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Pharmacological investigations of Tetrastigmabracteolatum(Wall.) Planch., an indigenous plant of Bangladesh, traditionally used for pain management

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Abstract:	Background: Tetrastigma bracteolatum (Wall.) Planch, an indigenous plant of Bangladesh, is traditionally used for pain management. So far no scientific report is available onits Pharmacological activities. The objectives of the study were to determine Pharmacological activities as well as to justify the traditional use of Tetrastigma bracteolatum for pain management. Methods: Crude methanol extract was prepared by maceration technique and partitioned by modified Kupchan method using petroleum ether, chloroform, dichloromethane and wateras extracting solvents. Anti-oxidant and cytotoxic activities were assessed by DPPH free radical scavenging and brine shrimp lethality bioassay methods, respectively. The thrombolytic and anti-inflammatory activities were measured by assessing the ability to lyse blood clots and membrane stabilizing bioassay, respectively. Central and peripheral analgesic activities were determined by tail flicking and writhing methods, respectively. Results: T he highest amount of phenolic content was found inCSF followed by DCMSF and and MESF (86.70, 40.07 and 33.01mg of GAE/gm of extractives, respectively).The CSF showed the highest level of inhibition against hypotonic solution (64.03%) and heat-induced hemolysis (51.95%) of erythrocytic cell membrane, compared to acetic acid (81.97%) and heat -induced solution (42.12%), respectively. The potential anti-oxidant activity was exhibited by AQSF (IC 50 7.48 µg/mL) and cytotoxic activity by PESF (LC 50 1.41 µg/mL). Crude methanol extract at 400 mg/kg reduced diarrheal defecation by 87.18% ($p<0.001$) which was higher than that of loperamide(3 mg/kg) by 82.05%. The hypoglycemic activity of methanol extract (200 mg/kg)(blood glucose reduction 71.74%) was comparable with glibenclamide(5 mg/kg) (glucose reduction 72.4%). The methanol extract at 200 mg/kg delayed the onset (54.67±15.19 min) (* $p<0.05$) and duration (137.33±34.68 min) of sleeping time compared to control (10±0.0 and 173.33±3.48 minutes, respectively). The methanolic extract at 400 mg/kg prolonged tail flick response time by 298.28% ($p<0.001$) and inhibited 67.74% writhing response induced by acetic acid ($p<0.001$). Conclusions: Our investigation demonstrated antioxidant, membrane stabilizing, antidiarrhoeal, hypoglycemic, antidepressant, and analgesic activities of methanol extract of Tetrastigma bracteolatum and/or its fractions. Thus, our findings support the traditional use of Tetrastigma bracteolatum for pain management. However, identification and isolation of bioactive compounds and comprehensive studies are suggested for future investigation.
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ORIGINAL RESEARCH ARTICLE

Pharmacological investigations of *Tetrastigma bracteolatum*(Wall.) Planch., an indigenous plant of Bangladesh, traditionally used for pain management

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Running title: **Pharmacological investigations of *Tetrastigma bracteolatum* (Wall.) Planch**

Abstract

Background:*Tetrastigma bracteolatum* (Wall.) Planch, an indigenous plant of Bangladesh, is traditionally used for pain management. So far no scientific report is available onits Pharmacological activities. The objectives of the study were to determine Pharmacological activities as well as to justify the traditional use of *Tetrastigma bracteolatum*for pain management.

Methods: Crude methanol extract was prepared by maceration technique and partitioned by modified Kupchan method using petroleum ether, chloroform, dichloromethane and water as extracting solvents. Anti-oxidant and cytotoxic activities were assessed by DPPH free radical scavenging and brine shrimp lethality bioassay methods, respectively. The thrombolytic and anti-inflammatory activities were measured by assessing the ability to lyse blood clots and membrane stabilizing bioassay, respectively. Central and peripheral analgesic activities were determined by tail flicking and writhing methods, respectively.

Results: The highest amount of phenolic content was found in CSF followed by DCMSF and and MESF (86.70, 40.07 and 33.01mg of GAE/gm of extractives, respectively).The CSF showed the highest level of inhibition against hypotonic solution (64.03%) and heat-induced hemolysis (51.95%) of erythrocytic cell membrane, compared to acetic acid (81.97%) and heat -induced solution (42.12%), respectively. The potential anti-oxidant activity was exhibited by AQSF (IC_{50} 7.48 µg/mL) and cytotoxic activity by PESF (LC_{50} 1.41 µg/mL). Crude methanol extract at 400 mg/kg reduced diarrheal defecation by 87.18% ($p<0.001$) which was higher than that of loperamide (3 mg/kg) by 82.05%. The hypoglycemic activity of methanol extract (200 mg/kg)(blood glucose reduction 71.74%) was comparable with glibenclamide (5 mg/kg) (glucose reduction 72.4%).

The methanol extract at 200 mg/kg delayed the onset (54.67±15.19 min) (*p<0.05) and duration (137.33±34.68 min) of sleeping time compared to control (10±0.0 and 173.33±3.48 minutes, respectively).

The methanolic extract at 400 mg/kg prolonged tail flick response time by 298.28% (p<0.001) and inhibited 67.74% writhing response induced by acetic acid (p<0.001).

Conclusions: Our investigation demonstrated antioxidant, membrane stabilizing, antidiarrhoeal, hypoglycemic, antidepressant, and analgesic activities of methanol extract of *Tetrastigma bracteolatum* and/or its fractions. Thus, our findings support the traditional use of *Tetrastigma bracteolatum* for pain management. However, identification and isolation of bioactive compounds and comprehensive studies are suggested for future investigation.

