

Phytochemical Screening and Anti-nociceptive Properties of the Ethanolic Leaf Extract of *Trema Cannabina* Lour

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ARTICLE INFO

Article Type:

Research Article

Article History:

Received: 22 September 2012

Accepted: 22 October 2012

ePublished: 7 February 2013

Keywords:

Trema cannabina

Anti-nociceptive

Hot plate test

Tail immersion test

Acetic acid induced writhing test

ABSTRACT

Purpose: The present study was designed to investigate the anti-nociceptive activity of ethanolic leaf extract of *Trema cannabina* Lour (family: Cannabaceae) in experimental animal models. **Methods:** The anti-nociceptive action was carried out against two types of noxious stimuli, thermal (hot plate and tail immersion tests) and chemical (acetic acid-induced writhing) in mice. **Results:** Phytochemical analysis of crude extract indicated the presence of reducing sugar, tannins, steroid and alkaloid types of secondary metabolites. Crude extract of *T. cannabina* (500 mg/kg dose) showed maximum time needed for the response against thermal stimuli (6.79 ± 0.15 seconds) which is comparable to diclofenac sodium (8.26 ± 0.14 seconds) in the hot plate test. Hot tail immersion test also showed similar results as in hot plate test. At the dose of 250 and 500 mg/kg body weight, the extract showed significantly and in a dose-dependent ($p < 0.001$) reduction in acetic acid induced writhing in mice with a maximum effect of 47.56% reduction at 500 mg/kg dose comparable to that of diclofenac sodium (67.07%) at 25 mg/kg. **Conclusion:** The obtained results tend to suggest the Anti-nociceptive activity of ethanolic leaf extract of *Trema cannabina* and thus provide the scientific basis for the traditional uses of this plant part as a remedy for pain.

Introduction

Bangladesh possesses rich floristic wealth and diversified genetic resources of medicinal plants. It has a widely ranging tropical and the agro climatic conditions, which are conducive for introducing and domesticating new and exotic plant varieties. The use of plant extracts and pure compounds isolated from natural sources provided the foundation to modern pharmaceutical compounds. *Trema cannabina* is one of the common medicinal plants grown in India and almost all districts of Bangladesh.

Trema cannabina (*T. cannabina*) is a tree of Cannabaceae family. The plant is used in traditional folk remedy by the rural people and shows various pharmacological activities.¹ The root of the plant is used in the treatment of asthma, diarrhoea and passing of blood in urine; the bark is used as poultice in muscular pain; the roots, barks and leaves are used in epilepsy.^{1,2} In Africa, this plant is used in various diseases including, hypertension and dysentery.³ The leaves are used to treat cough and sore throats and bark are used to make cough syrups in traditional medicine. It has been also reported to be used in bronchitis, gonorrhoea, malaria, yellow fever, toothache and intestinal worms.^{1,4}

Since no literature is currently available to substantiate Anti-nociceptive property from ethanolic extract of *T. cannabina*, therefore the present study was designed to provide scientific evidence for its use as a traditional folk remedy by investigating the Anti-nociceptive activity that also confirms its use as pain killer.

Materials and Methods

Collection and Identification of Plant Materials

The plant selected for present work was the leaves of *T. cannabina*, which were collected from Khulna, Bangladesh in March, 2011 at the daytime. The leaves were collected from the fresh plants. The samples were identified by Sarder Nasir Uddin, Senior Scientific Officer, Bangladesh National Herbarium, Mirpur, Dhaka, Bangladesh. A voucher specimen (DACB: 35921) has been deposited in the Herbarium for further reference.

Preparation of Ethanolic Extract

The leaves of *T. cannabina* were freed from any of the foreign materials. Then the leaves were air-dried under shed temperature followed by drying in an electric oven at 40 °C. The dried plant materials were then ground

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into powder. About 200 g of powdered material was taken in a clean, flat-bottomed glass container and soaked in 800 ml of 95% ethanol. The container with its contents was sealed and kept for a period of 7 days accompanying occasional shaking and stirring. The whole mixture then underwent a coarse filtration by a piece of clean, white cotton material. Then it was filtered through whatman filter paper (Bibby RE200, Sterilin Ltd., UK) which was concentrated with rotary evaporator at bath temperature not exceeding 40 °C to have gummy concentrate of extract (yield approx. 5.77%).

