

Evaluation of Antinociceptive, Antidiarrheal and Antimicrobial Activities of Leaf Extracts of *Clerodendrum indicum*

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ABSTRACT

Introduction: The methanolic extracts and its different partitioning fractions of leaves of *Clerodendrum indicum* were evaluated for their anti-nociceptive, anti-diarrheal and *in vitro* antimicrobial activities. **Methods:** The anti-nociceptive activity was evaluated using the acetic acid-induced writhing test in mice; the anti-diarrheal activity was investigated by the effect of extracts on castor oil-induced diarrhea while the *in vitro* antimicrobial activities were examined by the disc diffusion method. **Results:** In the acetic acid-induced writhing test, the methanolic extract at a dose of 200 and 400 mg/kg showed a significant ($p < 0.001$) and dose-dependent reduction in the number of writhes with 62.57% and 70.76% of inhibition, respectively, while the CCL₄ fraction at the same dose showed potent anti-nociceptive activity ($p < 0.001$) with 73.09% of inhibition of writhing which was even higher than that of standard diclofenac sodium (55.56% inhibition). The methanolic extract, CCL₄ and chloroform fraction showed moderate activity against the tested microorganisms in terms of both zones of inhibition (ranged from 9-13 mm, 10-13 mm and 10-13 mm, respectively, at a concentration of 400 µg/disc) and spectrum of activity. In castor oil-induced diarrhea testing, the methanolic extract and chloroform fraction at a dose of 400 mg/kg produced 21.74% and 26.96% inhibition of defecation, respectively, which were found to be comparable to that of standard drug loperamide (37.39% inhibition at 50 mg/kg) with regard to the severity of diarrhea. **Conclusion:** The results of the investigation demonstrated that the methanolic extract and its different fractions of leaves of *Clerodendrum indicum* possess significant anti-nociceptive, antimicrobial and antidiarrheal activities.

Key words: *Clerodendrum indicum*; leaves; anti-nociceptive; antimicrobial; anti-diarrheal; writhing

INTRODUCTION

Since ancient times, medicinal plants have been used for the treatment and management of various health problems. About 80% of the world's population relies on the use of traditional medicine, which is predominantly based on herbal products.^[1] To ensure the rational use of herbal medicine,

it is imperative to validate the folkloric claim of medicinal plants used in traditional medicine so that the beneficial ones can be deployed as phytomedicines and the bioactive constituents from such beneficial plants could be isolated and used as "leads" in drug discovery process.^[2]

Clerodendrum indicum (family: Verbenaceae; vernacular names: Bamunhatti, Nuli gach) is an annual shrub which is found in areas with moderate temperature. The species occurs variably in India, Nepal, Myanmar, Malaya, Indo-China, Indonesia, Java and Bangladesh. Leaves (aerial parts) and roots of *Clerodendrum indicum* are used for various medicinal purposes. In traditional system of remedies, the plant is mainly used in the treatment of asthma, bronchitis, cold fever, intestinal worms, arthritis, epilepsy, febrile convulsion, gastric tumor, hematuria, hysteria, impotence, lipoma, nasal polyps, painful micturation and rheumatism.^[3-5] Paste made out of its leaves is effective in application on wounds for early healing,

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and healing of the infected lymph node (lymphadenopathy). Leaf powder is used in digestive disorders and other GI-related ailments. It purifies blood, improves blood circulation and suppresses all kind of swelling of the body. It acts on the respiratory system thus expelling out the excessive mucus in the tract relieving cough, cold and asthma symptoms.

Although the leaves of the plant have been traditionally used in the treatment of various painful and anti-inflammatory conditions, gastrointestinal disorders and infectious disease, there is no extensive anti-nociceptive, antimicrobial and anti-diarrheal study of this valuable medicinal plant. Only Raihan et al.^[6] reported the analgesic activity of crude ethanolic extract of leaves and Rahman et al.^[7] reported the *in vitro* antibacterial activity of root and stem of the plant previously. To prove the ethno-medical claims, the present study was designed to evaluate the anti-nociceptive and anti-diarrheal activities of the methanolic extracts and its different partitioning fractions of leaves of *Clerodendrum indicum* in mice model. The *in vitro* antimicrobial activities of the methanolic extracts and its different partitioning fractions of leaves were also investigated.