Keywords:

Tetrastigma bracteolatum, Anti-inflammatory, Traditional medicine, Indigenous, Phytoconstituents, Kupchan, Herbal medicine, Natural medicine, Extract, *In vivo*, *In vitro*

Background

Regardless the revolution of modern medicine in the twentieth century, it has been estimated that about one-third of the world's population fail to receive affordable medicines. This has led to a surge in the use of traditional (herbal), complementary and alternative medicine in both developed and developing countries[1]. In countries like Bangladesh, the use of traditional medicine for primary health-care is extensive [2]. The practitioners, usually referred as the 'Kabiraj', commonly extend their services both in rural and urban areas however rural areas being the major locations where their practices are more popular. The Kabirajes mainly rely on medicinal plants for treatment [3]. Traditional medicine, herbal medicines, functional foods and nutraceuticals are prospective alternative and complementary

medicine for the treatment of diseases. Many scientific studies proved the effectiveness of prospective ingredients or substances *in vitro*, *in vivo* and clinical studies against diabetes [4-12], cancer [13-15], immunity [16-24], hyperlipidemia and obesity [25-26], analgesia [27], inflammation [28-29], oxidation [30], cognition [31], microbial infection [32], and many other diseases.

Tetrastigma bracteolatum (Wall.) Planch.(Family: Vitaceae) is one such plant that has been used for management of pain particularly in the remote hilly districts (Bandarban) of Bangladesh. It is a large woody climber, with slender branches and glabrous stems and leaves[33]. Tetrastigma is a genus of plants in the grape family, Vitacea e[34]. The local name of the plant is Golgotilata however certain tribes in Bangladesh (Chakma) call it Khurangulludi. The plant is mostly distributed in the hilly regions of Bangladesh such as Chittagong, Chittagong hill tracts, Cox's Bazar, Sylhet and Moulavi Bazar etc[3]. The Chakma tribal people frequently make a paste of the leaves and apply to the forehead for the treatment of headache [33][35]. Moreover, the Chiru tribe of Manipur, India, where use the boiled extracts of both the leaves and fruits against indigestion and stomach disorder[36]. However, literature survey reveals no scientific data yet being published to evaluate its traditional use or to estimate the dose or investigate any toxic effect of the plant in human body. In order to assess its traditional use and considering its medicinal property, it is necessary to conduct biological assays to justify its use, isolate compounds responsible for it activity (if any) as well as to assess its toxic effect. Therefore, the objective of the current study was to evaluate the traditional medicinal property of the *Tetrastigma bracteolatum* (Wall.) Planch. by conducting both *in-vitro* and *in-vivo* biological assays of the methanolic extract of the whole plant and its various organic partitions.

Methods

2.1 Collection and identification of the plant

Fresh whole plants of *Tetrastigma bracteolatum* (Wall.) Planch. were collected by Fatima Anjum Farooque from the hill tracts of Chittagong division, Bangladesh, in May 2018. The plant was then identified by a taxonomist of Bangladesh National Herbarium located at Mirpur, Dhaka, Bangladesh where the specimen sample has been preserved. The voucher number of the deposited plant specimen is 46512.

2.2 Preparation and fractionation of extracts

After collection and identification, the clean plants were sun dried and ground to coarse powders. Then 400 gm of this powder was soaked in 1600 mL of methanol in an amber glass bottle. The container was sealed with a cotton plug and kept for 7 days with occasional shaking to facilitate the extraction of phytoconstituents. The whole mixture was filtered by cotton plug followed by Whatman number 1 filter paper. The filtrate was further evaporated to dryness using a rotary evaporator at 40°C temperature and pressure. Thus, the crude methanol extract of *Tetrastigma bracteolatum* (METB) was prepared as concentrated gummy mass which was transferred to a clean beaker. This crude extract was partitioned using various organic solvents sequencing non-polar to polar gradient following the modified Kupchan method [8]. Five gm of solid crude extract was re-dissolved in 10% aqueous methanol and extracted consecutively with petroleum ether, dichloromethane, chloroform, and water to provide petroleum ether soluble fraction (PESF), dichloromethane soluble fraction (DCMSF), chloroform soluble fraction (CSF), and aqueous soluble fraction (AQSF).

2.3 Chemicals and reagents

Vincristine sulfate, morphine, glibenclamide, loperamide were generously donated by Square Pharmaceutical Ltd., Dhaka, Bangladesh. Diclofenac sodium and normal saline were donated by Incepta Pharmaceutical Ltd., Dhaka, Bangladesh. DPPH (2, 2-diphenylpicrylhydrazyl), BHT (Butylated hydroxytoluene) were purchased from Merck, India. Analytical grade acetic acid, methanol, tween-80, castor oil, streptokinase were locally purchased.

2.4 Experimental animals

Swiss Albino mice of both sexes weighing between 25-35 g having age of 5-6 weeks old were purchased from Animal breeding and Pharmacology laboratory of Jahangirnagar University, Bangladesh. The mice were housed at animal experiment facility of State University of Bangladesh at 22-24 °C temperature and fed with standard rodent feed and drink *ad libitum*. Environmental changes were strictly controlled and prior to any experiment, animals were kept for 1 week to adjust with the new housing environment. The ethical approval was obtained from the Animal Ethics Committee of State University of Bangladesh. All the experiments were conducted according to the approved Animal Use Protocol. The Federation of European Laboratory Animal Science Associations (FELASA) guidelines and recommendations were followed to reduce the pain and stress of the experimental animals. Twenty Swiss Albino mice were randomly divided into four groups (control group, 2 treated groups and a positive control group) consisting of five animals in each group. The tested groups received methanol extract 200 and 400 mg/kg, respectively; whereas the positive control group received standard drug of the respective interventions. At the end of the experiments, the animals were sacrificed by euthanasia.

2.5 Determination of total phenolic contents

The total phenolic content of *T. bracteolatum* extract was determined as describe by Skerget*etal.*,2005[37] using the Folin-Ciocalteu reagent as the oxidizing agent, gallic acid as the equivalent standard. The results were expressed as the mg of gallic acid equivalent, GAE/100 g of plant extract. For each reagent, it was diluted 10 times with distilled water. Later, 2.5 mL of the diluted reagent were taken and mixed with 7.5% Sodium carbonate (2 mg/mL water). Each sample was incubated for 15 min. at 45 °C and then absorbance was measured at 765 nm by using UV-visible spectrophotometer.

