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RESEARCH ARTICLE

Pharmacological studies on the antinociceptive, anxiolytic and antidepressant activity of *Tinospora crispa*

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Pharmacological studies were performed in mice on the methanol extract of *Tinospora crispa* (TC), and of its hexane (HF) and chloroform (CF) fractions. Significant antinociceptive activity was observed for TC, HF, and CF in the acetic acid-induced writhing and formalin-induced paw licking tests. Anxiolytic and antidepressant activities were assessed using the open field, hole board, and elevated plus maze (EPM) tests. TC, HF, and CF demonstrated a significant decrease in spontaneous locomotor activity. They also showed an increase in the number of head-dippings in the hole-board test, suggesting decreased fearfulness. TC, and most of its fractions, showed a significant increase of the time spent in the opened arm of the EPM, indicating reduced anxiety. This study provides some support to explain the traditional use of *T. crispa* as a remedy for pain.

KEYWORDS

antidepressant activity, antinociceptive activity, anxiolytic activity, *Tinospora crispa*

1 | INTRODUCTION

Tinospora crispa (L.) Hook. f. & Thomson (Menispermaceae) is an herbaceous vine with large heart-shaped leaves and small greenish yellow-colored flowers that grows commonly in the forests of South East Asia and Africa (Pathak, Jain, & Sharma, 1995). The plant is used in traditional medicine to treat a range of ailments, including to relieve inflammation, fever, and muscle pain (Khare, 2008; Kongsaktrakoon, Temsiririrkkul, Suvitayavat, Nakornchai, & Wongkrajang, 1984; Roosita, Kusharto, Sekiyama, Fachrurozi, & Ohtsuka, 2008). In Bangladesh, the plant is known as "Gulancha" (Ahmad, Jantan, & Bukhari, 2016). It is used as a tonic and blood purifier, and for intestinal disorders, jaundice, leprosy, fever, rheumatism, and pain (Ahmad et al., 2016; Yusuf, Chowdhury, Wahab, & Begum, 1994). *T. crispa* contains some flavonoids, sterols, terpenoids, alkaloids, and lignans. Its extracts and/or isolated compounds have demonstrated anti-inflammatory, immunomodulatory, cytotoxic, antioxidant, anti-nociceptive, antipyretic, hepatoprotective, antimalarial,

hypoglycaemic, antifilarial, and cardioprotective activities (Ahmad et al., 2016; Rakib et al., 2019). Another species of *Tinospora* (*T. cordifolia*) has been shown to exhibit sedative and anxiolytic effects (Barua et al., 2019; Mishra et al., 2016).

Pain is an unpleasant feeling—usually associated with tissue damage caused by noxious stimuli, inflammation, and disease processes—that can often be chronic (Hylands-White, Duarte, & Raphael, 2017; May et al., 2017). The management of pain mainly relies on the use of analgesics such as paracetamol, non-steroidal anti-inflammatory drugs (NSAIDs), and opioids, all of which present some significant adverse side effects (Carter et al., 2014). Other drugs, that can be prescribed, include adjuvants, such as anxiolytics that help reduce pain-related anxiety, and antidepressants that help to manage the depressive state encountered in chronic pain conditions and provide analgesia by interfering with the endogenous pain control mechanisms (Hylands-White et al., 2017). Unfortunately, many of the current anxiolytics and antidepressants exhibit some undesirable side effects too, which contribute to poor patient compliance (David & Gourion, 2016; Slee et al., 2019). There is a need to explore other sources, such as medicinal plants, for the discovery of new potential drug leads (Fajemiroye, Da Silva, De Oliveira, & Costa, 2016). This study was undertaken to assess the antinociceptive, anxiolytic, and antidepressant activity of the methanol extract of *T. crispa* (TC) and two of its fractions, namely the n-hexane fraction (NH) and the chloroform fraction (CF), in order to better understand the use of this plant in traditional medicine.

2 | MATERIALS AND METHODS

2.1 | Plant collection

The whole plant of *T. crispa* was collected at the mature stage from the Lawachara National Park, Moulavi Bazar, Bangladesh, in January 2018. The parts of the plant were cut into small pieces that were washed under tap water and then dried in the dark at 21–30°C for 15 days. The whole plant material was ground by a mechanical grinder and passed through a size of 60 mesh sieve to obtain a fine powder that was stored in an air-tight container.

