Published with Open Access at Journal BiNET
Vol. 04, Issue 01: 152-161
Bioscience and Bioengineering Communications
Journal Home: https://www.journalbinet.com/bbc-journal.html

Antioxidative and animal model-based pharmacological properties evaluation of leaves of *Trema cannabina* Lour.

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Article received: 19.07.2022; Revised: 09.09.2022; First published online: 30 September 2022.

ABSTRACT

Medicinal plants are of great importance since they exert beneficial pharmacological effects. In this study, phytochemical analysis of the ethanolic extract of the leaves of Trema (T.) cannabina Lour (family Cannabaceae) revealed the presence of carbohydrate, alkaloids, steroid, tannin and absence of gum, saponins, glycosides. Herein, a series of investigations were conducted with the extract to confirm the antioxidative, analgesic, antidiarrhoeal, and antimicrobial properties. T. cannabina extract showed antioxidant activity, both qualitatively and quantitatively. It displayed free radical scavenging activity in the 2,2-diphenylpicrylhydrazyl (DPPH) assay (IC_{50} = ≈201 µg/mL) which is comparable to that of ascorbic acid (IC_{50} = ≈13 µg/mL), a well-known standard antioxidant. Further, 250 and 500 mg/kg of the extract exhibited 28.95% (p=0.07) and 40.79% (p<0.02) inhibition of writhing reflex, respectively compared to the standard drug diclofenac (25 mg/kg, inhibition of writhing reflex 69.74%) in mice. In addition, we found significantly reduced number of stools with the extract at these doses (p<0.001) compared to the standard drug loperamide (3 mg/kg, p<0.001) in the castor oil-induced diarrhoeal mice. Interestingly, the extract (500µg/disc) suggested credible antibacterial properties against the bacterial strains; Enterococcus faecalis, Streptococcus pyogenes, Shigella dysenteriae, Vibrio cholera and no activity against Escherichia coli, Shigella boydii, Staphylococcus aureus, Staphylococcus epidermidis and Proteus mirabilis. For the comparison of antimicrobial properties, standard antibiotic discs of Kanamycin were used. Collectively, the obtained results provide compelling evidence for the role of T. cannabina in exerting antioxidant, analagesic, antidiarrhoeal, and antimicrobial effects, which rationale the use of this plant in traditional medicine. However, further investigations are required to confirm these effects at molecular level.

***Key Words**: Trema cannabina* Lour, Antioxidant, Analgesic, Antidiarrhoeal, Antimicrobial

Cite Article: Shah, V. K. and Rahman, M. A. (2022). Antioxidative and animal model-based pharmacological properties evaluation of leaves of *Trema cannabina Lour*.. Bioscience and Bioengineering Communications, 04(01), 152-161.
Crossref: https://doi.org/10.18801/bbc.040122.18

I. Introduction

Medicinal plants with potential phytochemical agents and those having important pharmacological properties have contributed to healthcare systems for centuries (Sofowora et al. 2013). *Trema (T.) cannabina* Lour. (family Cannabaceae) is a fast-growing shade tree can grow up to 18 m. It is widely distributed in tropical and subtropical regions of the world. There is a vast resource of different species of *Trema* in Nepal, India, Myanmar, Bangladesh, Australia, Malaysia, and China. Interestingly, *T. cannabina* is abundantly found in India and nearly all districts of Bangladesh (Hossain et al. 2013). Many of these are important medicinal plants and sources of bioactive chemical compounds. Some of the isolated compounds from *T. cannabina* include methylswertianin, decussatin, glycosides of decussatin, sweroside, scopoletin, (-)-epicatechin, lupeol, p -hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, adian-5-en-3-one, 2a, 3a, 23-trihydroxyurs-12-en-28-oic acid, 2a, 3ß-dihydroxyurs-12-en-28-oic acid, ß-sitosterol, 3- O -ß-glucopyranosyl-ß-sitosterol and hexacosanoic acid (Noungoué Tchamo et al. 2001). Also, a small amount of soluble haemoprotein with peroxidase activity has been isolated (Coventry et al. 1976).

