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Research article

Methanol, ethyl acetate and n-hexane extracts of *Tragia involucrata* L. leaves exhibit anxiolytic, sedative and analgesic activity in Swiss albino mice

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ABSTRACT

Introduction: *Tragia involucrata* L. have been utilized as traditional medicine in Indian subcontinent for the treatment of numerous illnesses such as inflammation, pain and skin infection. In this current study we sought to assess the anxiolytic, sedative and analgesic activity of *Tragia involucrata* L. leaves extract.
Materials and methods: We first performed a phytochemical screening test of the leaves extracts following standard phytochemical screening protocols. We next examined the anxiolytic and sedative activity of crude methanol (TIME), ethyl acetate (TIEAE) and n-Hexane (TIHE) extract of *Tragia involucrata* L. leaves using mouse behavioral models such as elevated plus-maze test and pentobarbital-induced sleeping time test, respectively. Likewise, we evaluated the analgesic activity using acetic acid induced writhing test and formalin induced paw licking test. Additionally, we performed a quantitative analysis of heavy metals content of *Tragia involucrata* L. leaves by overnight digestion in concentrated nitric acid (HNO_3).
Results: Phytochemical screening demonstrated that TIME, TIEAE and TIHE contain flavonoids, alkaloids, tannins, phenols, terpenoids and sterols. Administration of these extracts resulted in higher number of open arm entry, lower number of close arm entry and higher time spent in open arm compared to control treatment ($p < 0.05$). Moreover, these treatments decreased the onset of sleep time and increased the duration of sleep compared to control treated mice (all $p < 0.05$). Likewise, extracts treated mice exhibited decreased number of writhing as well as lower acute phase and late phase duration compared to control treatment (all $p < 0.05$). The average level of As and Fe in *Tragia involucrata* L. leaves was 5.16 ± 0.012 ppm and 2.76 ± 0.015 ppm, respectively.
Conclusion: Results from this study support that *Tragia involucrata* L. leaves extracts exhibit an anxiolytic, sedative and analgesic activity in mice.

1. Introduction

Wild plant parts are a rich source of phytochemicals and bioactive compounds that are used as a source of herbal preparation in treating numerous medical conditions in the Indian subcontinent [1, 2]. *Tragia involucrata* L., a member of Euphorbiaceae family of flowering plants, is a medicinal plant and widely used to treat skin infections, scabies, wounds, inflammation, pain, headache and eczema [3]. This plant is locally known as "Bichuti" in Bengali, "Stinging Nettle" in English. This perennial plant, with hispid herb and scattered stinging hairs, is a tropical and subtropical regional plant that has diaphoretic and duplicate root [3, 4]. Previous report suggests that *Tragia involucrata* L. root extracts have this plant has sedative activity [5]. The essential compounds Stigmasterol, Quercetin, Rutin and 3-(2,4-dimethoxyphenyl)-6,7-dimethoxy-2,

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3-dihydrochro-men-4-one have been identified and isolated from the ethyl acetate extract of *Tragia involucrata* L. leaves, demonstrated high antimicrobial activity [6]. Likewise, the methanol root extract of *Tragia involucrata* L. was shown to have anti-inflammatory, analgesic [7] and antibacterial activity [8]. However, the anxiolytic, sedative and analgesic effects of this plant are largely unknown. Previous studies suggest that presence of phytochemical components such as alkaloids, flavonoids, phenolic compounds, steroids, tannins, saponins, antioxidants and terpenoides are primarily responsible for beneficial effects of wild plants. For example, plant derived antooxidant compounds (i.e.flavonoids and polyphenols) were shown to be effective in treating diabetes, cancer, aging and cardiovascular disease [9]. Accumulating evidence suggests that wild plants may contain secondary toxic metabolites contaminated with environmental pollutants. Because of the environmental pollutants, heavy metal in the food chain and in living organisms is an increasing concern [10, 11]. Exposure to toxic materials and heavy metals may damage lipids, proteins, enzymes and DNA, resulting in organ dysfunctions and multiple diseases [12, 13].

Psychiatric disorder is a major public health problem with 450 million people affected globally [14]. It is an unfavorable impulsive condition characterized by chronic anxiety. Therefore, the condition is neither quickly recognized nor seen to be recalcitrant. It weakens the daily performances and it is related to numerous medical conditions, triggering insomnia. It is autonomously and unequivocally connected with low dimensions of life quality and chronic illnesses. Anxiety is also associated with helplessness, physiological arousal or somatic symptoms and is linked with anticipated threats whereas depression is categorized by hopelessness, anhedonia or social extraction [15]. Chronic inflammation and pain are other major health problems with one-fifth of the total global adults suffering from pain [16]. Because of the high cost and known side effects of current therapies for these treatments, an alternative approach to design new therapies is urgent [17].

We first performed phytochemical screening tests of methanol, ethyl acetate and n-hexane extracts of *Tragia involucrata* L. leaves. We next aimed to investigate the anxiolytic, sedative and analgesic properties of the leaves extracts of this plant. We assessed the anxiolytic activity using elevated plus-maze tests. Likewise, the sedative effects were measured using pentobarbital-induced sleeping time tests. The analgesic activity was examined using the acetic acid-induced writhing test and formalin induced paw licking test. Therefore, we assessed the presence and quantity of Copper (Cu), Arsenic (As), Lead (Pb), Zinc (Zn) and Iron (Fe) in our study samples.