2.6 Determination of antioxidant activity by free radical scavenging DPPH method

The antioxidant capacity (free radical scavenging activity) of the plant extractives on the stable radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH) was determined as per the method described by Brand-William *et. al.* [38]using tetra-butyl-1-hydroxytoluene (BHT) as standard antioxidant. For each sample, concentrations ranging from 500 μg/mL to 0.977 μg/mL were prepared using serial dilution with methanol. Two (2) mL from each concentration was then mixed with 3 mL DPPH (20 μg/mL) and kept in a dark space at normal room temp and pressure for 30 min. The absorbance of the sample was then analyzed by UV-visible spectrometer at a wavelength of 517 nm. The IC_{50} value (concentration of reagent required to neutralize 50% of the oxidant molecule) is calculated by using the following formula:

Percent inhibition = $(1 - A_1/A_0) \times 100$ %

Here, A_0 = absorbance of the blank (methanol)

A_1 = absorbance of the sample being analyzed

2.7 Determination of cytotoxic activity by brine shrimp lethality bioassay

Brine shrimp lethality bioassay was used for the determination of cytotoxic activity of methanol extract and its different fractions on brine shrimp following the experimental procedure as narrated by Meyer et al., 1982 [39]. The five fractionates were assessed for their cytotoxicity on Brine shrimp nauplii. In each case 4 mg sample from each fractionate were mixed with 100 μL of dimethylsulfoxide (DMSO) which were further diluted to get samples of concentrations ranging from 400 μg/mL to 0.781 μg/mL. Fifty (50)μL from each sample was mixed with 5 mL of simulated sea water containing 10nauplii. The test samples were then kept at normal temp for 24 hr and the numbers of a live nauplii were counted using a magnifying glass at the end of observation. LC_{50} value was calculated using the graph of log concentration of the sample versus the percentage mortality of the nauplii and compared with

the vincristine sulfate as a standard cytotoxic drug.

2.8 Determination of thrombolytic activity

The thrombolytic activity was determined following the method as used by Prasad et al. 2016 [40]in which streptokinase was used as positive control. Five (5) mL venous blood was taken and distributed in pre-weighed sterile micro-centrifuge tubes. Tubes were then incubated for 45 min at 37 °C temp. Serum was removed carefully after clot formation and the clot weight was measured. Then 100 μL aqueous solution of different samples (10 mg/mL water), 100 μL of streptokinase and 100 μL of distilled water (negative control) were added separately to tubes containing pre-weighed clot. After incubation at 37 °C for 90 min, the released fluid was removed carefully. Tubes were again weighed. The differences in weights were used to determine percentage of clot lysis according to the following equation:

$$\text{Percentage (\%) of clot lysis} = (\text{weight of released clot}/\text{weight of clot}) \times 100$$

2.9 Determination of membrane stabilizing activity in red blood cells

The membrane stabilizing activity of the extractives was determined by evaluating their ability to prevent hypotonic solution and heat-induced hemolysis of human erythrocyte cell membrane [41]. In case of hypotonic induced hemolysis, 0.50 mL erythrocyte suspension, 5 mL hypotonic solution (50 mMHCl), 10 mM sodium phosphate buffer saline (pH 7.4) were mixed with different extractives (2 mg/mL) once at a time. The mixture after incubating for 10 min, were centrifuged at 3000 g and the absorbance of the supernatant were measured at 540 nm in the UV spectrophotometer (Shimadzu). Acetyl salicylic acid (ASA) was used as the reference standard.

The amount of hemolysis of RBC was calculated by the following formula:

$$\text{Percentage of hemolysis} = 100 \times \{(OD_1 - OD_2)/OD_1\}$$

where,

OD_1 = Optical density of hypotonic-buffered saline solution alone (negative control)

OD_2 = Optical density of test solution in hypotonic solution

In case of heat-induced hemolysis, 0.5 mLfrom each of extractives (2 mg/mL) was transferred to two centrifuge tubes containing 4.5 mL of an isotonic buffer and 30 µL of erythrocyte suspension. Each of the centrifuge were kept in two different temperatures, one at 54 °C and the other at 0-5°C and both were kept for 20 min. The mixtures were later centrifuged for 3 min. at 1300 g and the absorbance of the supernatant was recorded at 540 nm with the isotonic solution and the buffer mixture as blank. In this case, the percentage of hemolysis was calculated by using the formula:

$$\text{Percentage (\%) of hemolysis} = 100 \times \left[1 - \left(OD_2 - \frac{OD_1}{OD_3} - OD_1\right)\right]$$

Where,

OD_1 = Optical density of the unheated test sample

OD_2 = Optical density of the heated test sample

OD_3 = Optical density of the isotonic buffer solution only (negative control)

2.10 Central and peripheral analgesic activity

In order to determine the central analgesic activity, the tail flicking method described by Pizziketti et al,1985 and for peripheral analgesic activity, the acetic acid induced writhing method as described by Kaushik et al, 2012 [42] were followed. Mice were divided into 4 groups(5mice/group): control, two treated groups (200 and 400mg/kg methanol extracts, respectively), and positive control group. The methanol extracts and 1% Tween 80 in saline mixture (0.1 mL/10 mg) was administered orally, and morphine(2 mg/kg) as positive control was injected subcutaneously. The tail tips the mice (1-2 cm) were submerged in hot water after 30, 60 and 90 min. of treatment and response (tail flick) time were recorded accordingly. The percentage of pain inhibition (PIP) was calculated by using the following formula:

$$\text{Pain inhibition percentage (PIP)} = \left[\frac{T_1 - T_0}{T_0}\right] \times 100$$