MATERIALS AND METHODS

Chemicals and reagents

The chemicals used were: acetic acid (Merck, Germany), castor oil (Sigma Chemicals, USA), diclofenac sodium and loperamide (Square Pharmaceuticals Ltd; Dhaka, Bangladesh), normal saline solution (0.9% NaCl; Orion Infusion Ltd, Bangladesh). Dimethylsulfoxide and Tween-80 were from Sigma–Aldrich and rests of the chemicals used were of BDH and E-Merck analytical grade.

Preparation of plant sample

Leaves of *Clerodendrum indicum* were collected from Modhupur, Tangail, Bangladesh, in November 2009 and authentication of the sample was confirmed by the taxonomist of the National Herbarium of Bangladesh, Mirpur, Dhaka. A voucher specimen no. has been deposited (accession No: DACB 34556) in the Herbarium for further reference. The leaves were sun dried for several days. After complete drying, the dried leaves were then ground to a coarse powder using high capacity grinding machine in the Phytochemical Research Laboratory, Faculty of Pharmacy, University of Dhaka. The coarse powder was then stored in an air-tight container marked for identification and kept in a cool, dark and dry place for future use.

Extraction and partitioning of the plant material and sample preparation

About 900 gm of powdered leave material was taken in a clean, round bottomed flask (5 liters) and macerated at

room temperature in 3 liters of methanol for 10 days with occasional shaking for better extraction. The whole mixture was then filtered through cotton followed by Whatman's No. 1 filter paper. After filtration, the filtrate was concentrated at 40 °C with a Heidolph rotary evaporator. The concentrated extract was then air dried to a solid residue. The weight of the crude methanolic extract of leaves obtained was 56 gm. Fractionation of the methanolic extracts was carried out by using solvent-solvent partitioning using the protocol designed by Kupchan^[8] and modified version by Wagenen et al.^[9] The crude extract (35 gm) was dissolved in 10% aqueous methanol which was subsequently extracted with petroleum ether, carbon tetrachloride and chloroform. All the three partitioning (pet ether fraction, carbontetrachloride fraction and chloroform fraction) fractions were evaporated to dryness by using rotary evaporator and kept in airtight containers for further analysis. The extracts and standard drug (diclofenac sodium, loperamide) were suspended in normal saline using 0.1% Tween-80.

Experimental animals

Swiss-albino mice (*Mus musculus*) of either sex, aged 4-5 weeks, obtained from the Animal Resource Branch of the International Center for Diarrheal Diseases and Research, Bangladesh (ICDDR, B) were used for the experiment. They were housed in polypropylene cages (30x20x13 cm) and kept in standard environmental conditions (temperature 23 ± 2 °C, relative humidity 55 ± 10% and 12 hours light/dark cycle). The animals were fed with standard rat food (ICDDR, B formulated) and water *ad libitum*. As these animals are very sensitive to environmental changes, they were kept in the environment where the experiment would take place 7 days before the test. The design and performance of research study involving mice was approved by the Ethical Review Committee, Faculty of Biological Science, University of Dhaka through the submission of a research protocol before the study.

Experimental procedures

Acetic acid-induced writhing response in mice

The methanolic crude extract and the different fractions of the methanolic extract of the leaves of *Clerodendrum indicum* were subjected to a screening for analgesic activity by acetic acid-induced writhing inhibition method.^[10] Initially, the Swiss albino mice were divided into five groups (n=5). Subsequently, vehicle (1% Tween-80 solution in normal saline, 10 ml/kg, as control group), diclofenac sodium (50 mg/kg, as standard), methanolic crude extract (200 and 400 mg/kg) and CCl₄ fraction of methanolic extract (200 mg/kg) were administered orally by means of a long needle with a ball-shaped end. After 40 minutes, acetic acid (0.7%, 0.1 mL/10 g) was administered intra-peritoneally to each of the animals of all the groups to induce pain. A forty-minute interval between the oral administration

of test materials and intra-peritoneal administration of acetic acid was given to assure proper absorption of the administered samples. Five minutes after the administration of acetic acid, the number of squirms or writhing characterized by contraction of the abdominal musculature together with turning of trunk and extension of hind limbs, were counted for each mouse for fifteen minutes. Percentage inhibition of writhing in comparison to control group was taken as an index of analgesia and was calculated using the following formula:

$$\text{Inhibition (\%)} = \frac{[(W_c - W_t) \times 100]}{W_c}$$

Where W_c is the average number of writhing reflex in the control group and W_t is the average number of writhing in the test groups.