T. cannabina has been traditionally used for different therapeutic purposes. Among the parts of the tree, its root is used to treat diarrhoea, asthma, and passing of bloody urine, while the bark is used as a poultice in muscular pain; the roots, barks and leaves are used in epilepsy (Kirtikar and Basu, 1935). In African folk medicine, it is used in many diseases, including dysentery and hypertension, etc (Uddin et al. 2008). Previously, it was reported to have analgesic, antioxidant and antibacterial effects from the ethanolic leaf extract (Uddin et al. 2008; Hossain et al. 2013).

Herein, we evaluated both the phytochemical and pharmacological properties of the ethanolic leaf extract using the *in-vitro* chemical, *in-vivo* animal and microorganisms' model. We confirmed previously performed studies on analgesic, antioxidant and antibacterial properties but presented the new finding for antidiarrhoeal effect and extended bacterial strains for antimicrobial activity.

II. Materials and Methods

Plant material collection and identification

For the present investigation, the fresh leaves of *T. cannabina* Lour. was collected from Khulna University, Bangladesh, in December 2009, which was then identified by Bangladesh National Herbarium, Mirpur, Dhaka (Accession number-34739) and a voucher specimen was also deposited there.

Preparation of the plant extract

The unwanted materials or undesirable plant parts were separated and then washed with water. After shade-dried for a week, the leaves were ground into a coarse powder with the help of a suitable grinder (Capacitor start motor, Wuhu motor factory, China). Then the powder was stored in an airtight container and kept in a cool, dark and dry place until used for further analysis. About 150 gm of powdered material was taken in a clean, flat-bottomed glass container and soaked in 700 mL of 80% ethanol. The container with its contents was sealed and kept for seven 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. The filtrate (ethanol extract) obtained was evaporated under ceiling fan and in a water-bath (35°C) until dried. It rendered a gummy concentrate of greenish-black color. The gummy concentrate was designated as crude extract of ethanol, having a final yield value of 3.75%.

Chemicals and drugs

Glacial acetic acid, ethanol and 2, 2-diphenyl-1-picryl hydrazyl (DPPH) were purchased from Sigma chemicals, USA. Diclofenac sodium, chloramphenicol, ascorbic acid, and loperamide were collected from Square Pharmaceuticals Ltd. Bangladesh. All other chemicals were of analytical grade.

Animals

Young Swiss-albino mice were aged 4-5 weeks with an average weight ranging from 20-28 gm collected from Animal Research Branch of the International Center for Diarrhoeal Disease and Research, Bangladesh (ICDDR), were used for all the experiments. Animals were kept in the animal house of

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Pharmacy Discipline, Khulna University, Bangladesh, for one week to allow the adaptation after the purchase. The animals were provided with standard laboratory food and tap water and maintained at a natural day-night cycle. Animal studies were carried out following the approval from Animal Ethics Committee, Pharmacy Discipline, Life Science School, Khulna University, Bangladesh. All the experiments were performed under isolated and noiseless conditions.

Phytochemical screening test

Preliminary phytochemical analysis of the *T cannabina* Lour. plant extract was carried out using the standard procedure reported earlier in the scientific articles (Hossain et al. 2017; Reddy et al. 2017).

Assessment of antioxidant effects

The antioxidant activity of the ethanolic extract was determined based on their scavenging activity of the stable 2, 2-diphenyl-1-picryl hydrazyl (DPPH) free radical reported previously (Sadhu et al. 2003). DPPH is a stable free radical with an odd electron in its structure and is usually used to detect radical scavenging activity in chemical analysis. The aliquots of the different concentrations (1-500 µg/mL) of the extract were added to 3 mL of a 0.004% w/v solution of DPPH. After 30 mins, the absorbance of each sample was determined by a UV spectrophotometer at 517 nm.