2. Materials and methods

2.1. Collection of plant

Tragia involucrata L. leaves, were collected from Bangladesh Agricultural Research Institute (BARI) (23.1634° N, 89.2182° E), Jashore Sador, Jashore-7400, Bangladesh, put into a sterile cloth bag and were shifted to the laboratory. The accession number of *Tragia involucrata* L. is DACB46915 from Bangladesh National Herbarium, Dhaka-1216, Bangladesh.

2.2. Extraction of Tragia involucata L. leaves

Tragia involucata L. leave was coarsely powdered. About 100 gm of powdered material was macerated with all solvents (Methanol, Ethyl Acetate and n-Hexane) as 1:4 ratio (W/V) at room temperature for 7 days period with occasional shaking and stirring. To get a viscous mass, whole mixture was filtered through a Whatman number 1 filter paper and then was filtrated by using a rotary evaporator (Bibby RE200, Sterlin Ltd, UK).

2.3. Chemicals and drugs

Methanol, Ethyl Acetate, n-Hexane, Ferric Chloride, Acetic Anhydride, Dinitrobenzene Solution was bought from Merck, Germany; Potassium Mercuric Iodide, Sulphuric Acid, Nitric Acid and Acetic Acid was bought Sigma-Aldrich, USA; Diazepam and Diclofenac Sodium was bought from Square Pharmaceuticals Ltd., Bangladesh; and Pentobarbitone Sodium was bought from Incepta Pharmaceuticals Ltd., Bangladesh.

2.4. Animal care

Swiss albino male mice (21 Days) weighing between 18-25 g, collected from the Department of Pharmacy, Jahangirnagar University, Savar, Dhaka-1342, were used for this study. Then, for adaptation, mice were kept 15 days in our laboratory. The animals were maintained in a room with controlled temperature (25 ± 1 °C), relative humidity (55–65%) and light: dark 12:12 h cycle in polypropylene cages with soft wood shaving. They were given a standard chow elevated by the International Centre for Diarrheal Diseases and Research, Bangladesh, Dhaka-1213 and water ad libitum. Before initiation of all experiments, all were acclimatized at least for 1 h period to laboratory conditions. All procedures were approved by the Ethical Committee of the Jashore University of Science & Technology, Jashore-7408 and laboratory animal use and care were conducted in accordance with the internationally accepted principles.

2.5. Experimental design

For each test group of sedative and analgesic activity, Swiss albino mice were divided into eight groups with 5 mice in each group. Every mouse was administered 10 ml/kg body weight (b.w) of dose solution. Control group was administered the vehicle (1% Tween 80 + Distilled Water), standard group was administered diazepam (1 mg/kg, b.w, i.p) for anxiolytic and sedative activity tests and diclofenac sodium (10 mg/kg, b.w, i.p) for analgesic activity tests. Based on previous studies as well as our toxicity screening test, we considered two doses (200 and 400 mg/kg, b.w) per extract. TIME-2 group was administered 200 mg/kg (b.w) of TIME, TIME-4 group was administered 400 mg/kg (b.w) of TIME, TIEAE-2 group was administered 200 mg/kg (b.w) of TIEAE, TIEAE-4 group was administered 400 mg/kg (b.w) of TIEAE, TIHE-2 group was administered 200 mg/kg (b.w) of TIHE, and TIHE-4 group was administered 400 mg/kg (b.w) of TIHE.

2.6. Phytochemical analysis of TIME, TIEAE and TIHE

Testing of different chemical groups present in TIME, TIEAE and TIHE represent the preliminary phytochemical studies. The chemical constituents of plant extract were identified following the procedures as described by Harborne, 1998 and Hossain et al., 2013.

Ferric chloride test for Phenols: The test solutions were treated with 3–4 drops of 0.1% (v/v) ferric chloride. Presence of phenols/phenolic compounds were confirmed by brownish green or blue color.

Mayer's test for Alkaloids: The test solutions were treated with Mayer's reagent (Potassium mercuric iodide) and mixed properly. Presence of alkaloids was confirmed by cream colored precipitate.

Ferric chloride test for Flavonoids: The test solution was mixed with a few drops of ferric chloride solution and the presence of flavonoids compounds were confirmed by an intense green color.

Liebermann Burchard test for Sterols: The test solution was treated with a few drops of acetic anhydride (Liebermann Burchard) solution and mixed properly. Concentrated sulphuric acid was added from the sides of the test tube. Presence of sterol was confirmed by the formation of a

brown ring at the junction of two layers and the green color of the upper layer.

Molisch's test for Carbohydrates: Test solution was mixed with a few drops of Molisch's reagent followed by addition of 2ml of concentrated sulphuric acid from the sides of test tube. Presence of Carbohydrates was confirmed by the purple ring at the junction of two liquids.

Xanthoproteic Test for Protein: The test solution was treated with concentrated nitric acid (Xanthoproteic reagent) solution and boiled properly. Presence of protein was confirmed by the showing of yellow color precipitation.

Ferric chloride test for Tannins: The test solution was mixed with a few drops of ferric chloride solution and the presence of Tannins was confirmed by a dark color.

Foam test for Saponins: The test solution was mixed with water and shaken properly. Presence of saponins was confirmed by formation of foam.

Raymond's test Glycosides: The test solution was treated with dinitrobenzene (Raymond's) solution and the presence of Glycosides was confirmed by a violet color.

Salkowski test for Terpenoids: Few drops of concentrated sulphuric acid was slowly added to the test solution and shaken properly and let stand at room temperature. Presence of Terpenoids was confirmed by a lower yellow layer.