In vitro Antimicrobial Screening

The *in vitro* antimicrobial activities of methanolic crude extracts and its different fractions from *Clerodendrum indicum* leaves were examined by the disc diffusion method.^[11-14] The bacterial and fungal strains used for the experiment were collected as pure culture from the Institute of Nutrition and Food Sciences (INFS), University of Dhaka. A measured amount of each test sample (methanolic crude extract, pet-ether fraction, chloroform fraction and CCl_4 fraction) was dissolved in a specific volume of the solvent (chloroform) to obtain the desired concentrations and applied to sterile discs (6 mm diameter) at a concentration of 400 $\mu\text{g}/\text{disc}$ followed by drying off the solvent in an aseptic hood. Discs containing the test material were placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic Kanamycin (30 $\mu\text{g}/\text{disc}$) discs and blank discs (impregnated with 10 μl of solvents) were used as positive and negative controls, respectively. These plates were then kept at a low temperature (4 °C) for 24 hours to allow maximum diffusion. During this time dried discs absorbed water from the surrounding media and then the test materials were dissolved and diffused out of the sample disc. The plates were then incubated at 37 °C for 24 hours to allow maximum growth of the organisms. If the test materials have any antimicrobial activity, it will inhibit the growth of the microorganisms and a clear, distinct zone of inhibition will be visualized surrounding the medium. The antimicrobial activity of the test agent was determined by measuring the diameter of zone of inhibition expressed in millimeter. The experiment was carried out in duplicate.

Effect of extract on castor oil-induced diarrhea

The effect of the methanolic crude extract and its different solvent-soluble fractions on castor oil-induced diarrhea was evaluated by the method described by Uddin et al.^[15] and Awouters et al.^[16] In this method castor oil is used to

induce diarrhea in all the experimental groups. The defecation is the primer to measure the anti-diarrheal effect. Thirty experimental mice were randomly selected and divided into six groups consisting of 5 mice in each group. Group I received vehicle (1% Tween-80 solution in normal saline, 10 ml/kg, as control group) and Group II received the standard anti-diarrheal agent loperamide (50 mg/kg p.o.). The third, fourth, fifth and sixth groups were the test groups and received methanolic crude extract and pet ether, CCl_4 and chloroform fractions of methanolic extract, respectively at a dose of 400 mg/kg body weight p.o. Prior to any treatment, each mouse was weighed properly and the doses of the test samples and control materials were adjusted accordingly. Each animal was then given 0.5 ml of castor oil orally after 30 min of treatment and placed in transparent cages to observe for consistency of fecal matter and frequency of defecation for 3 h. Feces were collected with an absorbent sheet of paper placed beneath the transparent cages.^[17] The wet feces were read at the end of the experiment by lifting up the upper part of the cage containing the sheet of paper and animals. The percent (%) inhibition of defecation was measured using the following formula.

$$\% \text{ Inhibition of defecation} = \left[\frac{(A - B)}{A} \right] \times 100$$

A = Mean number of defecation caused by castor oil

B = Mean number of defecation caused by drug or extract

Statistical analysis

The results obtained were expressed as mean \pm SEM. The data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's *t* test to determine the level of significance. A value of $P < 0.05$ was considered to be significant. The statistical analysis was carried out using the SPSS program (version 17).

RESULTS

Acetic acid-induced writhing response

In the acetic acid-induced writhing test, the methanolic crude extract of leaves of *C. indicum* at a dose of 200 and 400 mg/kg body weight showed a significant ($p < 0.001$) and dose-dependent reduction in the number of writhes with 62.57% and 70.76% of inhibition, respectively (Table 1) when compared to the control which were even higher than that of the standard drug diclofenac sodium (55.56% inhibition; $p < 0.001$). The carbon tetrachloride fraction of the crude extract at a dose of 400 mg/kg also showed potent anti-nociceptive activity ($p < 0.001$) with 73.09% of inhibition of writhing response when compared to control.