2.2 | Preparation of extracts

The dried *T. crispa* plant powder (600 g) was macerated in methanol (4 L) for 15 days at room temperature with occasional shaking and stirring. Following filtration, first with a cotton plug then with a Whatman no. 1 filter paper, the filtrate was evaporated to dryness under vacuum at 40°C to obtain a concentrated extract (30.55 g dry weight, 5.09% w/w). The methanolic extract of *T. crispa* whole plant (TC) was further fractionated using n-hexane and chloroform to obtain two fractions of (HF) and (CF), respectively. All extracts, fractions, and standards used in the in vivo tests were suspended in normal saline using dimethyl sulfoxide (DMSO) at the highest concentration of 1 and 1% Tween-80.

2.3 | Chemicals and reagents

Methanol, *n*-hexane, chloroform, formalin, and acetic acid were purchased from Merck (Darmstadt, Germany). Diclofenac sodium and diazepam (DZP) were purchased from Eskayef Bangladesh Ltd. (Tongi, Bangladesh). Normal saline solution (0.9% NaCl) was obtained from Orion Infusion Ltd. (Tejgaon, Bangladesh). DMSO and Tween-80 were from BDH Chemicals (Leicestershire, UK). All other chemicals were of analytical grade and were obtained either from BDH Fluka Chemie GmbH (Buchs, Switzerland) or Merck (Darmstadt, Germany).

2.4 | Experimental animals

Swiss albino mice (weighing 25–30 g, aged 4–5 weeks), of either sex, were used throughout the study. They were collected from the animal laboratory at Jahangirnagar University, Dhaka-1342, Bangladesh. The animals were kept in groups of five in a controlled laboratory environment (12-hr dark/12-hr light cycle; temperature 25 ± 2°C) for 7 days for acclimatization. The animals were given standard feed and water *ad libitium*. The animals fasted overnight and were weighed before the experiment. The design and performance of the research study involving mice were permitted by the Ethical Review Committee of the Faculty of Biological Science, University of Chittagong through the submission of a research protocol before the study, and ethical permission (Pharmacol/DPH/UC/01, 2018) was obtained.

2.5 | Acute toxicity studies

This was accomplished according to the OECD guidelines (OECD Guidelines, 2002). The test animals were abstained overnight prior to the experiment and continued under standard laboratory conditions. The animals were randomly chosen and divided into groups (n = 5) which were orally administered different increasing the doses (up to 2,000 mg/kg) of TC, HF, and CF. The control group received 1% Tween 80 in water (p.o.). The animals were kept under observation for 24 hr to record the general signs and symptoms of toxicity. The mortality rate was recorded for each group at the end of this period.

2.6 | Antinociceptive activity

2.6.1 | Acetic acid-induced writhing test

Peripheral analgesic activity was studied by observing the writhing response (i.e., contractions of the abdominal muscles and stretching of the hind limbs) of mice following the intraperitoneal administration of acetic acid (Koster, 1959). Forty experimental mice were randomly selected and divided into eight groups (n = 5). Normal saline with 1% DMSO (0.1 ml/10 g body weight, negative control), diclofenac sodium (100 mg/kg, positive control), TC, HF, and CF (at doses of 200 and 400 mg/kg) were administered *per os* (p.o.) to the animals 40 min prior

to the intraperitoneal administration of acetic acid (0.7%, 0.1 ml/10 g body weight). The 40-min interval was set to ensure the proper absorption of the administered samples. Five minutes after the administration of acetic acid, each animal was isolated in an individual observation chamber, and the cumulative number of writhing responses was recorded for 10 min. The percentage inhibition of writhing in comparison to the negative control group was taken as an index of analgesia and was calculated using the following formula:

$$\text{Inhibition}\,(\%) = \{(W_c - W_t)/W_c\} \times 100,$$

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

2.6.2 | Formalin-induced paw licking test

This was carried out according to a previous methodology (Okokon & Nwafor, 2010). Animals were divided into eight groups (five mice per group). A 2.5% formalin solution in saline (20 μl) was injected subcutaneously to a hind paw of mice 30 min after the subcutaneous administration of diclofenac sodium (10 mg/kg) (Positive control, Group 2), and of TC, HF, and CF (200 and 400 mg/kg, p.o.) (Test Group 3 to 10). The negative control group (Group 1) received only the 2.5% solution of formalin in normal saline (20 μl). The time spent licking and biting the injected paw was taken as an indicator of the response to pain, and the data were expressed as the total licking time in the early phase (0–5 min) and the late phase (15–30 min) after formalin injection. The percentage of inhibition of pain was determined using the following equation for early- and late-phase pain:

$$\text{Inhibition}\,(\%) = \{(TL_c - TL_t)/TL_c\} \times 100,$$

where TL_c is the average time spent licking in the control group, and TL_c is the average time spent licking in the test groups.

2.7 | Anxiolytic and antidepressant activity

2.7.1 | Open field test

This was conducted to evaluate the spontaneous locomotor activity of mice using an open field apparatus (Rauniar, Deo, & Bhattacharya, 2007). Forty animals were divided into eight groups (n = 5). The negative control group received a 1% Tween-80 solution in water (10 ml/kg; p.o). The test group was administered TC, HF, and CF (200 and 400 mg/kg, p.o.) and the positive control group received diazepam (1 mg/kg, i.p.). The mice were placed inside a box (50 cm × 50 cm × 40 cm h) with a floor separated into a series of lines forming 25 squares consecutively colored black and white. The number of crossed lines passed by each animal was counted for 3 min at 0, 30, 60, 90, 120 min during the study period. Different groups were used for different evaluation times. The percentage inhibition of movements was calculated as;

$$\text{Inhibition}\,(\%) = \{(M_c - M_t)/M_c\} \times 100,$$

where M_c is the average number of movements in the control group, and M_t is the average number of movements in the test groups.

2.7.2 | Hole board test

The study was conducted using a wooden board, measuring 20 cm by 40 cm with 16 evenly spaced holes (Sonavane, Sarveiya, Kasture, & Kasture, 2002). The animals were randomly divided into eight groups (five mice per group). The control group was treated with 1% Tween 80 (10 ml/kg, p.o.). The examined mice were treated with TC, HF, and CF (200 and 400 mg/kg), while those in the positive control group received diazepam (1 mg/kg). Thirty minutes after the treatment, the mice were placed singly on the board, and the number of times the mice dipped their head into the holes at the level of their eyes during a 5-min trial period was counted using a tally counter.

2.7.3 | Elevated plus maze test

The elevated plus maze (EPM) consisted of two opened arms (35 × 5 cm) crossed with two closed arms (35 × 20 cm). The arms were connected together with a central square (5 × 5 cm). The apparatus was elevated to a height of 25 cm in a dimly illuminated room. The animals, divided into 8 groups (n = 5 in each group), were treated with TC, HF, and CF (200 and 400 mg/kg, p.o.), and diazepam (1 mg/kg, i.p., positive control) or normal saline (negative control) 30 min before being placed individually in the center of the EPM, facing a closed arm. The time spent in both the opened and closed arms was recorded for 5 min (Pellow & File, 1986).

2.7.4 | Statistical analysis

The results obtained were expressed as mean ± *SEM* of five animals. For statistical analysis, one-way analysis of variance ANOVA followed by post-hoc Dunnett's test, was used to compare the test samples with the negative control. Effects were considered to be significant at the $p < .05$ level. The statistical analysis was carried out using SPSS v.16.0.