2.7. Acute oral toxicity studies of TIME, TIEAE and TIHE

All conform to OECD (Organization for Economic Cooperation and Development) guideline rules 423 (acute toxic class method). 20 Swiss albino mice were used for the study, since the plant extracts are relatively nontoxic; the starting dose level of TIME, TIEAE and TIHE was selected as 250, 500, 1000 and 2000 mg/kg and the extract was administered orally to mice which were fasted overnight with water ad libitum. In accordance with before and after treatment, body weights of mice were recorded. The onset of toxicity and signs of toxicity were recorded for 14 days. Any changes in the eyes, skin and mucous membrane as well as respiratory, autonomic, motor activity, circulatory, central nervous system (CNS), behavior patterns were observed and also signs of tremors, lethargy, convulsion, salivation, diarrhoea, sleep coma and death were noted [18].

2.8. Anxiolytic and sedative activity of TIME, TIEAE and TIHE

2.8.1. Elevated plus maze test (EPM)

EPM test was performed to observe the anxiolytic activity of TIME, TIEAE and TIHE. Control, standard, TIME-2, TIME-4, TIEAE-2, TIEAE-4, TIHE-2 and TIHE-4 group were administered their individual dose. The maze comprised of two open arms (30 × 5 × 0.2 cm) and two closed arms (30 × 5 × 15 cm) connected to a central partition in the middle (5 cm × 5 cm) and has an elevated height of 45 cm above the floor. Each mouse was placed on the central platform of the maze facing an open arm. The number of transitions in between the open arms and closed arms and time spent in open arms were observed for a period of 5 min. Two observers were still staying beside the elevated platform to observe the activity and the behavior of the mice. The parameters that were observed include total time spent and number of fecal boli in both open arms and closed arms of the mazes [19].

2.8.2. Pentobarbital induced sleeping time test

Pentobarbital induced sleeping time test was performed to observe the sedative activity of TIME, TIEAE and TIHE. Control, standard, TIME-2, TIME-4, TIEAE-2, TIEAE-4, TIHE-2 and TIHE-4 group were administered their individual dose. Then, thirty minutes later, pentobarbitone sodium (35 mg/kg, i.p) was used to induce sleep. Onset and duration of sleep was observed for every animal. The time from induction of sleep to loss of righting reflex was considered as onset of sleep, while that between loss and recovery of righting reflex was recorded as the duration of sleep [20].

2.9. Analgesic activity of TIME, TIEAE and TIHE

2.9.1. Acetic acid induced writhing test

The analgesic activity of TIME, TIEAE and TIHE was evaluated using acetic acid induced writhing as previously described. Briefly, acetic acid was administered intraperitoneally (0.7% v/v, 10 ml/kg, b.w.) to the mice to create pain sensibility. Control, standard, TIME-2, TIME-4, TIEAE-2, TIEAE-4, TIHE-2 and TIHE-4 group were administered their individual dose orally 30 min prior to administration of acetic acid solution. Each mouse of all groups was observed individually for counting the number of writhing they made in 15 min commencing just 5 min after the intraperitoneal 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 and two half-writhing was considered as one full writhing [21]. All data was calculated percent inhibition as follows:

Inhibition (%) = [Number of writhings (control) – Number of writhings (test)] × 100/Number of writhings (control).

2.9.2. Formalin induced paw licking test

The analgesic effects of each of TIME, TIEAE and TIHE were evaluated using formalin tests. Swiss albino mice were injected with 20μl of 3% formalin into the sub plantar space of the left hind paw. After 30 min, control, standard, TIME-2, TIME-4, TIEAE-2, TIEAE-4, TIHE-2 and TIHE-4 group were administered their individual dose. Anti-nociceptive effect was determined in two phases. The acute phase was recorded during the first 5 min, while the late phase was recorded during the last 15–30 min after formalin injection. Mice were observed and the amount of time spent licking the injected hind paw was measured in seconds during the acute phase (0–5 min) and late phase (15–30 min). The time (in second) spent in licking responses of the injected paw was considered as an indicator of pain response [22]. All data were calculated percent inhibition as follows:

Inhibition (%) = [Duration of paw licking (control) – Duration of paw licking (test)] × 100/Duration of paw licking (control).

2.10. Heavy metal detection of Tragia involucrata L. leaves

Sample preparation for heavy metal detection

Deionized water was used extensively to clean the leaf samples of *T. involucrata* L. to ensure fresh sample preparation followed by shade dry in appropriate air flow at ambient temperature for 15 days. The shade dried samples then grinded to fine powder in a mechanical grinder then homogenized and subsequently stored at room temperature in separate bottles prior to use.

Overnight digestion with concentrated HNO_3

1 g of plant material was macerated with 10 mL concentrated nitric acid (HNO_3; ultrapure 65%) and kept at ambient temperature overnight. The sample extract was then subjected to heat for 4 h at 120 °C, followed by heating in increased temperature at 140 °C and continued to concentrate to dryness at this temperature until 1 ml of acid left. The crude extract was then cooled to room temperature and transferred to a 50 ml volumetric flask for filtration followed by dilution up to volume. Stock solutions of Arsenic (As), Copper (Cu), Lead (Pb), Zinc (Zn) and Iron (Fe) containing 1000 ppm of each were prepared by adding distill water with weighed quantity of metal salt (analytical grade) to dissolve. Calibration standard of each element was made by dilutions of the mixed stock solutions. Flame atomic absorption spectrometry (FAAS) was used to determine the concentrations of the elements. The elements were

measured by aspirating the samples into air acetylene flame and under optimum operating conditions [23].

