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# *In vivo* pharmacological activities of methanolic extract of *Tabernaemontana recurva* Roxb.

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ABSTRACT

**Objective:** To evaluate analgesic, hypoglycemic, anxiolytic, and anthelmintic activities with phytochemical screening of methanolic extract of *Tabernaemontana recurva* (*T. recurva*) whole plants.

**Methods:** The plant parts of *T. recurva* were collected, dried, powdered and extracted with methanol. Then the extracts were subjected to *in vivo* analgesic, hypoglycemic, anxiolytic activity in mice model and *in vitro* anthelmintic activity.

**Results:** The analysis of phytochemical screening confirmed the existence of alkaloid, saponin, tannins, carbohydrate, phytosterols, glycosides and phenol. In analgesic test, a significant level of percentage inhibition of abdominal constriction was observed with concentration of 200 and 400 mg/kg of body weight of extract and this was found better with formalin induced hind paw licking test rather than acetic acid induced writhing test. In hypoglycemic test, it was observed that concentration 200 mg/kg reduced blood sugar level slightly while concentration 400 mg/kg increased glucose level by 22.95%. A significant level of anxiolytic activity was observed for the study plant extract. The extract revealed potent anthelmintic activity at different concentrations.

**Conclusions:** In light, the methanolic extract of *T. recurva* exhibited excellent anthelmintic, anxiolytic and analgesic activity. This plant showed moderate hypoglycemic effect at lower concentration but higher concentration increased blood glucose level.

## 1. Introduction

Medicinal plants play an important role in total health care system by being sources of medicines for both human and animals. At still most common people choose herbal medicines rather than conventional medicines[1]. The medicinal and pharmacological properties of these plants are due to the presence of active constituents. *Tabernaemontana recurva* (*T. recurva*) which is known as rupa-tola in Bengali belongs to the genus *Tabernaemontana* and family Apocynaceae. It is a small shrub widely distributed in India and Myanmar. In Bangladesh, this plant is found in Chittagong, Cox's Bazar and Chittagong Hill Tracts. The genus *Tabernaemontana* which occurs in all tropical and subtropical parts of the world, consists of a large number of species; they have been used extensively for medicinal purposes especially against all sorts of infections[2]. In the chemical screening of *Tabernaemontana* species usually alkaloids are found and only occasionally other important secondary plant metabolites[2]. During selection of plant for this study, survey about the plant rupa-tola in Chittagong Hill Tracts revealed that root part is used to cure throat problem due to cold among Khumi, Marma and Tripura communities; leaf part in the form of paste is used to cure inflammation in Chakma and Marma people and steam juice is taken to treat diarrhea among Marma communities.

However, the plant has not yet scientifically explored for its phytoconstituents and pharmacological activities. Therefore, in the light of its use in ethnomedicine, the present investigations were carried out to study the analgesic, hypoglycemic, anxiolytic, and anthelmintic activities of the methanolic extract of *T. recurva*.

## 2. Materials and methods

### 2.1. Collection of the plant parts and other reagents

The plant *T. recurva* was collected from Balipara in Bandarban district, Bangladesh during January 2015 and was identified by National Herbarium, Bangladesh with a reference number of DACB 37789 for future utility.

Acetyl salicylic acid, glibenclamide, diazepam, albendazole,

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All experimental procedures involving animals were conducted in accordance to NC3Rs ARRIVE Guidelines and approved by the NSTU ethical committee.

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glacial acetic acid, and formalin were purchased from local market of our country.

### 2.2. Experimental design and management of animals

A total of 73 mice were used for experiments in which 16 mice were treated as control and the remaining were used either as sample or standard group as per requirement of the experiment. Mice were selected randomly for different experiments and treated as per designed method of the experiment. Swiss-albino mice of aged 4–5 weeks and weight between 20–30 g were obtained from the animal house of Jahangirnagar University, Bangladesh. Because of high homological resemblance with human, ease of maintenance and low price, mice are selected for experiment. They were kept in standard environmental condition and fed ICDDR, B formulated rodent food and water (*ad libitum*). The animals were adapted for 4 days in the environment of actual experiments. All experimental procedures involving animals were conducted in accordance to NC3Rs ARRIVE Guidelines and approved by the NSTU ethical committee.