3 | RESULTS

3.1 | Acute toxicity studies

No lethal effects were observed within the 24 hr, following the administration of TC, HF, and CF, even at the highest dose of 2,000

TABLE 1 Antinociceptive activity of TC, HF, and CF in the acetic-acid induced writhing test in mice[a]

Group	Number of writhes (mean ± *SEM*)	% of inhibition of writhing
Control	28.73 ± 0.47	—
Standard (diclofenac)	9.98 ± 0.46***	65.27
TC (200 mg/kg)	13.94 ± 1.47**	51.48
TC (400 mg/kg)	12.96 ± 1.69**	54.89
HF (200 mg/kg)	15.35 ± 3.29**	46.57
HF (400 mg/kg)	12.56 ± 1.47**	56.28
CF (200 mg/kg)	20.56 ± 2.57	28.44
CF (400 mg/kg)	17.49 ± 2.78*	39.12

Abbreviations: CF, chloroform fraction; HF, hexane fraction; TC, methanolic extract of *T. crispa*.
[a]Values are mean ± *SEM* ($n = 5$).
*$p < .05$, **$p < .01$ and ***$p < .001$ vs. control (one-way ANOVA followed by Dunnett's test).

mg/kg. With that in mind, dose levels of 200 and 400 mg/kg were selected for the present study.

3.2 | Antinociceptive activity

3.2.1 | Acetic acid-induced writhing test

The methanol extract of *T. crispa* dose-dependently induced a significant ($p < .01$) decrease in the number of writhes with 51.48 and 54.89% of inhibition at doses of 200 and 400 mg/kg, respectively, compared to the negative control. This was comparable to the standard drug, diclofenac (65.27% inhibition). The hexane fraction (HF) derived from TC exhibited a comparable antinociceptive effect (56.28% inhibition) which was significant ($p < .01$) at a dose of 400 mg/kg (Table 1).

3.2.2 | Formalin-induced paw licking test

TC exhibited a substantial and significant inhibition of licking responses at both doses of 200 and 400 mg/kg compared to the negative control. At the dose of 400 mg/kg, it showed 57.37% inhibition in the early phase ($p < .001$) and 56.43% ($p < .01$) in the late phase. At a dose of 400 mg/kg, CF showed significant inhibition in the late phase (64.36%) that was comparable to the standard drug, diclofenac (69.45%) (Table 2).

3.3 | Anxiolytic and antidepressant activity

3.3.1 | Open field test

At doses of 200 and 400 mg/kg, TC demonstrated a dose-dependent and significant decrease in locomotion in the test animals during the experiment period, which was comparable to the effect of the standard drug, diazepam. Treatments with HF and CF at the same doses also induced a significant decrease in locomotion (Table 3).

3.3.2 | Hole board test

TC-treated mice showed a significant increase in the number of head-dipping compared to the negative control (46.05 and 83.51% at 200 and 400 mg/kg, respectively). The number of head-dipping also increased significantly ($p < .001$) in DZP-treated mice compared to the control, as did treatments with HF and CF at both doses. The highest significant increase (73.54%, $p < .01$) was observed for CF at 400 mg/kg (Table 4).

3.3.3 | Elevated plus maze (EPM) test

Diazepam significantly increased the time spent in the opened arm. Mice treated with TC, HF, and CF, at doses of 200 and 400 mg/kg,

TABLE 2 Effects of TC, HF, and CF on the formalin-induced paw licking in mice[a]

Group	Early phase (0–5 min)	% inhibition	Late phase (15–30 min)	% inhibition
Control	57.46 ± 1.38	—	41.32 ± 1.47	—
Standard (diclofenac)	14.68 ± 0.49***	74.37	13.37 ± 0.48***	69.45
TC (200 mg/kg)	35.35 ± 0.39**	35.32	26.91 ± 1.03**	36.57
TC (400 mg/kg)	25.47 ± 1.28***	57.37	18.34 ± 0.437**	56.43
HF (200 mg/kg)	39.85 ± 1.29*	31.47	29.37 ± 0.46**	29.47
HF (400 mg/kg)	34.95 ± 1.38*	40.36	24.57 ± 0.91***	42.67
CF (200 mg/kg)	39.36 ± 1.28*	32.47	20.34 ± 0.328*	51.46
CF (400 mg/kg)	33.23 ± 0.329**	42.58	15.57 ± 0.23**	64.36

Abbreviations: CF, chloroform fraction; HF, hexane fraction; TC, methanolic extract of *T. crispa*.
[a]Values are mean ± *SEM* ($n = 5$).
*$p < .05$, **$p < .01$ and ***$p < .001$ vs. control (one-way ANOVA followed by Dunnett's test).