2.11. Statistical analysis

Results are expressed as mean ± S.E.M and statistical significance of the study was evaluated by one-way ANOVA followed by Dunnett t-test in SPSS software (Version 16.0). $P < 0.05$ were considered as statistically significant.

3. Results

3.1. Phytochemical screening of TIME, TIEAE and TIHE

The phytochemical screening of TIME, TIEAE and TIHE demonstrated the presence of some secondary metabolites such as phenols, alkaloids, flavonoids, sterols, carbohydrates, proteins, tannins, saponins, glycosides and terpenoids (Table 1). The carbohydrates and terpenoids were present in all extracts.

3.2. Acute oral toxicity of TIME, TIEAE and TIHE

In acute oral toxicity assessment, TIME, TIEAE and TIHE extract did not exhibit any toxicity and mortality up to a dose 2000 mg/kg.

3.3. Anxiolytic and sedative activity of TIME, TIEAE and TIHE

3.3.1. Elevated plus maze test

Anxiolytic activity of *Tragia involucrata* L. leaves was evaluated by elevated plus-maze test. In the study, the administration of TIME-4, TIEAE-4 and TIHE-4 significantly ($p < 0.001$) elevated the number of open arm entries compared with control (3.40 ± 0.51) (Figure 1A). Increased duration in the open arm entries was also observed ($p < 0.01$) for TIME-4 and TIEAE-4 in a dose-dependent manner compared with control (64.40 ± 2.06) (Figure 1B). TIHE-4 was most significant in terms of the number of arm entries (9.60 ± 1.82) and the duration in open arm (89.80 ± 4.60 min) when compared with the control group. Similarly, the standard group exhibited a marked increase ($p < 0.001$) in the number of arm entries (13.40 ± 1.36) and the duration in the open arm entries (114.60 ± 1.72) compared with control (Figure 1A-B). However, numbers of close arm entries were not different among the groups (Figure 1C). Collectively, these findings clenched the anxiolytic effects of *Tragia involucrata* L. leaves.

3.3.2. Pentobarbital induced sleeping time test

The sedative activity of the leaves of the plant was assessed by the pentobarbital-induced sleeping time test. According to the results, the administration of TIEAE-4 and TIHE-4 (7.60 ± 1.44) significantly decreased ($p < 0.01$) in the onset of sleeping compared with control (Figure 2A). Regarding the duration of sleeping time, a significant increase ($p < 0.001$) was observed following the administration of TIME-4 (40.40 ± 2.62), TIEAE-4 and TIHE-4 compared with control (15.60 ± 2.51) (Figure 2B). The standard group showed a pronounced reduction in latency to sleep and an increase in time spent in sleeping in comparison to control (Figure 2A-B). All the aforementioned findings clinch the presumption that *Tragia involucrata* L. leaves extract has significant anxiolytic and sedative activity and has therapeutic potentials.

3.4. Analgesic activity of TIME, TIEAE and TIHE

3.4.1. Acetic acid induced writhing

We next sought to examine the analgesic effect of *Tragia involucrata* L. leaves using acetic acid-induced writhing test, where oral administration of TIME-4 and TIHE-4 demonstrated anti-nociceptive activity with a significant ($p < 0.01$) decrease in the number of writhing compared with control (25.40 ± 1.96) (Figure 3A). TIEAE-4 showed the highest decrease in number of writhing (13.20 ± 1.39; $p < 0.001$) and increased in the percentage of inhibition (48.03%) (Figure 3A-B). The standard group also showed a significant reduction ($p < 0.001$) in the number of writhing compared with control and elevation in the percentage of inhibition (Figure 3A-B).

3.4.2. Formalin induced paw licking test in mice

To evaluate the reliability of our initial findings in acetic acid induced writhing test, we performed another analgesic activity test known as formalin-induced paw licking test. Standard group demonstrated significant ($p < 0.001$) reduction in paw licking time both at acute phase (49.80 ± 2.52) and late phase (1.80 ± 1.80) compared to control (Figure 4A and Figure 4C). In acute phase, TIEAE-4 and TIHE-4 demonstrated a significant ($p < 0.001$) reduction in paw licking time compared to control (Figure 4A); percentage of inhibition of TIEAE-4 and TIHE-4 was 46.41 and 36.21, respectively (Figure 4B). In late phase, TIME-4, TIEAE-2, TIEAE-4 (11.09 ± 2.51), TIHE-2, TIHE-4 was statistically significant ($p < 0.001$) reduction in paw licking time compared to control (Figure 4C); TIEAE-4 had the highest number of percentage inhibition (74.80%) (without standard group; 95.91%) (Figure 4D). Taken together, these results support that *Tragia involucrata* L. leaves has significant analgesic activity.

3.5. Heavy metal analysis of Tragia involucrata L. leaves

Tragia involucrata L. leaves part showed off five different heavy metal (Table 2).

4. Discussion

This is the first study to examine the anxiolytic, sedative and analgesic activity of the leaf extracts of a medicinal plant *Tragia involucrata* L. In

Table 1. The Phytochemical Screening of n-Hexane, ethyl acetate and methanol extract of *Tragia involucrata* L. leaves.