Earthworms (*Pheretima posthuma*) for anthelmintic study were collected from moist soil in the campus of Noakhali Science and Technology University. Adult earth worm (*Pheretima posthuma*) was used to perform the test because of its anatomical and physiological resemblance with intestinal round worm parasite[3]. Collected earthworms were 3–5 cm in length and 0.1–0.2 cm in width weighing 0.80–3.04 g. They were thoroughly washed with saline water.

### 2.3. Preparation of the plant extracts

The collected plants were washed, cut into small pieces and then air-dried in the shade for about 5 weeks. Then the dried parts were micronized into coarse powder with a suitable grinder. About 250 g of powdered material was taken in a clean glass beaker and soaked in 99% methanol (1 500 mL). This beaker was then sealed with aluminum foil and kept for a period of 15 days followed by occasional shaking and stirring. After that period, the supernatant liquid was filtered using clean white cloth and Whatman filter paper. The filtrate obtained was evaporated using a rotary evaporator followed by room drying under ceiling pan. The resulted crude extract was then stored for further study.

### 2.4. Phytochemical analysis

The freshly prepared crude extract was subjected for qualitative analysis for the presence of active constituents, namely, carbohydrate, alkaloids, phenol, saponins, glycosides, tannin, phytosterols and fixed oils and fats. Presence of these constituents was confirmed by characteristics of color changes following standard phytochemical testing procedures[4,5].

### 2.5. Analgesic activity test

#### 2.5.1. Acetic acid induced writhing test

Four groups of five mice in each group were formed and fasted for 18 h. Later, Group I and II mice were given 10 mL/kg of distilled water (control) and 100 mg/kg of acetylsalicylic acid (standard) intraperitoneally respectively while Groups III and IV were received 200 and 400 mg/kg of methanolic extract *i.p.* respectively. After one hour of administration, 0.6% glacial acetic acid (10 mL/kg) was given intraperitoneally to all groups of mice to induce pain[6]. The number of abdominal contraction was then counted at 5 min interval for 30 min. The percentage of analgesic activity was then calculated by using following formula[7].

Inhibition (%) = [Number of writhes (control) – number of writhes (test)]/Number of writhes (control) × 100

#### 2.5.2. Formalin induced hind paw licking test

After one hour of administration of 10 mL/kg of distilled water (control) and 100 mg/kg of acetylsalicylic acid (standard) and 200 mg/kg and 400 mg/kg of methanolic extract, mice were administered 20 μL of 2.5% formalin subcutaneously under the plantar surface of the hind paw of all groups. The time spent in licking and biting responses of the injected paw was taken as an indicator of pain response. Antinociceptive effect was determined in two phases. The early phase was recorded during the first 5 min while the last phase was recorded during the last 20–30 min after formalin injection[8].

### 2.6. Hypoglycemic activity test

Fasted mice were grouped into four groups of three mice each and serum glucose level was measured. Then all mice of each group were administered glucose (3 g/kg of body weight) orally. After one hour of administration, blood sample was collected for the measurement of glucose level. After that, these four groups were treated differently. Control group received Tween 80 (1%) 10 mL/kg of body weight, standard group received glibenclamide 10 mg/kg of body weight. Remaining two groups received extract at a dose of 200 and 400 mg/kg of body weight. After 2 h of administration of glucose, blood samples were collected and serum glucose level was determined by glucometer using glucose estimation kit. This method for determination of hypoglycemic activity was previously adopted by Ahmed *et al.*[9].