TABLE 3 Locomotor activity TC, HF, and CF in the open field test in mice[a]

Group	Number of movements			
	0 min	30 min	60 min	90 min
Control	278.21 ± 4.85	284 ± 3.69	290.04 ± 4.20	298.56 ± 5.60
DZP standard	130.45 ± 5.62	125.65 ± 1.23***	119.68 ± 3.45***	115.56 ± 4.21***
TC (200 mg/kg)	152.46 ± 2.34	150.34 ± 1.45*	147.36 ± 1.34**	145.54 ± 0.32*
TC (400 mg/kg)	145.34 ± 1.39	143.46 ± 2.49**	142.56 ± 1.93**	140.36 ± 1.34***
HF (400 mg/kg)	149.34 ± 1.36	146.35 ± 1.64**	142.47 ± 1.47**	140.35 ± 1.73***
HF (200 mg/kg)	157.37 ± 2.74	153.57 ± 2.45**	150.36 ± 1.83*	147.35 ± 2.91*
CF (200 mg/kg)	155.79 ± 6.97	143.48 ± 2.65*	141.58 ± 3.15*	139.79 ± 5.26*
CF (400 mg/kg)	141.48 ± 8.12	135.58 ± 3.12**	133.78 ± 4.12**	122.84 ± 3.75**

Abbreviations: CF, chloroform fraction; HF, hexane fraction; TC, methanolic extract of *T. crispa*.
[a]Values are mean ± *SEM* (n = 5).
*p < .05, **p < .01 and ***p < .001 vs. control (one-way ANOVA followed by Dunnett's test).

TABLE 4 Activity of TC, HF, and CF on the exploratory behavior in the hole-board test in mice[a]

Group	Number of head-dipping	% increase
Control	29.1 ± 0.75	—
DZP standard (1 mg/kg)	66.8 ± 1.30***	127.15
TC (200 mg/kg)	42.5 ± 1.76*	46.05
TC (400 mg/kg)	53.4 ± 2.49**	83.51
HF (200 mg/kg)	33.6 ± 1.86**	15.46
HF (400 mg/kg)	44.9 ± 2.61***	54.30
CF (200 mg/kg)	36.4 ± 1.57*	25.09
CF (400 mg/kg)	50.5 ± 2.30**	73.54

Abbreviations: CF, chloroform fraction; HF, hexane fraction; TC, methanolic extract of *T. crispa*.
[a]Values are mean ± *SEM* (n = 5).
*p < .05, **p < .01 and ***p < .001 vs. control (one-way ANOVA followed by Dunnett's test).

TABLE 5 Anxiolytic activity of TC, HF, and CF in the EPM test in mice[a]

Group	Time spent in the closed arm	Time spent in the opened arm
Control	285.38 ± 1.47	10.17 ± 1.18
DZP Standard (1 mg/kg)	246.45 ± 2.457**	48.36 ± 1.55***
TC (200 mg/kg)	265.38 ± 3.26*	29.36 ± 2.35**
TC (400 mg/kg)	252.37 ± 2.47**	41.237 ± 2.34***
HF (200 mg/kg)	275.38 ± 2.47	19.34 ± 2.55*
HF (400 mg/kg)	263.36 ± 2.346*	30.56 ± 2.43**
CF (200 mg/kg)	270.456 ± 2.47*	25.36 ± 2.46**
CF (400 mg/kg)	260.35 ± 3.36*	34.36 ± 2.43***

Abbreviations: CF, chloroform fraction; HF, hexane fraction; TC, methanolic extract of *T. crispa*.
[a]Values are mean ± *SEM* (n = 5).
*p < .05, **p < .01 and ***p < .001 vs. control (one-way ANOVA followed by Dunnett's test).

also showed a trend toward increased time spent in these arms, although this did not reach significance for all treatments. The highest, and significant, time increase vs. the negative control was observed for CF at 200 mg/kg in the closed arm (p < .05) and TC at 400 mg/kg (p < .001) in the opened arms (Table 5).