Phytochemical constituents	Name of the test	Observation	Methanol extract	Ethyl Acetate Extract	n-Hexane Extract
Phenols	$FeCl_3$ test	Brownish green or blue colour	++	−−	−−
Alkaloids	Mayer's test	Cream colored precipitate	++	−−	++
Flavonoids	$FeCl_3$ test	Intense green color.	++	−−	++
Sterols	Liebermann-Burchard test	Brown ring at junction of two layers and upper layer turns green.	−−	++	++
Carbohydrates	Molisch test	The purple ring at the junction of two liquid.	++	++	++
Proteins	Xanthoproteic test	Yellow color precipitate	++	−−	−−
Tannins	$FeCl_3$ test	Dark color	++	−−	−−
Saponins	Foam test	Formation of foam.	++	−−	−−
Glycosides	Raymond's test	Violet color	−−	−−	++
Terpenoids	Salkowski test	Lower layer turns yellow.	++	++	++

Note: "++" = Present, "−−" = Absent.

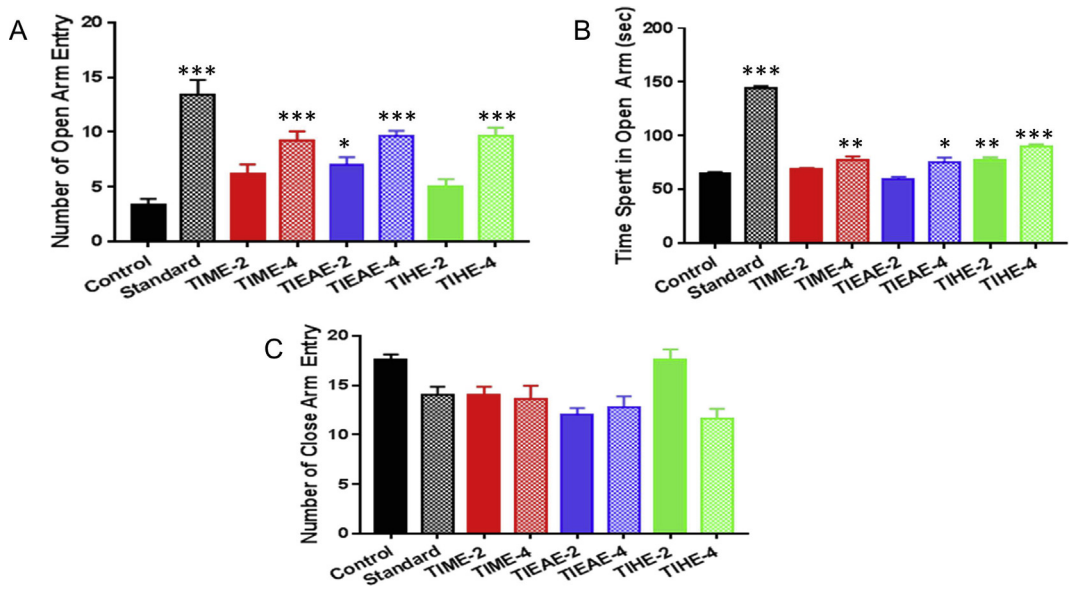

Figure 1. Anxiolytic effects of *Tragia involucrata* L. leaves. (A) Number of open arm entry, (B) Time spent in open arm, (C) Number of close arm entry. Data are shown as mean ± SEM. * denotes $p < 0.05$ vs control, ** denotes $p < 0.01$ vs control, *** denotes $p < 0.001$ vs control (One way ANOVA followed by Dunnett *t*-test).

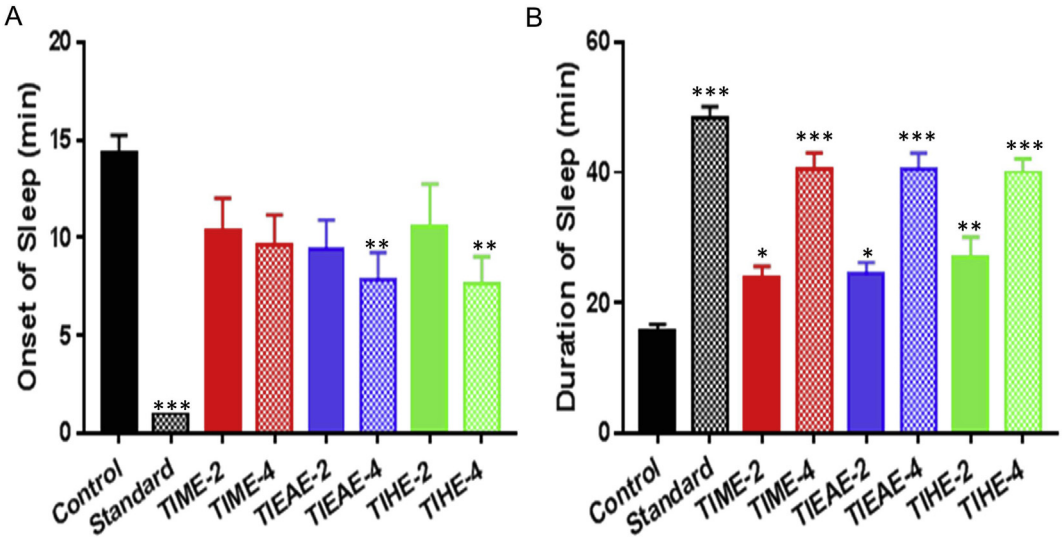

Figure 2. Impact of *Tragia involucrata* L. leaves extracts on onset and duration of sleep. (A) Onset of sleep, (B) Duration of sleep. Data are shown as mean ± SEM. * denotes $p < 0.05$ vs control, ** denotes $p < 0.01$ vs control, *** denotes $p < 0.001$ vs control (One Way ANOVA followed by Dunnett *t*-test).