Where,

$T_1 =$ Post-drug latency period

$T_0 =$ Pre-drug latency period

For acetic acid induced writhing method (peripheral analgesic activity), methanol extracts (200 and 400mg/kg) were orally administered 40 min. before the administration of 1% (v/v) of acetic acid (10 mL/kg, i.p.) to induce writhing response. Diclofenac sodium (50mg/kg, oral) was used as positive control. After administration, the number of writhing movement were recorded for the next 10 min. Percentage (%) inhibition of writhing movement was proportional to the analgesic activity and was calculated using the following formula:

$$\text{Percentage (\%) inhibition} = \frac{\text{Control licking response} - \text{Test licking response}}{\text{Control licking response}} \times 100$$

2.11 Anti-diarrheal activity

Anti-diarrheal effect of the crude extract was assessed by using castor oil-actuated loose bowels and gastrointestinal motility tests. Swiss albino mice of both sexes were fasted overnight for 14 hr. Mice were divided into 4 groups (n=5): control group received normal saline (2 mL/kg, oral), treated two groups received METB (200 mg/kg, oral and 400 mg/kg, oral, respectively), and positive control group received loperamide (2 mg/kg, oral) as standard antidiarrheal drug. One hour after treatment, 1 mL castor oil was fed orally to all mice and then placed in cages lined with adsorbent papers in order to calculate characteristic diarrheal droppings for the next 4 h. Anti-diarrheal activity was determined with the percentage inhibition of castor oil induced diarrheal response by the following formula:

$$\text{Percentage inhibition} = \frac{\text{Number of diarrheal droppings (Control group)} - \text{Number of diarrheal droppings(sample group)}}{\text{Number of diarrheal droppings (Control group)}} \times 100$$

2.12 Oral glucose tolerance test to measure the antihyperglycemic activity of METB

Overnight fasted mice were divided into 4 groups (n=5): control group (received water only), treated groups received METB 200 mg/kg and 400 mg/kg, and positive control group received glibenclamide (5 mg/kg) through oral routes. Twenty min after the treatment, 100 mg of glucose (2 mg/kg) in 1 mL glucose solution (100 mg/mL) was orally administered to all groups of mice. Blood glucose levels were measured at before treatment and 30 min, 1 hr, 2 hr, and 3 hrs after the administration of oral glucose by tail tipping method [43] using a Glucometer (Accu-Check, Roche, Switzerland).

2.13 CNS stimulant activity: Thiopental sodium-induced sleeping time bioassay

In thiopental sodium-induced sleeping time test [44], the mice were divided into 3 groups (n=5). The control group received saline water containing 1% tween 80 while the other 2 test groups received the crude extracts 200 and 400 mg/kg, respectively. After 30 min, thiopental sodium (25 mg/kg) was injected intraperitoneally to all the mice in order to induce sleep. Later, all the mice were then monitored to record the time of onset of sleep and total sleeping time after the administration of the test samples.

Statistical analysis

The data were analyzed by one way analysis of variance (ANOVA) using Statistical Package for the Social Science (SPSS) software (version 25). In order to determine statistical significance between groups, Dunnett's test was performed. The data are means ± S.E.M. (standard error mean) of 3 independent observations or five mice. $p < 0.05$ was considered as statistically significant.

Results

3.1 Total phenolic contents in crude METB and in its different fractions

The total phenolic content varied from lowest as 0.38 mg of GAE/gm of PESF to as high as 86.70 mg GAE/gm of CSF. The highest phenolic content was found in CSF (86.70 ± 0.29) followed by DCMSF (40.07 ± 0.86), MESF (33.01±0.32), and AQSF (20.52±0.65) mg of GAE/gm of extractives (Table 1).

3.2 Antioxidant activity of METB and its different fractions

The antioxidant activity of METB and its different fractions by measuring its ability to scavenge DPPH free radicals. Our investigation resulted with the highest to the lowest free radical scavenging ability of AQSF, CSF, DCMSF, PESF, and MESF showing IC_{50} values 7.48±0.05, 27.74±0.55, 74.49±2.79, 80.38±0.43, and 170.13±2.40 µg/mL, respectively. The IC_{50} value of AQSF (aqueous soluble fraction of extract) (7.48±0.05) was found to be stronger than that of BHT (a standard antioxidant used in this experiment) (21.68±0.41). In addition, the antioxidant capacity of CSF (IC_{50}:27.74±0.55) was comparable with that of BHT (IC_{50}: 21.68±0.41) (Table 1).

3.3 Effect of METB and its different fractions on cytotoxic activity on Brine shrimp nauplii

The cytotoxic effects of METB and its different fractions on the lethality on Brine shrimp have been presented in Table 1. Among the tested extracts and fractions, the highest cytotoxic activity was exhibited by PESF followed by CSF, MESF, AQSF, and DCMSF having LC_{50}

values of 1.41±0.01, 12.41±0.16, 31.59±0.26, 39.34±0.05, and 51.47±0.29 µg/mL, respectively. The LC_{50} value of petroleum ether soluble fraction (PESF) 1.41±0.01 was comparable with vincristine sulphate (VS: an anticancer drug used as standard in this exp.) (0.45 ±0.00).

3.4 Effect of METB and its different fractions on membrane stabilizing of RBC

The membrane stabilizing activities of METB and its different fractions were determined by hypotonic-solution-induced as well as heat-induced hemolysis. In case of hypotonic-solution-induced-hemolysis, the highest membrane stabilizing activity was showed by CSF (64.03±1.16%) inhibition of RBC membrane compared to control) followed by DCMSF (63.94±1.77%), MESF (38.76 ±1.49), PESF (29.34±1.06), and AQSF (23.84±1.35), respectively; whereas the standard membrane stabilizing agent, aspirin, showed 81.97±2.37 % inhibition of hemolysis (Fig. 1).

Similarly, in case of heat-induced-hemolysis experiments, the highest membrane inhibition was exhibited by CSF (51.95±2.82%) followed by PESF (38.18±3.11%), DCMSF (31.20±1.21%), MESF (24.35±0.77%), and AQSF (7.02±1.11%), respectively. Our investigation resulted with the higher membrane stabilizing activity of chloroform soluble fraction of METB (51.95 ±2.82%) than that of standard membrane stabilizing drug, aspirin (42.12 ±2.04%) (Fig. 1).