Chemicals

Gallic acid, Folin-ciocalteu phenol reagent and atropine were obtained from Sigma Chemical Co. [(St. Louis, MO, USA)]. Bromocresol green, Phosphate buffer, Sodium hydroxide, hydrochloric acid, sodium carbonate, ethanol and chloroform were of analytical grade and purchased from Merck (Darmstadt, Germany).

Test Animals and Drug

Swiss albino mice of either sex and male rats of Wister strain weighing between 20-25 g and 175-202 g respectively were used for in-vivo pharmacological screening. The mice and rats were collected from the animal research branch of the International Center for Diarrhoeal Disease and Research, Bangladesh (ICDDR, B). The animals were housed under standard laboratory (at Pharmacology Laboratory of BCSIR, Chittagong) conditions maintained at 25±1 °C and under 12/12 h light/dark cycle and feed with Balanced Trusty Chunks and water ad libitum (Chatterjee 1993) during acclimatization period. The animals were acclimatized to this laboratory condition for a period of 14 days prior to performing the experiments. The animals were fasted overnight before the experiments. All the experimental animals were treated following the Ethical Principles and Guidelines for Scientific Experiments on Animals (1995) formulated by The Swiss Academy of Medical Sciences and the Swiss Academy of Sciences. All experimental protocols were approved by the BCSIR Ethics Committee.

The standard drug diclofenac Na used for this study and purchased from Square Pharmaceuticals Ltd, Bangladesh.

Phytochemical Screening

The freshly prepared crude extract was qualitatively tested for the presence of chemical constituents, by using the following reagents and chemicals, for example, alkaloids were identified by the Dragendorff's reagent, flavonoids with the use of Mg and HCl, tannins with ferric chloride and potassium dichromate solutions, steroids with Libermann-Burchard reagent and reducing sugars with Benedict's reagent.⁵⁻⁷

Quantification of Total Phenolic Content

The total phenolic content of the extract was determined by the modified Folin-Ciocalteu method.⁸ 1.0 ml of extract (1 mg/ml) was mixed with 5 ml of Folin-Ciocalteu reagent (1:10 v/v distilled water) and 4 ml (75 g/l) of sodium carbonate solution. The mixture was then allowed to stand for 30 min at 40 °C for color development. The absorbance was read at 765 nm against blank using a double beam UV/Visible spectrophotometer (Analykjena, Model 205, Jena, Germany). Total phenolic content was determined as mg/g of gallic acid equivalent using the equation obtained from a standard gallic acid calibration curve $y=6.2548x - 0.0925$, $R^2=0.9962$.

Quantification of Total Alkaloid Content

Total alkaloid content was determined by the Fazel *et al.*, method.⁹ The plant extract (1mg/ml) was dissolved in 2 N HCl and then filtered. The pH of phosphate buffer solution was adjusted to neutral with 0.1 N NaOH. One ml of this solution was transferred to a separating funnel and then 5 ml of bromocresol green solution along with 5 ml of phosphate buffer were added. The mixture was shaken and the complex formed was extracted with chloroform by vigorous shaking. The extracts were collected in a 10 ml volumetric flask and diluted to volume with chloroform. The absorbance of the complex in chloroform was measured at 470 nm. Atropine was used as reference standard for this study. All experiments were performed thrice; the results were averaged and reported in the form of mean ± S.E.M.