this study, we utilized methanol, ethyl acetate and n-Hexane solvent extraction methods. We first performed a phytochemical screening and found that the presence of numerous secondary metabolites such as phenols, alkaloids, flavonoids, sterols, tannins, terpenoids etc. TIME demonstrated the higher number of secondary metabolites compared to TIEAE and TIHE. TIME contains phenols, alkaloids, flavonoids, carbohydrates, proteins, tannins, saponins and terpenoids. Because of diverse biological functions, these metabolites have particular interest in drug discovery research and thus advance the medicinal research field [24]. For instance, the plant derived phenolic compounds have been reported to be anti-inflammatory, anti-atherosclerotic, anti-carcinogenic, cardiac- and vasculo-protective [25]. Likewise, the alkaloids have anti-microbial, anti-inflammatory, anti-spasmodic and analgesic properties and have growing interest in pharmacological industries [26]. Flavonoids, synthesized by plants, are hydroxylated phenolic substances which response to microbial infection [27]. These compounds form a complex with the extracellular and transmembrane protein of bacterial cell wall and thus exhibit anti-microbial properties against a wide array of microorganisms [28]. Furthermore, the plant of this study contains substantial quantities of flavonoids to exhibit anxiolytic and analgesic properties [29]. Steroids or terpenoids derived from plants are known to have cardiotonic, anti-bacterial and insecticidal properties and are often used as analgesics [30]. Tannins have antitumor antibacterial and antiviral activities [31]. The plant extracts were also revealed to contain saponins which are known to produce inhibitory effect on inflammation and peripheral analgesic activity [26] and glycosides are widely used to treat congestive heart failure and cardiac arrhythmia [32].

Numerous plant extracts have been shown to have anxiolytics properties, a compound of substance that inhibits anxiety. EPM is a widely used assay to examine anxiety in laboratory animals [33]. Using EPM assay, we found that administration of different group of TIME, TIEAE and TIHE induced an anxiolytic-like effect in mice as demonstrated by increased open arm entries and the time spent in the open arms compared to control. Mostly dysfunction of neurotransmitters (dopamine, GABA, and serotonin) or dysregulation of glutamatergic, serotonergic, GABA-ergic, and noradrenergic transmission cause anxiety. In the study,

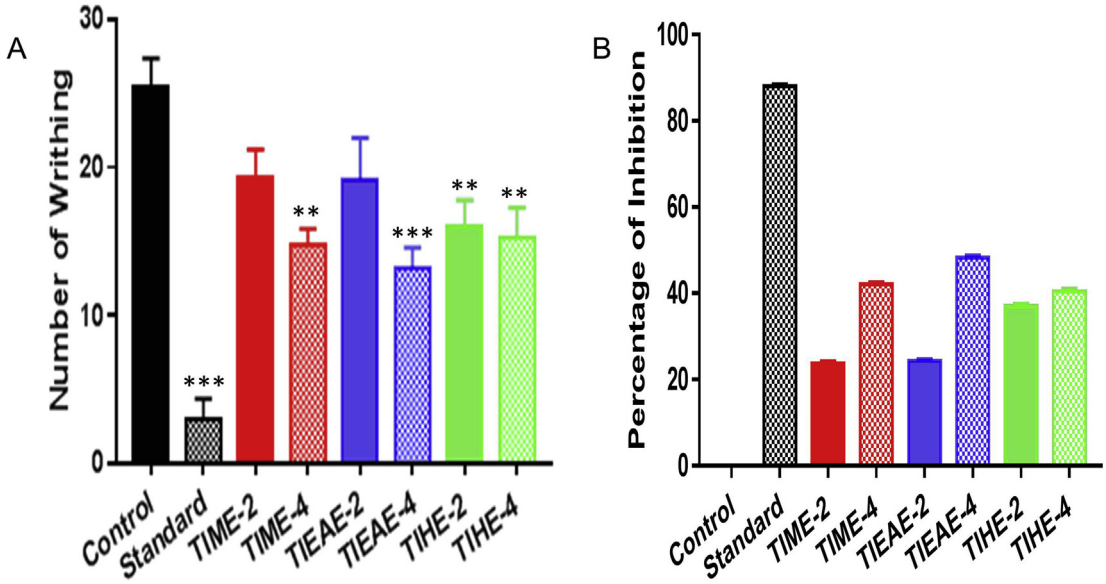

Figure 3. Effects of *Tragia involucrata* L. leaves extracts on acetic acid induced writhing. (A) Number of writhing, (B) percentage of inhibition. Data are shown as mean ± SEM. * denotes $p < 0.05$ vs control, ** denotes $p < 0.01$ vs control, *** denotes $p < 0.001$ vs control (One-way ANOVA followed by Dunnett *t*-test).