3.5 Thrombolytic activity of METB and its different fractions

All the extractives showed inhibition of blood clotting in the thrombolytic bioassay but in all the cases the inhibition ability were lower than that of standard thrombolytic drug Streptokinase (67.36±2.31%). The highest inhibition was found to be exhibited by the CSF (46.46± 1.02%), followed by MESF (33.3± 2.03%) and DCMSF (24.7± 1.87%)(Fig. 2).

3.6 Anti-diarrheal activity

The methanol extract of *T. bracteolatum* showed dose dependent castor oil induced diarrhea in Swiss albino mice. The methanol extract at doses 200 and 400 mg/kg reduced diarrhea related stool defecation by 80.78% ($p<0.001$) and 87.18% ($p<0.001$), respectively (Table 2).

3.7 Evaluation of METB for oral glucose tolerability

Oral glucose tolerance ability of METB was assessed in Swiss albino mice with the oral administration of glucose (2 mg/kg). The glucose levels in mice were determined after 30, 60, 120 and 180 min. after the administration of oral glucose. METB extract at the dose of 200 mg/kg significantly controlled the boosting of post-prandial plasma glucose levels compared to control keeping the glucose levels at 6.40±1.39 vs. 14.57±5.14 ($p<0.05$) and 5.40±0.56 vs. 15.73 ±0.03 ($p<0.001$) after 60 and 120 minutes, respectively. Similarly, the plasma blood glucose levels were found to be significantly controlled by METB 400 mg/kg keeping the glucose levels at 5.67±0.93 vs. 14.57±5.14 ($p<0.05$) and 5.87±0.23 vs. 15.73 ±0.03 ($p<0.001$) after 60 and 120 minutes, respectively. The METB at the doses of 200 and 400 mg/kg reduced the boosting of plasma blood glucose levels 2 hours after the administration of oral glucose by 65.67% and 62.68%, respectively compared to untreated group; whereas the standard antidiabetic drug glibenclamide at the dose of 5 mg/kg reduced plasma glucose levels by 68.02% (Table 3).

3.8 Effect of METB on CNS stimulating activity in thiopental sodium-induced sleeping time bioassay

In Thiopental sodium–induced sleeping time test, the onset of sleeping were found to be significantly delayed in treated groups compared to control: 54.67±15.2 min vs. 10.00±0.0 ($p<0.05$) at METB dose 200 mg/kg, and 37.67±10.11 min vs. 10.00±0.0 at METB dose 400 mg/kg (Table 4). The total sleeping time in the treated groups were found to be 137.3±34.7

min(200 mg/kg) and 138.4±14.5 min(400 mg/kg) which were insignificantly lower compared to untreated group (173.3±3.5 min) (Table 4).

3.9 Central analgesic activity of METB

In case of central analgesic activity assay by tail-flick method, the tail flick response time to heat was measured at 0, 30, 60 and 90 min. The latency periods of METB 200 mg/kg, 400 mg/kg treated mice groups were found to be significantly prolonged compared to control. The latency period of 200 mg/kg METB treated mice were found to be 3.58±0.27 ($p<0.01$)at 30min, 5.50±0.33 ($p<0.001$) at 60 min, 8.71±0.37 ($p<0.001$) at 90 min, respectively. The percent (%) elongation of response time in mice groups treated with METB 200 mg/kg, 400 mg/kg, and morphine (2 mg/kg) at 90 min were found to be 275.43%, 298.28% and 334.05%, respectively (Table 5).

3. 10 Peripheral analgesic activity METB

In case of acetic acid induced writhing assay, the number of writhing in mice treated with METB at the dose of 200 mg/kg (8.67±0.67, $p<0.001$), 400 mg/kg (6.67±0.33, $p<0.001$), and standard analgesic drug diclofenac sodium at a dose of 50 mg/kg (4.67±0.33, $p<0.001$) were significantly reduced compared to untreated mice group. The highest inhibition of writhing was recorded in mice treated with METB 400 mg/kg (67.74%) which was comparable with that of standard analgesic drug, diclofenac sodium (77.42%) (Table 6).

Discussion

The highest phenolic contents were observed in the CSF and DCMSF fractionates of *T. bracteolatum*. This high phenolic content could be the cause for the antioxidant activities of the extracts [45-47].

The highest cytotoxic activity in brine shrimp bioassay where observed by CSF (LC_{50}: 1.41) whereas the LC50 of standard anticancer drug vincristine sulphate was 0.45 (Table 1). This finding indicates that the CSF possesses a potential bioactive compound(s) responsible for the cytotoxic activity. The potential antioxidant activity and the presence of high amount of phenolic compounds may be the possible reason behind the strong cytotoxic activities of CSF fraction of *T. bracteolatum.*

The membrane stabilizing activity is likely to hamper the inflammatory response and thus expected to exhibit anti-inflammatory activity. Even though, in both the cases the results were lower but promising when compared to the standards but the different fractionates were lower compared the standard Streptokinase. One of the main factors responsible for the inflammatory response in the body is the release of the cytokines which increase the migration of WBC's and further contribute to the inflammatory cascade. The lysosomal membrane storing the cytokines and other inflammatory mediators, can be compared to the membrane of an erythrocyte [48]. Stabilization of this membrane could be an important factor in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil such as bactericidal enzymes and proteases, which cause further tissue inflammation and damage upon extra cellular release [49].

The CSF and MESF fractionates exhibited promising thrombolytic activity which may be valuable for the discovery of new drugs in the management of cardiovascular diseases [50]. In case of both the hypotonic solution and heat-induced hemolysis the CSF showed the potential membrane stabilizing activities (Fig. 1).