Assessment of analgesic effect

Analgesic effect of the plant extract was investigated by the acetic acid-induced writhing method (Hossain et al. 2013). Briefly, a set of mice (n=5 per group) received 250 and 500 mg/kg of the plant extract orally. Control animals receive 1% v/v tween 80 at 10 mL/kg while positive control animals receive diclofenac-Na at 25 mg/kg. Test samples, control, and Diclofenac-Na were given orally using a feeding needle. 30 mins interval was given to ensure proper absorption of the administered substances. Then, the writhing inducing chemical acetic acid solution (0.7%, 10 mL/kg) was administered intraperitoneally to each group of animals. After an interval of 5 mins which was given for absorption of acetic acid, number of squirms (writhing) was counted for 15 mins.

Assessment of antidiarrheal effects

Castor oil-induced diarrheal method in mice was performed for *T. cannabina* extract for the assessment of antidiarrheal activity as performed previously (Jahan et al. 2021). Test samples, control, and loperamide, a standard drug, were given orally through a feeding needle. Animals received the samples, control, and loperamide treatment 40 mins prior to the oral administration of castor oil (0.3mL per mouse). Individual animals of each group were placed in separate cages having adsorbent paper beneath and examined for the presence of diarrhoea every hour for the tenure 4 hour after the castor oil administration. Number of stools or any fluid material that stained the adsorbent paper were counted at each successive hour during the 4 hours and were noted for each mouse. The latent period of each mouse was also counted. At the beginning of each hour, new papers were placed for the old ones. During an observation period of 4 hours, the total number of faecal (stools) output, including diarrheic faeces excreted by the animals was recorded. A numerical score based on stool consistency was assigned as follows: normal stool=1 and watery stool=2.

Assessment of antimicrobial effects

Antimicrobial effect of the ethanolic extract of *T. cannabina* Lour. was determined by the disc diffusion method (Ahmed et al. 2003). Briefly, filter paper discs (5 mm in diameter) were impregnated with the crude extract at the concentration of 250 and 500 µg/disc and then placed onto the agar plates previously inoculated with the test microorganisms. The test microorganisms included *Enterococcus faecalis, Streptococcus pyogenes, Shigella dysenteriae, Vibrio cholera*, *Escherichia coli, Shigella boydii, Staphylococcus aureus, Staphylococcus epidermidis* and *Proteus mirabilis*. The Petri dishes were kept at 4 °C for 2 hours. The plates were incubated at 37 °C for 16 hour to allow the growth of the microorganisms. The diameters of the zones of inhibition (clear circular area) were measured in millimeters using a calibrated scale.

Statistical analyses

An unpaired *t*-test was used to compare data as described by Glasnapp *et al.* (Glasnapp and Poggio, 1985) and values are expressed as mean ± SEM (Standard Error of the Mean). GraphPad Prism (version

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8; GraphPad Software Inc, San Diego, CA) was applied for data analysis. P< 0.05 was considered statistically significant.

III. Results

Phytochemical test

We found that the extract of *T. cannabina* leaves possesses organic compounds like- carbohydrates, alkaloids, steroids, and tannins which can show extensively pharmacologic and other activities as presented in Table 01.

Table 01. Phytochemical screening of ethanolic extract of leaves of *T. cannabina* Lour

Phytochemical groups	Results
Reducing sugars	+
Saponins	-
Alkaloids	+
Glycosides	-
Flavonoids	-
Tannins	+
Gums	-
Steroids	+

Here, + = Presence; - = Absence

Antioxidant activity

To observe if the ethanolic extract of *T. cannabina* Lour. have antioxidant effects, we studied this property with the TLC plates. Herein, the TLC plates were observed under UV detector at 254 nm and 366 nm wavelength, respectively where colored and fluorescent positive components were detected that indicated the presence of UV positive substances in the plant extract and marked (as shown in the images provided, Figure 01). The qualitative test for antioxidant activity revealed the presence of yellowish spots at both 254 nm and 366 nm. Furthermore, in the quantitative assay, *T. cannabina* Lour. leaves displayed a prominent free radical scavenging activity in the DPPH assay (IC_{50} = ≈201 µg/mL, Figure 02) which is comparable to that of ascorbic acid (IC_{50} = ≈13 µg/mL), a well-known standard antioxidant.