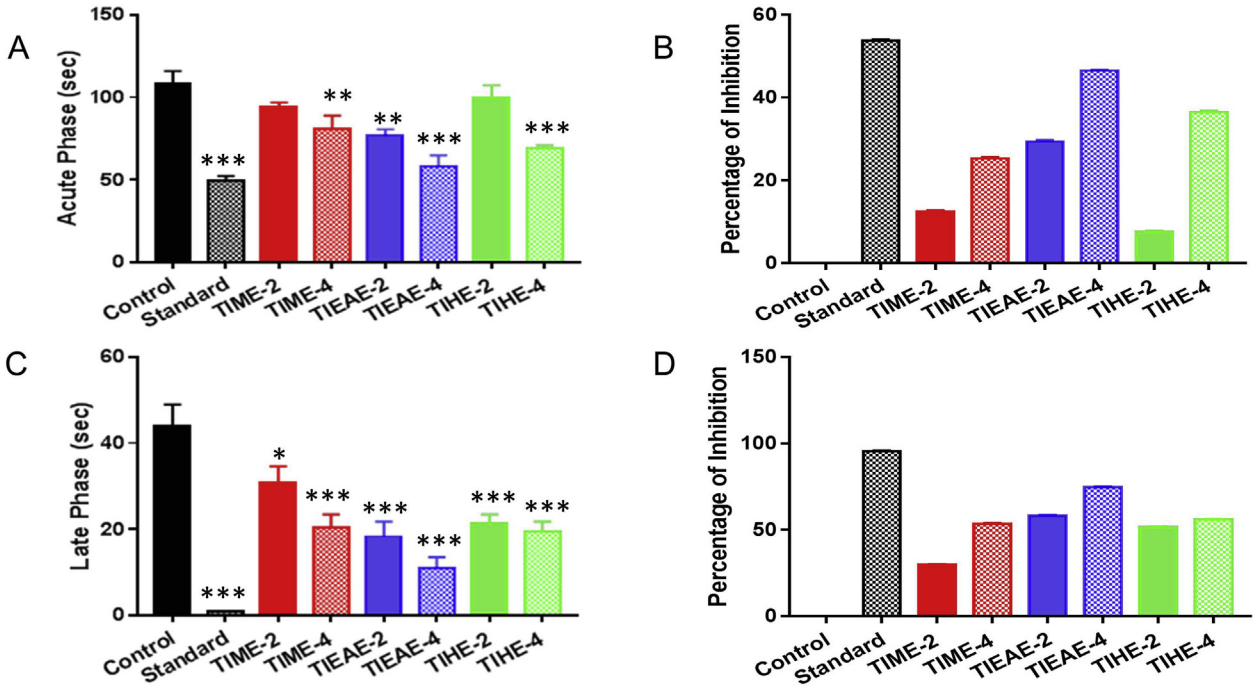

Figure 4. Effects of *Tragia involucrata* L. leaves extracts on formalin-induced paw licking tests. (A) Duration of acute phase, (B) Percentage inhibition of acute phase, (C) Duration of late phase, (D) Percentage inhibition of late phase. Data are shown as mean ± SEM. * denotes $p < 0.05$ vs control, ** denotes $p < 0.01$ vs control, *** denotes $p < 0.001$ vs control (One-way ANOVA followed by Dunnett *t*-test).

Table 2. Heavy metal analysis of *Tragia involucrata* L. leaves.

Name of Heavy Metal	Amount (ppm)
Cu	0.33 ± 0.003
As	5.16 ± 0.012
Pb	0.19 ± 0.001
Zn	0.59 ± 0.001
Fe	2.76 ± 0.015

Data are presented as Mean ± SEM.

extracts of *Tragia involucrata* L. may induce anxiolytic activity by altering the function and synthesis of neurotransmitters. It is assumed that active components of the plant interact either with neurotransmitters or with neuromodulator receptors. These receptors are responsible for the regulation of neuronal communication, stimulation of the CNS activity and development of endocrine systems function [34, 35, 36]. Moreover, our phytochemical screening reported that the plant contains flavonoids, terpinoids, tannins and saponins which may exhibit anxiolytic activity by interacting with the neurotransmitter or neuromodulator receptors. Thus, it can be hypothesized that the plant contains potential compounds

that show anxiolytic effect.The chemical compounds that reduce the time of onset of sleep and/or extend the duration of sleeping time, often exhibit sedative and CNS depressant activity [37]. Numerous studies report that the bioactive compounds work through gamma-aminobutyric acid (GABA) receptors [38, 39]. Thus, there is a possibility that the TIME, TIEAE and TIHE act on GABA receptors to mediate the downstream actions. In general, brain cells make GABA using glutamate that works as inhibitory neurotransmitter resulting in reduced firing rate of nerve cells [40]. GABA receptor is a ligand-based ion channel whose activation leads to the cellular inhibition in the CNS [40]. Mechanistically, the phytochemical constituents saponins and alkaloids have been revealed to be able to stimulate GABA receptors [41, 42]. Our findings evidenced that *Tragia involucrata* L. has a pronounced effect on both induction and duration of sleep. Our phytochemical screening reveals the presence of a multitude of secondary metabolites such as flavonoids, alkaloids and saponins that may underlie the observed effects. Thus, it can be hypothesized that the plant contains potential compounds that show sedative and anxiolytic effect. Future investigation need to be performed to identify the bioactive compounds that inducing the sedative properties.

Therefore, this study investigates the analgesic properties of various doses of TIME, TIEAE and TIHE when all is said in the non-narcotic peripheral acting model by acetic acid induced writhing test and central acting model by formalin induced paw licking test.