Diarrhea refers to the excess passage of watery stools resulting due to the decrease in consistency or increased frequency of the movement of the bowl. Castor oil initiates diarrhea by stimulating intestinal motility and secretory processes [51] and induction of diarrhea by castor oil is a common method that is being followed in this current study. The methanol extract of the

plant at dose 200 and 400 mg/kg showed significant inhibition of diarrhea by 80.78% ($p<0.001$)and 87.18% ($p<0.001$) respectively whereas loperamide (3 mg/kg) was used as the positive control which inhibited diarrhea by 82.05%. This dose dependent inhibition of diarrheal stool indicates the presence of bioactive compounds capable of antidiarrheal activity.

Diabetes is a diseased condition caused when the body's ability to process blood glucose is impaired due to either lack of secretion or action of insulin [52]. Antihyperglycemic agents decrease blood glucose level either by increasing glucose uptake or by potentiating insulin secretion from the pancreas [53]. The crude extract of the plant showed significant ($p<0.001$) time dependent hypoglycemic activity at both doses (200 and 400 mg/kg) The extract at two different doses reduced the blood glucose level by 71.74% and 69.93% when compared to the control and the standard glibenclamide showed 72.4% inhibition. This result indicates that the crude extract of the plant *T. bracteolatum* may be a potential source of anti-hyperglycemic agents.

The CNS stimulating activity of the crude methanol extract of the plant (METB) was also promising. The extract at the dose of 200 mg/kg potently delayed the sleeping time of experimental rats (54.67±15.2 min vs. 10.00±0.0) (Table 4).

In the current study the crude extract of the plant showed significant ($p<0.001$) dose dependent inhibition of acetic acid induced writhing *in vivo.* However the results are lower compared to the NSAID diclofenac sodium. The writhing response initiated by the acetic acid indicates that it may cause the release of inflammatory mediators like prostaglandins, prostacyclines etc. which are responsible for the sensitization of the nerve fibres resulting in pain sensations [54]. Inhibition of the writhing response is an indication that the plant extract of *T. bracteolatum* may produce non-narcotic analgesic activity due to the inhibition of prostaglandin synthesis by blocking of lipooxygenase and cyclooxygenase activities.

The crude extract of *T. bracteolatum* showed dose dependent inhibition of analgesic activity both centrally and peripherally. The 'tail flick' method for the central and the 'writhing technique' for the peripheral analgesic activity are very useful techniques for the evaluation of analgesic activity of any plant extract [55]. The central analgesic action is mediated via inhibition of central pain receptors [56-58]. It is now evident that μ, κ3, d and d2 are the opioid receptor sub-types primarily responsible for the supra-spinally mediated analgesic action of opiates and spinal analgesia appears to be mediated through μ2, d2 and κ1 receptors [58-59]. Opiates such as morphine and its derivatives (plant origin) exert their analgesic activity by interacting with various receptors both at spinal and supraspinal sites. Morphine at a dose of 2 mg/kg.b.wt. caused significant analgesic effect in the tail flick method and the effect became more prominent as time increased. Similar effects were recorded in case of the methanolic extracts of the plant. On the other hand, the peripheral analgesic effect is generally mediated through inhibition of cyclooxygenase and/or lipoxygenase and other inflammatory mediators) or inhibition of pain responses mediated by nociceptors peripherally [59-60]. Therefore, it is possible that the methanolic extract of this plant may show the analgesic effect through these mechanisms. Both the central and peripheral analgesic effect increased with increase in dose and duration of action in the mouse model. Even though the chemical(s) responsible for this analgesic activity and the mechanism of action (interactions with receptors or other mediators) is still unknown, the promising result demands further chemical investigation to figure out the active principle(s) responsible for the analgesic activity.

Conclusions

The aim of the current study was to assess the traditional use of *T. bracteolatum* using various bioassays. The findings of the current study demonstrated that CSF is the potential candidate

which is responsible for the major bioactivities of the plant including cytotoxicity, membrane stabilizing, and thrombolytic activities in case of *in vitro* assays. The methanolic crude extract showed strong antidiabetic, antidiarrheal, CNS stimulating and analgesic activities. Our results for the first time provided the comprehensive scientific evidences that the reason behind the popularity of this plant concoctions for pain management is due to the presence of phytoconstituents with analgesic and anti-inflammatory activity. However, further investigations for the identification and isolation of bioactive phytochemicals responsible for those activities should be addressed including the re-evaluation of those compounds *in vitro* and *in vivo* Pharmacogical studies for the discovery of new drug molecules from the plant.

List of abbreviations

MESF: Methanol extract soluble fraction; PESF: Petroleum ether soluble fraction; CSF: Chloroform soluble fraction; DCMSF: Dichloromethane soluble fraction; AQSF: Aqueous soluble fraction; VS: Vincristine sulfate; BHT: Butylated hydroxytoluene; GAE: Gallic acid equivalent; DPPH: 1, 1-diphenyl-2-picrylhydrazyl; DMSO: Dimethyl sulfoxide; *T. bracteolatum*: *Tetrastigma bracteolatum.*

Declarations

Ethics approval and consent to participate

The experimental procedures on laboratory animals were approved by the Animal Ethical Committee of Pharmacy Department, State University of Bangladesh, Bangladesh

Consent of publication

All the authors of this manuscript have consented to publish the article and they don't have any conflict of interest on this article.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

All data generated or analyzed during this study have been included in the article and its supplementary files.

Competing interests

The authors declare that they have no competing interests on the research work and to publish the article.

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Authors' contributions

TA and MMRS designed the study. FAF, MSK and MSI carried out the experiments. TA, FAF, MSK and MSI analyzed the data. TA and MMRS were the major contributors in writing the manuscript. INM and LCM critically revised the draft, specially for important intellectual contents, and contributed in the data analysis as well. All the authors read the manuscript and agreed to be accountable for all aspects of the work and approved the final manuscript.