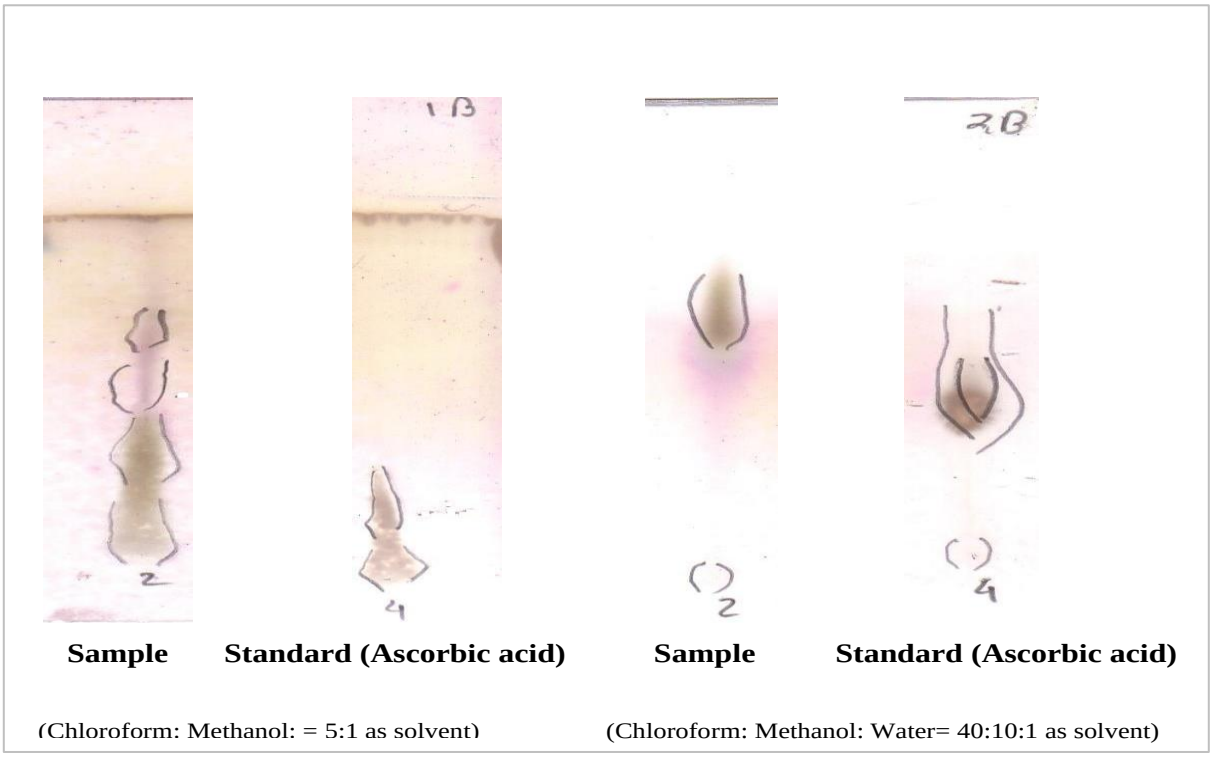

Figure 01. Comparison of TLC plate for *T. cannabina* Lour. with standard (Ascorbic acid) after applying DPPH