In vitro antimicrobial screening

The zones of inhibition produced by methanolic crude extract, carbon-tetrachloride fraction and chloroform fraction of the methanolic extract of *Clerodendrum indicum*

(leaves) ranged from 9-13 mm, 10-13 mm and 10-13 mm, respectively at a concentration of 400 µg/disc (Table 2). The methanolic crude extract, carbon-tetrachloride fraction and chloroform fraction showed moderate activity against the tested organisms in terms of both zone of inhibition and spectrum of activity against some gram-positive, gram-negative bacteria and fungi. But the pet ether fraction showed no antimicrobial activity.

Table 1: Effects of methanolic crude extract and its CCl₄ fraction of leaves of *C. indicum* on acetic acid-induced writhing response in mice

| Treatment | Dose (mg/kg) | Writhing ^a | % inhibition |
|-------------------|--------------|-----------------------|--------------|
| Control (Vehicle) | 10 mL/kg | 34.2 ± 2.81 | - |
| Diclofenac sodium | 50 | 15.2 ± 1.93* | 55.56 |
| MCE | 200 | 12.8 ± 1.43* | 62.57 |
| MCE | 400 | 10 ± 1.30* | 70.76 |
| CTF | 200 | 9.2 ± 1.48* | 73.09 |

^avalues represent mean ± SEM (n=5). One-way ANOVA followed by Dunnett's t test; *p<0.001: significantly different from control. MCE: methanolic crude extract of leaves of *C. indicum*; CTF: Carbon-tetrachloride fraction of methanolic extract of leaves of the plant.

Screening of antidiarrheal activity

The chloroform fraction of the crude extract exhibited significant anti-diarrheal activity, while the methanolic crude extract exhibited moderate anti-diarrheal activity (Table 3). The percent of inhibition of defecation produced by the chloroform fraction of the crude extract was 26.96%, while methanolic crude extract showed 21.74% inhibition at a concentration of 400 mg/kg body weight. The results were found to be comparable to that of standard drug loperamide

Table 2: Antimicrobial activity of test samples of *Clerodendrum indicum* (leaves)

| Test microorganisms | Diameter of zone of inhibition (mm) | | | | |
|--------------------------------|-------------------------------------|-----|-----|-----|-----------|
| | MCE | CTF | CHF | PEF | Kanamycin |
| Gram positive bacteria | | | | | |
| <i>Bacillus cereus</i> | 12 | 13 | 10 | - | 27 |
| <i>Bacillus megaterium</i> | 10 | 10 | 10 | - | 31 |
| <i>Bacillus subtilis</i> | 10 | 10 | 13 | - | 34 |
| <i>Staphylococcus aureus</i> | 10 | 13 | 13 | - | 31 |
| <i>Sarcina lutea</i> | 10 | 10 | 13 | - | 34 |
| Test microorganisms | Diameter of zone of inhibition (mm) | | | | |
| | MCE | CTF | CHF | PEF | Kanamycin |
| Gram negative bacteria | | | | | |
| <i>Escherichia coli</i> | 13 | 13 | 10 | - | 29 |
| <i>Pseudomonas aeruginosa</i> | 10 | 13 | 13 | - | 31 |
| <i>Salmonella paratyphi</i> | 10 | 10 | 10 | - | 35 |
| <i>Salmonella typhi</i> | 10 | 10 | 10 | - | 28 |
| <i>Shigella boydii</i> | 10 | 10 | 10 | - | 31 |
| <i>Shigella dysenteriae</i> | 9 | 9 | 10 | - | 27 |
| <i>Vibrio mimicus</i> | 9 | 10 | 13 | - | 31 |
| <i>Vibrio parahemolyticus</i> | 9 | 10 | 10 | - | 34 |
| Fungi | | | | | |
| <i>Candida albicans</i> | 10 | 10 | 10 | - | 35 |
| <i>Aspergillus niger</i> | 10 | 10 | 10 | - | 34 |
| <i>Sacharomyces cerevisiae</i> | 10 | 10 | 13 | - | 34 |

MCE = Methanolic crude extract of leaves, CTF = Carbon-tetrachloride fraction of crude extract of leaves, CHF = Chloroform fraction of crude extract of leaves, PEF = Pet-ether fraction of crude extract of leaves. (-): No inhibition.