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Figure Legends

Figure 1: Membrane stabilizing activity of the different extractives of *Tetrastigma bracteolatum*

Figure 2: Thrombolytic activity of the different extractives of *Tetrastigma bracteolatum*

Table 2. Effects of *Tetrastigma bracteolatum* crude methanol extract on castor oil-induced diarrhea in mice

Group	n	Number of stools after 4 h (mean±SEM)	Inhibition of defecation (%)
Control (1% tween-80)	5	26.0±0.58	-
Loperamide(std.) (3 mg/kg)	5	4.67±0.67***	82.05%
MESF (200 mg/kg)	5	5.0±1.53***	80.78%
MESF (400 mg/kg)	5	3.33±0.88***	87.18%

Here, *p < 0.05, **p < 0.01 and ***p < 0.001 when compared with the control group. SEM: Standard error of mean, MESF: Methanol soluble fraction, n: Sample size

Table 3. Effects of *Tetrastigma bracteolatum* crude methanol extract on the oral glucose tolerance test (hypoglycemic activity) in mice

Group	n	Blood glucose level (mean±SEM)					% inhibition	
		Fasting	30 min	60 min	120 min	180 min	120 min	180 min
Control (1% tween-80)	5	4.33±0.82	20.97±1.88	14.57±5.14	15.73±0.03	14.40±0.35	-	-
Glibenclamide (std.)(5 mg/kg)	5	4.13±0.20	12.13±3.03	6.57±0.69*	5.03±1.76**	3.97±0.09***	68.02%	72.4%
MESF(200 mg/kg)	5	4.87±0.27	10.53±1.16**	6.40±1.39*	5.40±0.56***	4.07±0.69***	65.67%	71.74%
MESF(400 mg/kg)	5	5.70±0.57	12.6±2.14*	5.67±0.93*	5.87±0.23***	4.33±0.62***	62.68%	69.93%

Here, *p < 0.05, **p < 0.01 and ***p < 0.001 when compared with the control group. SEM: Standard error of mean, MESF: Methanol soluble fraction, n: Sample size

Table 4. Effects of *Tetrastigma bracteolatum* crude methanol extract on Thiopental sodium-induced sleep in mice (CNS stimulant activity)

Groups	n	Time of onset of sleep (minutes)	Total sleeping time (minutes)
Control (1% tween-80)	5	10±0.0	173.33±3.48
MESF (200 mg/kg)	5	54.67±15.19*	137.33±34.68
MESF (400 mg/kg)	5	37.67±10.11	138.35±14.52

Here, *p < 0.05, **p < 0.01 and ***p < 0.001 when compared with the control group. SEM: Standard error of mean, MESF: Methanol soluble fraction, n: Sample size

Table 5. Effects of *Tetrastigma bracteolatum* crude methanol extract on tail flicking time of mice (Central analgesic activity)

Group	n	Latency period				% Elongation		
		0 min	30 min	60 min	90 min	30 min	60 min	90 min
Control (1% tween-80)	5	2.38±0.11	1.97±0.08	2.32±0.14	2.32±0.14	-	-	-
Morphine (2 mg/kg)	5	2.34±0.01	7.74±0.29***	10.07±0.33***	10.07±0.33***	292.89	334.05	334.05
MESF(200 mg/kg)	5	2.25±0.12	3.58±0.27**	5.50±0.33***	8.71±0.37***	81.73	137.07	275.43
MESF(400 mg/kg)	5	2.26±0.18	3.00±0.32*	6.55±0.01***	9.24±0.10***	52.28	182.33	298.28

Here, *p < 0.05, **p < 0.01 and ***p < 0.001 when compared with the control group. SEM: Standard error of mean, MESF: Methanol soluble fraction, n: Sample size

Table 6. Effects of *Tetrastigma bracteolatum* crude methanol extract on acetic acid-induced writhing in mice (Peripheral analgesic activity)

Groups	n	Number of writhing by 10 minutes	Inhibition of writhing (%)
Control (1% tween-80)	5	20.67±3.48	-
Diclofenac sodium (Std.) (50 mg/kg)	5	4.67±0.33***	77.42
MESF (200 mg/kg)	5	8.67±0.67***	58.06
MESF (400 mg/kg)	5	6.67±0.33***	67.74
Here, *p < 0.05, **p < 0.01 and ***p < 0.001 when compared with the control group. SEM: Standard error means, MESF: Methanol soluble fraction, n: Sample size			

