogistic agent. Ethanol extract of the *A. indica* showed a significant dose depended reduction at 500 mg/kg body weight. However, maximum inhibition of edema was found to be 22.22% at eight hour of study at a dose of 500 mg/kg body weight weight respectively (Tables 7 and 8).

**Discussion**

This research reported the phytochemical, antimicrobial, cytotoxic, analgesic and anti-inflammatory properties of *A. indica* both in *in vitro* and *in vivo* models. Phytochemical constituents play the major role guiding the pharmacological effects of individual plant source. Chemical screening is thus performed to allow localization and targeted isolation of new or useful phytochemical constituents with potential activities. This procedure recognizes occurrence of the known phytochemical metabolites in extracts either in preliminary stage or at the earliest stages of separation and this is very important in economic context.

Successful prediction of botanical compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. Traditional medicinal plants are used primarily water as the solvent but in our studies we found that plant extracts in organic solvent (ethanol) provided more consistent antimicrobial activity compared to those extracted in n-hexane or chloroform extract. So we have decided to continue our experiments by using ethanol extract. These observations can be rationalized in terms of the polarity of the compounds being extracted by each solvent and in addition to their intrinsic bioactivity [25]. Ethanolic extracts of plants generally possess terpenes and phenolics, which are reported by different workers as antimicrobial compounds [26,27]. Medicinal plants containing different types of phytochemical have a long history of serving people throughout the world. Actually phytochemicals show a variety of pharmacological actions in human body [28].

The result of the phytochemical investigation of the ethanol extract of *A. indica* indicates the presence of alkaloids, flavonoids, steroids, gum and carbohydrates, reducing sugar, saponins and tannins. Results of n-hexane and chloroform extracts indicate the presence of steroids, gum and carbohydrates, reducing sugar, terpenoids and alkaloids. The presence of alkaloids represents the possibility of some biological activity of the extracts of *A. indica*; such as anti-arrhythmic, anti-cholinergic, anti-tumor, vasodilating, anti-hypertensive, cough expectorant, anesthetic, analgesic, muscle relaxant, anti-pyretics, anti-malarial, anti-protozoal agent [29-35]. The presence of flavonoids represent...
The plant from various extraction processes showed their potential activity against the given test organisms. The variation in BSLA results may be due to the difference in the amount and kind of cytotoxic substances (e.g. tannins, flavonoids or terpenoids) present in the extracts. Moreover, this significant lethality values less than 100 ppm or μg/ml) to brine shrimp is indicative of the presence of potent cytotoxic and probably insecticidal compounds which warrants further investigation. BSLA results may be used to guide the researchers on which plant extracts/fractions to prioritize for further fractionation and isolation of these bioactive compounds. Other cytotoxicity tests and specific bioassays may be done on the isolated bioactive compounds later.

The antimicrobial potential of plants was compared according to their zone of inhibition against the several pathogenic organisms. The analgesic activity was expressed as mean increase in latency time as compared to control. Positive control diclofenac Na showed significant (p<0.05) increase in latency time as compared to control. Positive control diclofenac Na showed significant (p<0.05) analgesic activity at the dose of 10 mg/kg.

The variation in BSLA results may be due to the difference in the amount and kind of cytotoxic substances (e.g. tannins, flavonoids or terpenoids) present in the extracts. Moreover, this significant lethality values less than 100 ppm or μg/ml) to brine shrimp is indicative of the presence of potent cytotoxic and probably insecticidal compounds which warrants further investigation. BSLA results may be used to guide the researchers on which plant extracts/fractions to prioritize for further fractionation and isolation of these bioactive compounds. Other cytotoxicity tests and specific bioassays may be done on the isolated bioactive compounds later.

Table 6: Analgesic effect of the ethanol extract of A. indica using the acetic acid induced writhing test in mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean ± SEM</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>42.80 ± 3.10</td>
<td>-</td>
</tr>
<tr>
<td>Positive control (Ketorolac sodium)</td>
<td>20.80 ± 0.86</td>
<td>51.40%</td>
</tr>
<tr>
<td>A. indica (250 mg/kg)</td>
<td>30.80 ± 2.47*</td>
<td>28.03%</td>
</tr>
<tr>
<td>A. indica (500 mg/kg)</td>
<td>20.40 ± 2.83</td>
<td>52.33%</td>
</tr>
</tbody>
</table>

Values in the results are expressed as mean ± SEM, significantly different in comparison with control at *p<0.05.

Table 7: Anti-inflammatory effect of ethanolic extract of A. indica on carrageenan induced rat paw inflammation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.73 ± 0.03</td>
</tr>
<tr>
<td>Positive control</td>
<td>0.73 ± 0.01</td>
</tr>
<tr>
<td>A. indica (250 mg/kg)</td>
<td>0.87 ± 0.03</td>
</tr>
<tr>
<td>A. indica (500 mg/kg)</td>
<td>0.72 ± 0.02</td>
</tr>
</tbody>
</table>

Values in the results are expressed as mean ± SEM, significantly different in comparison with control at *p<0.05.
The acetic acid-induced writhing is a sensitive method to evaluate peripherally acting analgesics. Ethanol extract of *A. indica* possesses analgesic effects in the model of acetic acid-induced writhing test. Acetic acid induced writhing in mice finds much attention in the screening of analgesic drugs. Acetic acid-induced abdominal writhing and the visceral pain model release prostanoids, via cyclooxygenase pathway, which plays a role in the nociceptive mechanism. This model of response is thought to be mediated by peritoneal mast cells acid sensing ion channels and the prostaglandin pathway. In other words, the acetic acid induced writhing has been associated with increased level of PGE2 and PGF2α in peritoneal fluids as well as lipooxygenase products [37]. The increase in prostaglandin levels within the peritoneal cavity then enhances inflammatory pain by increasing capillary permeability. Results of the present studies show that ethanol extract of *A. indica* produced significant analgesic effect which may be due to the inhibition of the synthesis of the arachidonic acid metabolite.

Carrageenan-induced edema involves the synthesis or release of mediators at the injured site. These mediators include prostaglandins, especially the E series, histamine, bradykinins, leukotrienes and serotonin all of which also cause pain and fever [46,47]. Inhibitions of these mediators from reaching the injured site or from bringing out their pharmacological effects normally ameliorate the inflammation and other symptoms [48,49]. In the present study, it has been shown that the ethanol extract of the *A. indica* possessed a significant anti-edematogenic effect on paw oedema induced by carrageenan.

**Conclusion**

The present study indicated that the ethanol extract of *A. indica* may have potential use in medicine. From the previous study and our investigation it may be concluded that, ethanol extract possesses more pharmacological properties than n-hexane and chloroform extracts. In our study, the ethanol extracts of *A. indica* showed significant dose dependent inhibition of analgesic effect. These current studies suggested ethanol extract of *A. indica* demonstrated significant result and possess analgesic properties. Now our next aim is to isolate the leading compounds and to establish their chemical structure.

**References**