Anti-nociceptive Activity

Hot Plate Test

Albino mice were placed in aluminum hot plate kept at a temperature of 55±0.5 °C for a maximum time of 10 second.¹⁰ The animals were divided into control, positive control and test groups with five mice in each group. The animals of test groups received test substance at the dose of 250 and 500 mg/kg body weight. Positive control group was administered with diclofenac Na (standard drug) at the dose of 25 mg/kg body weight and vehicle control group was treated with 1% tween 80 in water at the dose of 10 ml/kg body weight. Reaction time was recorded when animals licked their forepaws and jumped at before and at 0, 15, 30 and 45 min followed by oral administration of control and crude extract. Diclofenac sodium (25 mg/kg) was administered intra-peritoneally.

Tail Immersion Test

Anti-nociceptive effect of the test substances was determined by the tail immersion test method described by Sewell and Spencer.¹¹ Mice were treated with diclofenac sodium (25 mg/kg) and two doses of the crude extract (250 and 500 mg/kg). One to two centimeter of the tail of mice was immersed in warm water kept constant at 50 °C. The reaction time was the

time taken by the mice to deflect their tails. The first reading is discarded and the reaction time was taken as a mean of the next two readings. The latent period of the tail-flick response was taken as the index of antinociception and was determined at 0, 30 and 60 min after the administration of drugs. The maximum reaction time was fixed at 10 seconds.

Acetic Acid-Induced Writhing Test

The Anti-nociceptive activity of the crude ethanolic leaf extract of *T. cannabina* was studied using acetic acid induced writhing model in mice.^{12,13} The animals were divided into control, positive control and test groups with five mice in each group. The animals of test groups received test substance at the dose of 250 and 500 mg/kg body weight. Positive control group was administered with diclofenac Na (standard drug) at the dose of 25 mg/kg body weight and vehicle control group was treated with 1% tween 80 in water at the dose of 10 ml/kg body weight. Test samples, standard

drug and control vehicle were administered orally 30 min before intra-peritoneal administration of 0.7% acetic acid. After an interval of 15 min, the mice were observed writhing (constriction of abdomen, turning of trunk and extension of hind legs) for 5 min.

Statistical analysis

The results are presented as mean±SEM. The statistical analysis of the results was performed using one way analysis of variance (ANOVA) followed by Dunnett's test using SPSS 11.5 software. Differences between groups were considered significant at a level of $p < 0.05$.

Results

Chemical Group Test

Results of different chemical tests on the ethanolic extract of leaves of *T. cannabina* showed the presence of alkaloids, reducing sugars, tannins and steroids (Table 1).

Table 1. Results of different group tests of ethanolic extract of leaves of *T. cannabina*

Serial No.	Chemical Constituents	Test	Extract	Result
1.	Test for Reducing Sugar	Benedict's Test	Ethanolic	+
		Fehling's Test	Ethanolic	+
		Alpha Naphthol Solution Test	Ethanolic	+
2.	Test for Tannins	Ferric Chloride Test	Ethanolic	++
		Potassium dichromate Test	Ethanolic	+
3.	Test for Flavonoids	Hydrochloric Acid Test	Ethanolic	-
4.	Test for Saponins	Foam Test	Ethanolic	-
5.	Test for Gums	Molisch Test	Ethanolic	-
6.	Test for Steroids	Liebermann-Burchard Test	Ethanolic	+
		Sulphuric acid Test	Ethanolic	+
7.	Test for Alkaloids	Mayer's Test	Ethanolic	+
		Wagner's Test	Ethanolic	+
		Dragendroff's Test	Ethanolic	+
		Hager's Test	Ethanolic	+

+: Positive result; -: Negative result; ++: significantly positive

Total Phenolic & Alkaloid Content

The amount of total phenolic content was calculated as quite high in the ethanolic crude extract of *T. cannabina* (152.37±1.32 mg/g of gallic acid equivalent). The alkaloid content was also found high in ethanolic extract (28.59±0.73 mg/gm) (Table 2).

Table 2. Total phenolic and alkaloid content of *T. cannabina* extracts.