Table 3: Effect of leaf extracts of *Clerodendrum indicum* on castor oil-induced diarrhoea

| Test Sample | Animal Group | Dose mg/Kg | Number of defecation (Mean ± SEM) | % Inhibition of defecation |
|----------------------|--------------|------------|-----------------------------------|----------------------------|
| Control | I | 0.2 ml | 115 ± 0.48 | - |
| Loperamide | II | 50 | 72 ± 0.37* | 37.39% |
| Crude extract | III | 400 | 90 ± 0.32* | 21.74% |
| Pet-ether | IV | 400 | 95 ± 0.20* | 17.39% |
| Carbon-tetrachloride | V | 400 | 97 ± 0.79 | 15.65% |
| Chloroform | VI | 400 | 84 ± 1.68* | 26.96% |

Values are expressed as mean ± SEM (n=5); One-way ANOVA followed by Dunnett's t test; *p<0.05 compared to control.

(37.39% inhibition at 50 mg/kg body weight) with regard to the severity of diarrhea. The carbon-tetrachloride soluble fraction and pet-ether soluble fraction of the methanolic crude extract exhibited poor antidiarrheal activity having 15.65% and 17.39% inhibition of defecation, respectively, at a concentration of 400 mg/kg body weight.

DISCUSSION

The writhing response of the mouse to intraperitoneally injected noxious chemicals such as acetic acid is widely used to screen for peripheral analgesic activity.^[18] The intra-peritoneal administration of a 0.7% solution of acetic acid induces endogenous pain mediators, such as prostaglandins, histamine and bradykinin, which stimulate the pain sensation locally.^[19] It has been reported that the level of prostanoids, particularly PGE₂ and PGF_{2α}, as well as lipoxygenase products significantly increased in the peritoneal fluid during writhing test.^[20-21] The ability of the extracts to attenuate the acetic acid-induced writhing in mice suggests that they possess analgesic activity. So the observed analgesic activity of the methanolic crude extracts and carbon-tetrachloride fraction of the plant might be due to its possible interference in the biosynthesis, release and/or action of some chemical agents such as prostaglandins and leukotrienes from cyclo-oxygenase and lipo-oxygenase pathway, respectively, which are mainly responsible to block the pain sensation and thereby showed pain-inhibitory activity. Phytochemical study of ethanolic extracts of leaves of *C. indicum* revealed the presence of alkaloids, steroid, saponin, tannin etc.^[6] The presence of steroids, saponins and tannins may be major contributors to the anti-nociceptive activity as previously it has been observed that tannins, saponins and steroidal compounds possess good analgesic activity by inhibiting prostaglandin synthesis.^[22-23]

The data from the *in vitro* antimicrobial screening test showed that the methanolic extract and its CCl₄ and chloroform partitionates showed moderately potent and broad spectrum antimicrobial activities. It has been documented that saponins, alkaloids and tannins are known to have curative activity against several pathogenic bacteria and fungus.^[24] The broad antibacterial activities of the leaf extracts of *C. indicum* might be due to the presence of these compounds (saponins, alkaloids and tannins).

Castor oil-induced diarrheal model is widely used for the evaluation of anti-diarrheal property of drugs. The anti-diarrheal property of the methanolic crude extract and chloroform fraction of methanolic extract of *Clerodendrum indicum* leaves found in the present study could be owing to the presence of tannins, alkaloids, saponins and terpenes in

this plant as previous studies have shown that anti-dysenteric and anti-diarrheal properties were mostly due to tannins, alkaloids, saponins, steroids and triterpenes.^[25-27]

CONCLUSION

In conclusion, the results of this study demonstrated that the methanolic extract and different partitioning fractions of leaves of *Clerodendrum indicum* possess significant anti-nociceptive, antimicrobial and anti-diarrheal activities. The findings of the study validated the traditional use of the plant in the treatment of different painful conditions, gastro-intestinal disorders and infectious diseases. Further works are required to isolate and characterize the bioactive compound(s) responsible for the observed analgesic, antimicrobial and anti-diarrheal activities and to evaluate the mechanism(s) of action of these activities.

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