### 2.7. Anxiolytic test

#### 2.7.1. Elevated plus maze (EPM) test

This study was conducted using a wooden EPM consisting of two open arms (30 cm × 5 cm) crossed with two closed arms (30 cm × 5 cm × 15 cm). The arms were connected together with a central square of 5 cm × 5 cm. The apparatus was elevated to the height of 40 cm in a dimly illuminated room. To conduct this test, first was to prepare four groups with three mice in each group which were fasted overnight. Distilled water (10 mL/kg), diazepam (1 mg/kg), plant extract (200 and 400 mg/kg of body weight) were administered to four group of mice respectively. After 30 min, mice were individually placed in center square of the EPM facing either one of the open arms. The time spent in both the open and closed arms was recorded for 5 min[7].

#### 2.7.2. Hole board test

The study was conducted using a wooden hole-board apparatus measuring 40 cm × 40 cm × 25 cm with 16 evenly spaced holes (each of diameter 3 cm). The apparatus was elevated to a height of 25 cm. One hour after treatment with distilled water (10 mL/kg), diazepam (1 mg/kg), plant extract (200 and 400 mg/kg of body weight), each mice was placed in turn at one corner of the hole board. The number of head dips during a 5 min period was recorded

for individual mice[10].

### 2.8. Anthelminthic activity test

Anthelmintic activity of the plant extract was evaluated by the previously described method[3] with necessary modifications. At first, different concentrations (10, 20, 30, 40 and 50 mg/mL) of solution using methanol extract of *T. recurva* and reference standard solution of albendazole (25 mg/mL) were prepared separately. Then earthworms were divided into six Petri dish each containing three earthworms. Five groups were used to the five concentrations of methanol extract and one group was applied to reference standard. Continuous observation was made to notice any physical change in the earthworms. 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[11].

### 2.9. Statistical analysis

A statistical approach was designed and the experimental data were evaluated using SPSS software, version 20. All data were expressed as mean ± SEM with their corresponding *P* values. Comparison of the investigated parameters between test sample and control/standard was performed by One-way ANOVA followed by Dunnett's *t*-test.

## 3. Results

The crude extract of *T. recurva* plant was initially screened for the presence of active constituents which confirmed the presence of alkaloids, saponin, tannin (strongly), carbohydrate (strongly), phytosterol, glycosides and phenol. The antinociceptive activity test was performed by two chemical stimulation method: acetic acid (0.6%) induced writhing test and formalin (2.5%) induced hind paw licking test. Results of analgesia of crude methanolic extract of *T. recurva* in four groups mice model (control, standard and two test sample groups) at two concentrations are presented in Tables 1 and 2. In acetic acid induced antinociceptive activity test, the extract at doses of 200 and 400 mg/kg body weight reduced the number of abdominal constrictions induced by 44.26%, and 58.2%. A standard antinociceptive drug, acetyl salicylic acid, when administered at a dose of 100 mg/kg body weight, reduced the number of abdominal constriction by 64.72%. In formalin induced hind paw licking test, antinociceptive effect was determined in two phases and the time (s) spent in licking and biting responses was noted as an indicator of pain responses. Administration of standard at a concentration of 100 mg/kg of body weight reduced licking and biting responses by 50.00% at early phase and 67.00% at later phase. When crude extract of *T. recurva* was administered at two doses 200 mg/kg and 400 mg/kg of body weight, a potent action of inhibition of licking was observed in later phase with 89.66% and 86.16% of inhibition respectively. Thus the extract exhibited more potent analgesic activity than the standard, acetyl salicylic acid. The results of the effects of two doses (200 mg/kg and 400 mg/kg) of *T. recurva* whole plant extract, glibenclamide and control groups in glucose administered diabetic mice are presented in Figure 1. The significant reduction in blood glucose level by the treatment of standard drug was found to be 29.4% as compared to control group. While negligible reduction (4%) in glucose level was found at a dose 200 mg extract per kg of body weight, on the other hand methanol extract at a dose of 400 mg/kg of body weight showed increase in blood glucose level by 22.95% as compared to control group. In this research study, evaluation of anti-anxiety property of methanol extract of *T. recurva* was performed with EPM and hole board and results are summarized in Tables 3 and 4. In EPM, methanol extract of *T. recurva* (200 mg/kg and 400 mg/kg) showed anxiolytic effect by increasing the time spent on open arms and decreasing the time spent in closed arm as compared to the control group. In hole board, the number of head dipping was counted during 5 min of test period and it is a well-established method for the determination of anxiolytic and sedative effects of plant extract by observing behavioral responses. Results are summarized in Table 4. It was observed that the number of head dipping was significantly higher as compared to control group ($P < 0.001$) both for doses 200 mg/kg and 400 mg/kg. The gradual increase of sample concentration of methanolic extract of *T. recurva* demonstrated paralysis as well as death of worms in shorter time. At doses of 10, 20, 30, 40 and 50 mg/mL, the methanolic extract of *T. recurva* showed paralysis time of 24.67, 22.33, 14.67, 13.00, 10.67 min and death time of 33.67, 29.33, 21.67, 19.67 and 14.33 min, respectively (Figure 2). These results were compared to that of the standard drug of albendazole at dose 15 mg/kg of body weight and paralysis time 37.33 min and death time 67.67 min were observed.