Fig. 1

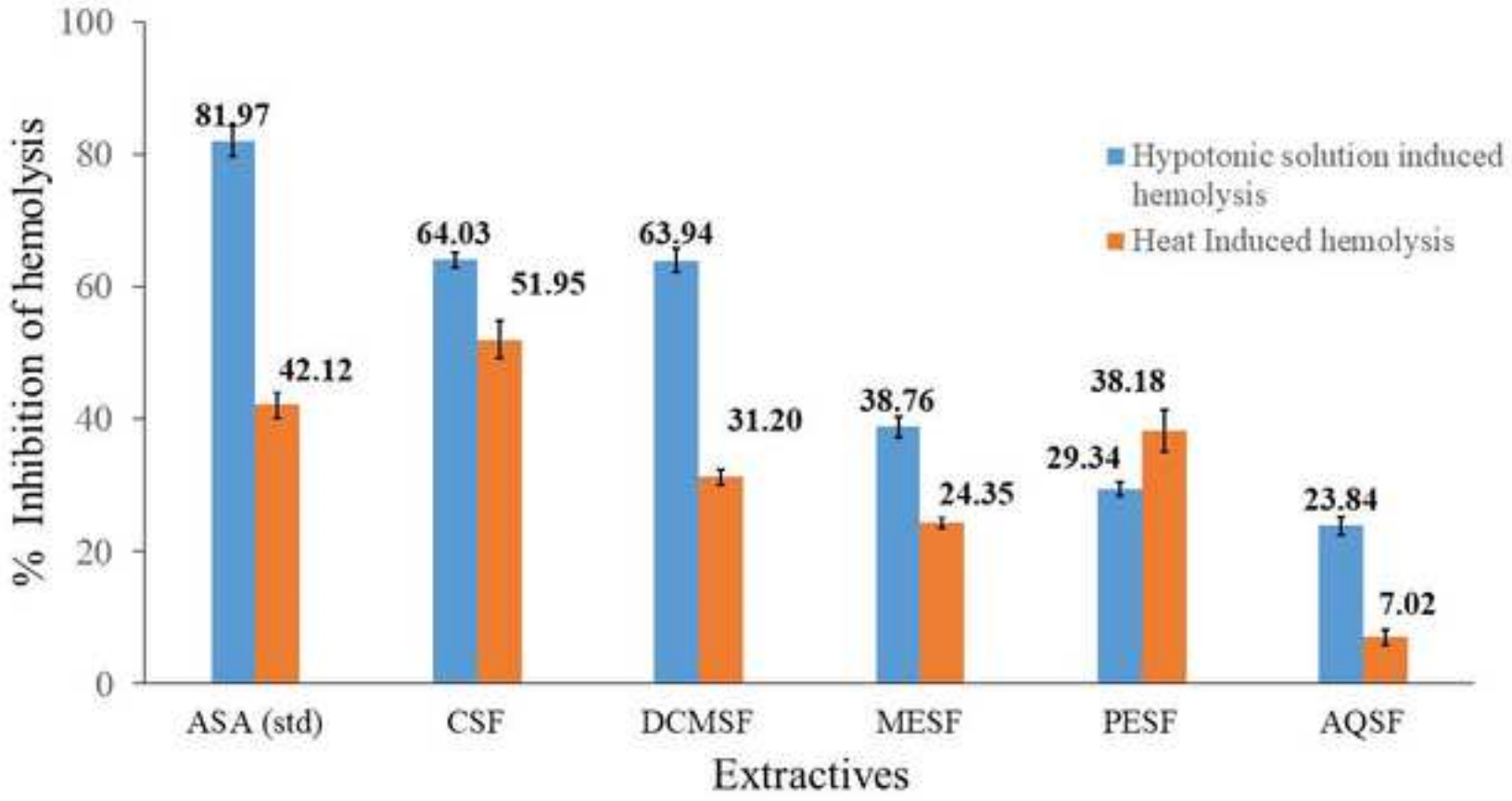
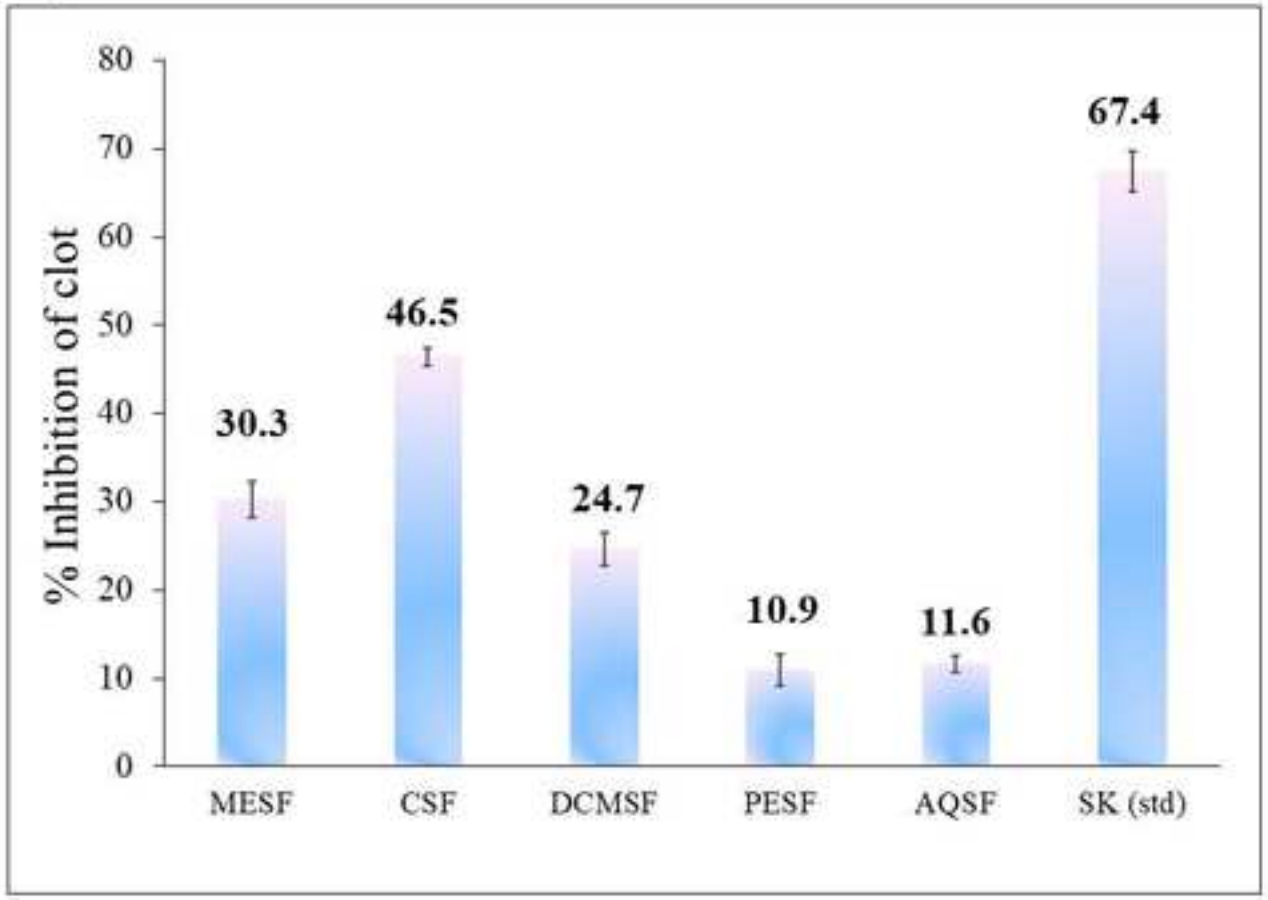


Fig. 2



Cover letter