Extract	Total phenolic content (mg/gm of gallic acid equivalent)	Total alkaloid content (mg/gm)
Ethanol extract of <i>T. cannabina</i>	152.37±1.32	28.59±0.73
The values are expressed as mean ± SEM (n=3)		

Analgesic Activity

Hot Plate Test

Two doses of ethanolic extract of leaves of *T. cannabina* increased the reaction time in a dose dependent manner to the thermal stimulus which was summarized in Table 3. The highest nociceptive inhibition of thermal stimulus was exhibited at a higher dose 500 mg/kg of crude extract which has maximum time needed for the response against thermal stimuli (6.79±0.15 seconds) which is comparable to diclofenac sodium (8.26±0.14 seconds) and found statistically significant ($p < 0.001$).

Tail Immersion Test

The Anti-nociceptive activity of *T. cannabina* and diclofenac Na demonstrated in tail immersion test are given in Table 4. *T. cannabina* at all two doses (250 and 500 mg/kg) significantly increased the latency period to hot-water induced thermal stimuli ($p < 0.001$)

in a dose-dependent manner. The highest nociceptive inhibition of thermal stimulus was exhibited at a higher dose 500 mg/kg of crude extract (8.14 ± 0.11 seconds) comparable to diclofenac sodium (8.17 ± 0.11 seconds) and was also statistically significant ($p < 0.001$).

Table 3. Effect of ethanolic extract of leaves of *T. cannabina* on hot plate test in mice.

Treatment	Dose (mg/kg, p.o.)	Response Time (sec)			
		0 min (Latency)	15 min	30 min	45 min
Control (1% aq. Tween 80)	10 ml/ kg	1.80±0.08	2.56±0.25	2.96±0.21	3.13±0.16
Diclofenac-Na	25	2.08±0.06	5.84±0.31*	6.75±0.17*	8.26±0.14*
Et. of leaves of <i>T. cannabina</i>	250	1.96±0.04	3.92±0.16*	4.11±0.10*	4.90±0.28*
	500	2.02±0.09	5.19±0.21*	6.20±0.21*	6.79±0.15*

Values are expressed as mean±SEM (Standard Error Mean); Et.:Ethanolic; *indicates $P < 0.001$; one-way ANOVA followed by Dennett's test as compared to control; p.o.: per oral

Table 4. Effect of ethanolic leaf extract of *T. cannabina* on tail immersion test in mice

Treatment	Dose (mg/kg, p.o.)	Response Time (sec)		
		0 min	30 min	60 min
Control(1% aq. Tween 80)	10 ml/ kg	2.22±0.22	3.19±0.19	3.76±0.20
Diclofenac-Na	25	4.20±0.26*	7.89±0.16*	8.17±0.22*
Et. of leaves of <i>T. cannabina</i>	250	3.21±0.11*	4.89±0.17*	5.79±0.16*
	500	4.04±0.33*	7.07±0.35*	8.14±0.11*

Values are expressed as mean±SEM; Et.:Ethanolic; *indicates $P < 0.001$; one-way ANOVA followed by Dennett's test as compared to control; p.o.: per oral.

Acetic Acid-Induced Writhing Test

Table 5 showed the effect of the ethanolic extract of leaves of *T. cannabina* on acetic acid induced writhing in mice. At the dose of 250 mg/kg and 500 mg/kg of body weight, the extract produced 34.15% & 47.56 %

writhing inhibition in test animals respectively. The results were statistically significant ($p < 0.001$) and was comparable to the standard drug diclofenac Na, which showed 67.07% at a dose of 25 mg/kg weight.

Table 5. Effects of the ethanolic leaf extract of *T. cannabina* on acetic acid induced writhing of mice (n=5)

Group	Treatment and Dose	Number of writhes (% Writhing)	% Writhing Inhibition
Control	1% tween 80 solution 10 ml/kg, p.o.	16.4± 1.72 (100)	---
Positive Control	Diclofenac Na 25 mg/kg, p.o.	5.4 ± 0.68 * (32.93)	67.07
Test Group- 1	Et. Extract of <i>T. cannabina</i> 250 mg/kg, p.o.	10.80± 0.58 * (65.85)	34.15
Test group- 2	Et. Extract of <i>T. cannabina</i> 500 mg/kg, p.o.	8.60 ± 0.68 * (52.44)	47.56