**Table 1**

Antinociceptive effect of methanolic extract of *T. recurva* in the acetic acid induced pain mice model.

| Treatment | Dose (mg/kg) | Writhing count | % of inhibition |
|---|---|---|---|
| Group I (control group) | 10 mL/kg | 40.67 ± 1.76 | - |
| Group II (standard group) | 100 | 14.33 ± 1.53[a] | 64.72 |
| Group III (sample) | 200 | 22.67 ± 3.00[a] | 44.26 |
| Group IV (sample) | 400 | 17.00 ± 3.00[a] | 58.20 |

Values are represented as mean ± SEM ($n = 5$). [a]: $P < 0.05$ compared with control done by One-way ANOVA followed by Dunnett's *t*-test.

**Table 2**

Effect of *T. recurva* extract on formalin induced hind paw licking mice model.

| Treatment | Dose (mg/kg) | Early phase | | Late phase | |
|---|---|---|---|---|---|
| | | Mean time to licking response | % of inhibition | Mean time to licking response | % of inhibition |
| Group I (control group) | 10 mL/kg | 19.67 ± 0.88 | - | 9.67 ± 0.33 | - |
| Group II (standard group) | 100 | 9.67 ± 1.45[b] | 50.00 | 3.00 ± 1.52 | 67.00 |
| Group III (sample) | 200 | 8.00 ± 1.53[b] | 59.33 | 1.00 ± 0.58[b] | 89.66 |
| Group IV (sample) | 400 | 13.67 ± 2.33 | 30.50 | 1.33 ± 0.33[b] | 86.16 |

Values are represented as mean ± SEM ($n = 3$). [b]: $P < 0.001$ compared with control done by One-way ANOVA followed by Dunnett's *t*-test.

**Table 3**

Anxiolytic effect of *T. recurva* on mice model in the open and closed arm of the EPM.

| Treatment | Dose (mg/kg) | Time spent in open arm during 5 min test period (s) | Time spent in closed arm during 5 min test period (s) |
|---|---|---|---|
| Group I (control group) | 10 mL/kg | 18.33 ± 5.21 | 281.67 ± 5.21 |
| Group II (standard group) | 1 | 101.00 ± 6.35[b] | 199.00 ± 6.35[b] |
| Group III (sample) | 200 | 50.33 ± 2.91[b] | 249.67 ± 2.91[b] |
| Group IV (sample) | 400 | 61.67 ± 3.48[b] | 253.33 ± 3.48[b] |

Values are represented as mean ± SEM ($n = 3$). [b]: $P < 0.001$ compared with control done by One-way ANOVA followed by Dunnett's *t*-test.