Pain is usually induced by endogenous inflammatory mediators, as like prostaglandin, serotonin and bradykinin that elevate the stimuli of peripheral nerve results in activation of nociceptors [43]. Among the experiments, it was found that the writhing method was effective for the analysis of peripherally active analgesics by the acetic acid induced. So, the molecules that reduce the number of writhing will express analgesic effect mostly via the interference of prostaglandin synthesis, a peripheral mechanism of pain inhibition [44]. In this peripheral model, pain sensation is stimulated by localized inflammatory response. It was assumed in the earlier that arachidonic acid regulates the release of prostaglandins after the induction of inflammatory response by activating phospholipase A_2. It is now evident that the process of local prostaglandin production from arachidonic acid is stimulated by local interleukin-1β (IL-1β) and facilitated by cyclooxygenase enzymes (COX-1 and COX-2) and membrane phospholipid. In addition, elevated peritoneal fluids of prostaglandin (PGE_2 and PGF 2α) and elevated lipoxygenase level has been found in acetic acid induced mice [45]. TIME, TIEAE and TIHE administration significantly reduced the number of abdominal constrictions induced by acetic acid in mice. So, the experimental outcome clenches the hypothesis that all the extracts of *Tragia involucrata* L. may act by blocking cyclooxygenase enzymes (COX-1 and COX-2) resulting in reduction of PG synthesis from arachidonic acid since the nociceptive mechanism of acetic acid induced abdominal constriction involves the prostanoid biosynthesis pathway. Another possible mechanism could be involved in neurotransmitter systems such as catecholaminergic, cholinergic, serotonergic, opioids, GABAergic system as well as ATP gated potassium channels. We assume that phytochemical compounds alkaloids and flavonoids (e.g: apigenin, terniflorin) may exert the analgesic and anti-inflammatory activity via arachidonic acid metabolism pathway [46, 47]. Thus, it can be hypothesized that the plant contains potential compounds that show analgesic effect.

In pharmacological studies, it is found that two distinct phases (early phase and late phase) of pain are provided by formalin test. The possible mechanisms of analgesic effects can be determined by this useful design [48]. This formalin test can also explain the difference between the peripheral and central antinociceptive mechanism of action. Cytokines can elevate pain in different ways. One way (in early phase) is these can directly stimulate the nociceptive neuron and so an acute inflammatory response might be noticed at the early phase right away the formalin injection. Another way (in late phase) is cytokine-induced release of inflammatory mediators particularly serotonin, prostaglandins, bradykinin and histamine and activation of the nociceptive neuron of spinal dorsal horn which elicit delayed inflammatory response observed in late phase [49, 50]. The pain sensation in both phases was inhibited significantly by different doses of TIME, TIEAE and TIHE, and late phase was more prominent to reduce the licking behavior. It can be said that TIME, TIEAE and TIHE may have inflammatory properties because of inhibition of licking behavior in late phase. The phytochemical screening revealed that the *Tragia involucrata* L. leaves extracts contain flavonoids, alkaloids and saponins which perhaps interact with the COX receptors and other inflammatory and neurogenic pain modulators. Thus, it can be hypothesized that the plant contains potential compounds that show analgesic effect.

Heavy metal contamination in medicinal plants is an increasing concern [51]. The anthropogenic release of heavy metals into the environment by mining, manufacturing, agriculture, and smelting is the major cause of heavy metal entry into the food chain and plant constituents. Certain heavy metals are essential for fundamental biological processes. However, when in excess heavy metals may induce substantial detrimental effects both as cellular and organismal level. For instance, copper works as a cofactor in numerous cellular reactions including metabolic pathways; however, in excess it can cause toxicity and may exhibit carcinogenic activities [52]. Likewise, lead is a highly poisonous metal, affecting almost every organ and tissue in the human body including brain, kidneys and liver. By mimicking calcium, lead can cross the blood–brain barrier and degrades the myelin sheaths of neurons, reduces their numbers, interferes with neurotransmission routes and decreases neuronal growth. In the human body, lead inhibits porphobilinogen synthase and ferrochelatase, resulting in ineffective heme synthesis causing anemia [53, 54]. The role of arsenic in humans is not well understood; however, excess intake of arsenic has detrimental effects on human health [55]. Zinc is an important micronutrient for many biochemical metabolisms in the plants. It is required for the optimum crop growth and is taken in divalent form by the plants. Zinc deficiency causes the gastrointestinal, central nervous, epidermal, immune, skeletal, and reproductive disorders. Zinc supplement can reduce episode of malaria, prevalence of pneumonia and diarrhea [56]. Iron is an important component or molecule of hemoglobin in red blood cells that carry oxygen from our lungs to transport it throughout our body. A lack of red blood cells is called “iron deficiency anemia” which is dangerous for the human body [57, 58]. The soil has been drastically polluted and becomes reservoir of metal contents because of excessive use of synthetic fertilizers and industrial discharge to the agricultural land and other intentional or unintentional hazardous activities by human. These heavy metals in turn transferred to human and other animals by consumption of plants parts which store metal contents. Consequently, heavy metals enter to the ecosystem and lead to elevated bioaccumulation and storage in plants, animal and human.

5. Conclusion

This study examined various pharmacological properties of the *Tragia involucrata* L. leaves using elevated plus maze, pentobarbital induced sleeping time, acetic acid induced writhing and formalin induced paw-licking tests. Findings from this study demonstrate that *Tragia involucrata* L. has significant anxiolytic, analgesic and sedative effects. Furthermore, this study detects the presence of numerous secondary metabolites along with the presence of heavy metals in *Tragia involucrata* L. leaves that may be insightful for real-life applications. To identify the potential therapeutic agent, further investigation is required to isolate and purify the bioactive compound of the plant.

Declarations

Author contribution statement

S. Sana and Md. S. Islam: Conceived and designed the experiments; Analyzed and interpreted the data.

Md. E. Haque, S.M. Mushiur Rahman, A. Samad, A. Al Noman, R. Alam, S. Rana and R.I. Meem: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

D. Mondol, Md. S. Islam, Md. Torikul Islam and K. Mazumder: Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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