Values are expressed as mean ± SEM; Et.:Ethanolic; * indicates $P < 0.001$; one-way ANOVA followed by Dennett's test as compared to control; n = Number of mice; p.o.: per oral

Discussion

A number of natural products are used in various traditional medical systems to treat relief of symptoms from pain. The crude extracts of leaves of *T. cannabina* demonstrated significant anti-nociceptive activity at

two different dose levels in various animal models of pain. Acetic acid-induced writhing response elucidated peripheral activity, while the hot plate tests, hot tail flick test investigated both peripheral and central activity.^{14,15} Nociceptive reaction towards thermal

stimuli in hot plate test and tail immersion in hot water test using mice is a well-validated model for the detection of opiate analgesic as well as several types of analgesic drugs from spinal origin.^{11,16} Nociceptive pain inhibition was noticed highest in both the test at 45 minutes after administration of the extracts and the response time is increased from 2.02 seconds to 6.79 seconds in hot plate test at dose 500 mg/kg while it was also increased from 4.04 seconds to 8.14 seconds in tail flick test at the same dose level. Other doses used in this study also increases the latent period significantly with the time being in both tests. Acetic acid-induced writhing test has been used as a model of chemo-nociceptive induced pain, which increases PGE2 and PGF2a peripherally. The crude ethanolic extract of leaves of *T. cannabina* showed significant reduction of abdominal contraction in mice. Local peritoneal receptors were postulated to be partly involved in the abdominal constriction (writhing) response.^{11,17} The method has been associated with prostanoids in general, i.e. increased levels of PGE2 and PGF2a in peritoneal fluids¹⁷ as well as lipoxygenase products by some researchers.¹⁵ In the present study, the reduction of the Anti-nociceptive process obtained within the first hour is probably related to reduction in the release of preformed inflammatory agents, rather than to a reduced synthesis of the inflammatory mediators by inhibition of cyclooxygenases and/or lipoxygenases (and other inflammatory mediators). Thus the anti-nociceptive activity shown by crude extracts of leaves of *T. cannabina* in hot plate, hot tail-flick and acetic acid induced writhing test indicate that ethanolic extract of the plant might possess centrally and peripherally mediated Anti-nociceptive properties. Furthermore, phytochemical screening of the ethanol extract of *F. hispida* reveals the presence of steroid, reducing sugars, alkaloid and significant amount of phenolic compounds (tannins). Inhibition of pain is associating with presence of steroidal constituents.¹⁸ Tannins also play a role in Anti-nociceptive and anti-inflammatory activities in some studies.¹⁹ Because tannins inhibit prostaglandin synthesis by modifying the production of cyclooxygenase (cox-1 and cox-2) and lipoxygenase involved in the prostaglandin synthesis.^{20,21} Besides, alkaloids are well known for their ability to inhibit pain perception.²² So these phyto-constituents might be responsible for its Anti-nociceptive activity.

Conclusion

In conclusion, it can be revealed that the crude ethanolic leaf extract of *T. cannabina* plant possess significant Anti-nociceptive activity. The potential of the extract of *T. cannabina* as Anti-nociceptive agent may be due to the presence of phyto-constituents like tannins, alkaloids etc and might be responsible for its activity and justify its use as a traditional folk remedy. However, a more extensive study is necessary to

determine the exact mechanism(s) of action of the extract and its active compound(s).

Acknowledgements

The authors are thankful to Prof. Dr. Samir Kumar Sadhu, Pharmacy Discipline, Khulna University; Dr. Jamil Ahmed Shilpi, Associate professor, Pharmacy Discipline, Khulna University; Dr. Mahiuddin Alamgir, Research Scientist, National Measurement institute (NMI), Australia, for their encouragement during the research time. All the informants of the study area are cordially acknowledged for their valuable cooperation.

Conflict of Interest

There is no conflict of interest in this study.

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