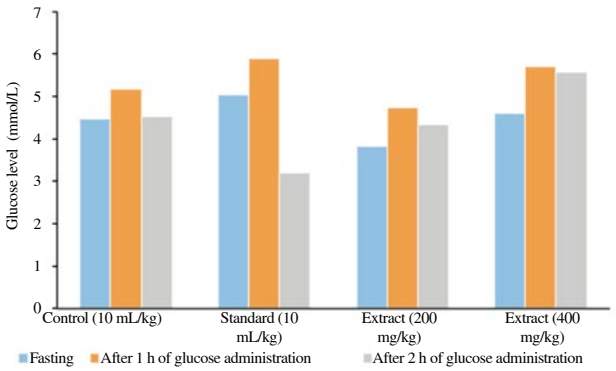


**Figure 1.** Glucose level of mice on glucose tolerance study.

**Table 4**

Anxiolytic effects of methanolic extract of *T. recurva* on mice model in the hole board.

| Treatment | Dose (mg/kg) | Number of head dipping during 5 min test period |
|---|---|---|
| Group I (control group) | 10 mL/kg | 37.33 ± 3.38 |
| Group II (standard group) | 1 | 38.33 ± 7.96 |
| Group III (sample) | 200 | 59.67 ± 5.03[b] |
| Group IV (sample) | 400 | 63.33 ± 5.51[b] |

Values are represented as mean ± SEM ($n$ = 3). [b]: $P < 0.001$ compared with control done by One-way ANOVA followed by Dunnett's $t$-test.

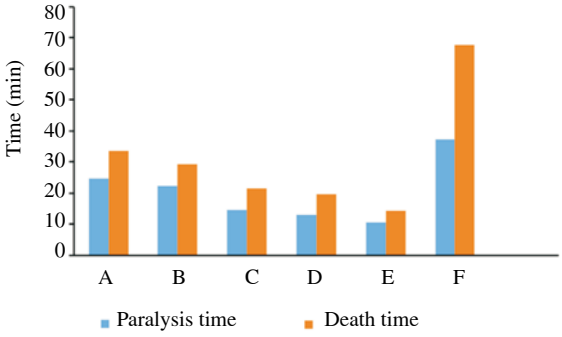


**Figure 2.** Anthelminthic activity of crude methanol extract of *T. recurva*. A: 10 (Sample1); B: 20 (Sample2); C: 30 (Sample2); D: 40 (Sample4); E: 50 (Sample5); F: 15 (Standard).

## 4. Discussion

In acetic acid induced writhing method, pain is triggered by release of free arachidonic acid from tissue phospholipid via cyclo-oxygenase and prostaglandin biosynthesis. This pathway associated with increased level of prostaglandin $E_2$ and prostaglandin $F_{2\alpha}$ in peritoneal fluids as well as lipoxygenase products which enhances inflammatory pain by increasing capillary permeability. Acetic acid induced writhing response in mice is reliable and affords rapid evaluation of peripheral type of analgesic action[12]. The plant extract of *T. recurva* showed dose dependent reduction of abdominal constriction and significantly reduced writhing as compared to control ($P < 0.05$). The analgesic effect produced by the *T. recurva* and acetyl salicylic acid might be via central inhibition or cyclooxygenase or via peripheral mechanisms involved in the inhibition of prostaglandins, leukotrienes, and other endogenous substances that stimulate pain. In formalin induced licking and biting, neurogenic pain occurs due to a direct effect of formalin on nociceptors which cause stimulation of afferent C fibers resulting from bradykinin and substance P release, in the early phases. In late phase, inflammatory pain results from release of inflammatory mediators such as histamine and prostaglandins[13]. The methanolic extract of *T. recurva* showed significant inhibition of hind paw licking both in early and late phase as compared to control ($P < 0.001$). In late phase, the plant extract showed significantly higher inhibition as compared to standard ($P < 0.05$). The inhibitory effect of the extract in the later phase is due to its peripheral action. Previously various phytochemicals *e.g.* alkaloids, flavonoids, saponins and tannins were reported to exhibit analgesic activity by other researcher[14-16]. Therefore, from the preliminary phytochemical screening, it can be assumed that the extract of *T. recurva* showed analgesic activity due to the presence of alkaloids, saponins and tannins and this might be due to the blockade of the release of inflammatory mediator.