4 | DISCUSSION

Many medicinal plants are employed in traditional medicine worldwide to relieve pain (Wirth, Hudgins, & Paice, 2005) and treat depression and anxiety (Sarris, Panossian, Schweitzer, Stough, & Scholey, 2011). The present study was conducted to investigate the antinociceptive, anxiolytic, and antidepressant effects of a methanolic extract of *Tinospora crispa* (TC), and of its hexane (HF) and chloroform (CF) fractions, using in vivo and in silico approaches. In order to support the traditional use of *T. crispa* for the relief of inflammation and pain, peripheral antinociceptive activity was first evaluated using the acetic acid-induced writhing test. The latter measures abdominal muscle contractions (writhing) that are induced following the release of inflammatory mediators, such as prostaglandins and bradykinins in peripheral tissues (Sakiyama, Sujaku, & Furuta, 2008). The formalin-induced paw licking test was also used to assess the analgesic activity. In this test, formalin induces pain in two distinct phases that relate to different nociceptive mechanisms (Dubuisson & Dennis, 1977). In the early phase, formalin directly acts on the nociceptors and the pain can be controlled with centrally acting analgesics. The late phase, on the other hand, involves an inflammatory pain response that can be inhibited by non-steroidal anti-inflammatory drugs such as diclofenac (Clavelou, Dallel, Orliaguet, Woda, & Raboisson, 1995). We observed that TC, HF, and CF showed significant antinociceptive activity in both tests.

The rationale for screening TC and its fractions for their activity on the central nervous system (CNS) was based on the knowledge that some sedative and anxiolytic effects had previously been reported for another species of *Tinospora* (Barua et al., 2019; Mishra et al., 2016). The open field test was used to measure the behavioral and locomotor activity of mice (Tatem et al., 2014). Locomotor activity is considered an indicator of alertness, and any decrease in the locomotor performance indicates a CNS-depressant effect (Gahlot, Lal, & Jha, 2013; Hunskaar & Hole, 1987; Sousa et al., 2004). TC, and its fractions, demonstrated a significant decrease in the locomotor activity of animals, which may be as a result of interfering with the neural mechanisms underlying locomotion (Côté, Murray, & Knikou, 2018). In the hole-board test, which evaluates exploratory behavior independently from locomotor activity, TC-treated mice showed a significant increase in the number of head-dippings, suggesting a decreased fearfulness (Brown & Nemes, 2008; Crawley, 1985; Takeda, Tsuji, & Matsumiya, 1998). Anxiety disorders are thought to originate from a dysregulation of a range of neuronal systems, involving gamma-aminobutyric acid (GABA), serotonin, melatonin, adrenalin, dopamine, glutamate, neuropeptides, and the endocannabinoids (Murrough, Yaqubi, Sayed, & Charney, 2015) Anxiety-like behavior was assessed in the elevated-plus maze (EPM) test (Rodgers, 1997). TC and most of its fractions showed a significant reduction in the time spent in the closed arm and increase of the time spent in the opened arm, indicating reduced anxiety (Gagan, Richa, Avninder, Sandeep, & Vivek, 2010).

It remains to be seen if the observed pharmacological effects are attributable to the presence of any specific phytochemicals, although it is worth mentioning that some previously isolated compounds in *T. crispa* have demonstrated antinociceptive activity through interfering with the release of nitric oxide, prostaglandin E2, and tumor necrosis factor (TNF)-alpha (Choi et al., 2004; Yuanyuan, Yuan, & Hongquan, 2010). This warrants further detailed investigation.

5 | CONCLUSION

We observed that the whole plant of TC exerted significant antinociceptive, anxiolytic, and antidepressant activity on mice. This study provides some support for the observed in vivo antinociceptive, anxiolytic, and antidepressant effects of this plant.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

A.R., S.A., M.A.I. conducted the in vivo studies. A.R., S.A., T.B.E., and V.S. wrote the manuscript. A.R., M.M.N.U., A.P., M.N.U.C. analyzed the data, organized the references and performed the statistical calculations. T.B.E. and M.M.N.U. supervised the project and V.S. critically reviewed the manuscript. All the authors reviewed the content of the manuscript.

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