Dual characteristics were observed for whole plant extract of *T. recurva*. At low doses (200 mg/kg), it slightly reduces blood glucose level in glucose induced mice and in high doses (400 mg/kg) it increases blood glucose level. Previous studies have suggested that a 25% lowering in blood glucose levels was considered a significant hypoglycemic effect[9]. In preliminary phytochemical screening, strong presence of carbohydrate was found in methanolic extract of *T. recurva* with glycosides and saponins. Study by Feinman *et al.*[17] supported that dietary carbohydrate restriction reliably reduces high blood glucose level with the elimination of need for medication. Literature study also revealed that the constituent's saponins are good antidiabetic metabolites[18].

Psychological anxiety and distress engender generalized anxiety disorder, social phobia and post-traumatic stress disorder. Several researches indicate that neurobiological abnormalities in serotonergic, noradrenergic, glutamatergic and GABAergic transmission may be involved in pathophysiology of anxiety disorder[19]. These pathways in turn contribute to the anxiolytic action by maximizing the action of selective serotonin reuptake inhibitors, selective serotonin and noradrenalin reuptake inhibitors and benzodiazepines. Other mechanisms that may be involved include GABA transaminase or glutamic acid decarboxylase inhibition and binding with subunit of benzodiazepine receptor sites. In consequence, increased GABA neurotransmission has a damping effect on stimulatory pathways, which ultimately provides a psychologically calming effect[19]. The EPM is a valid animal model to investigate anxiolytic activity of phytoconstituents. Anxiolytic potential is here characterized by reluctance to enter open and high space and liking to stay on closed place[20]. Thus the EPM test in this research work suggested the anxiolytic activity of methanol extract of *T. recurva*. Increase in head dipping behavior is the expression of anxiolytic state and decrease in the number of head dipping reflects the depressant effects in hole board test[19,21,22]. The hole board showed that the methanolic extract of *T. recurva* causes a dose dependent increase in the number of head dips in mice which is statistically significant ($P < 0.001$) as compared to control group. It was also observed that the plant extract is more potent than the standard drug for anxiolytic activity ($P < 0.001$). The EPM and hole board method give consistent result for anxiolytic effect in this research work. Several study reported that the plants containing alkaloids, glycosides, phytosterols, flavonoids, saponins and tannins possess good anxiolytic activity[7,13,23]. The methanolic extract of *T. recurva* also contains alkaloids, glycosides, tannin (strongly), saponin and phytosterol. Alkaloids, glycosides, and flavonoids in

plant extract possess anxiolytic effect through the interaction with $GABA_A$ receptors[24].

In anthelmintic activity test, the paralysis and death time were much lower than the standard drug even for low concentration of crude extract *i.e.* this medicinal plant is a potent source of anthelmintic agent. Significant anthelmintic activity was observed for the presence of some phytochemicals like alkaloids, tannins, phenols *etc.*[25]. Alkaloids cause paralysis of the worms by acting on its central nervous system while tannins bind to the free protein of the gastrointestinal tract or block energy generation of worms by uncoupling oxidative phosphorylation and lead to death[25-27]. From Figure 2, it is clearly manifested that the methanolic extract of *T. recurva* showed dose dependent antihelmintic activity showing maximum efficacy at 50 mg/mL as compared to standard drug albendazole. Finally, the methanolic extract of *T. recurva* exhibits excellent anthelmintic, anxiolytic and analgesic activity. This plant showed moderate hypoglycemic effect at lower concentration but at higher concentration increased blood glucose level.

## Conflict of interest statement

We declare that we have no conflict of interest.

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