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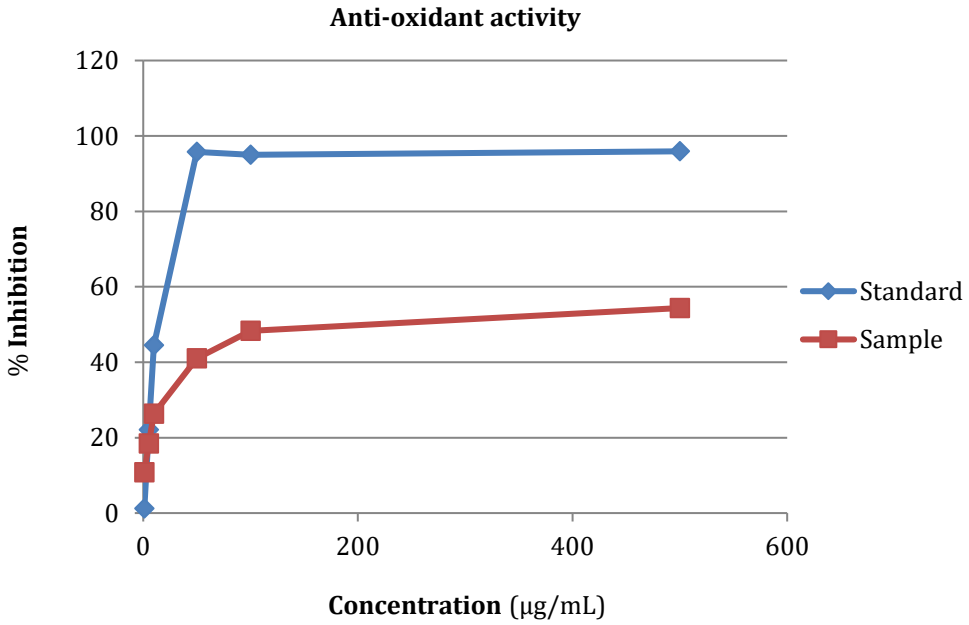

Figure 02. Comparison of % inhibition vs concentration graph for standard (Ascorbic acid) vs ethanolic extract of *T. cannabina* leaves (Sample)

Analgesic activity

The ethanolic extract of leaves of *T. cannabina* showed significant inhibition of writhing reflex by 28.95% (at 250 mg/kg, p<0.01) and 40.79% at 500 mg/kg (p<0.02), respectively. On the other hand, the standard drug diclofenac inhibition was found to be 69.74% at a dose of 25 mg/kg (Table 02).

Table 02. Effects of the ethanolic extract of leaves of *T. cannabina* Lour. on acetic acid-induced writhing in mice

Treatment group (n=5)	Mean of writhing (% Writhing)	% Inhibition of writhing
Blank control	15.2±1.74 (100)	00.00
Diclofenac Na (25 mg/kg)	4.6±0.68 (30.26) ***	69.74
Extract I: (250 mg/kg)	10.8±1.28 (71.05) ns	28.95
Extract II: (500 mg/kg)	9±0.89 (59.21) *	40.79

Here, Values are expressed as mean ± SEM, SEM=Standard error of mean; *P<0.05, ***P<0.001 vs. control, Student's t-test, ns=no significant

Antidiarrhoeal activity

The present study indicated significantly reduced number of stools with the extract at 250 and 500 mg/kg doses (p<0.001) compared to loperamide (3 mg/kg) in the castor oil-induced diarrhoeal mice. Interestingly, the extract produced a moderate increase in the latent period compared to loperamide at a dose of 500 mg/kg and the effect of the dose of 250mg/kg was also moderately significant (Table 03).

Table 03. Effects of the ethanolic extract of leaves of *T. cannabina* Lour. on the latent period and mean number of stools in castor oil induced-diarrhoea in mice.

Treatment group (n=5)	Mean latent period (hour)	Mean number of stools
Blank control	0.74±0.05	9±0.7
Diclofenac Na (25 mg/kg)	2.16±0.15 ***	3.4±0.05 ***
Extract I: (250 mg/kg)	1.3±0.13 **	4.2±0.48 ***
Extract II: (500 mg/kg)	1.52±0.19 **	4±0.63 ***

Here, Values are expressed as mean ± SEM, SEM=Standard error of mean; **P<0.01 ***P<0.001 vs. control, Student's t-test

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Antibacterial activity

The crude extract of the *T. cannabina* Lour. reflected antibacterial activity against the bacterial strains *Enterococcus faecalis, Streptococcus pyogenes, Shigella dysenteriae, Vibrio cholera,* and *no activity against Escherichia coli, Shigella boydii, Staphylococcus aureus, Staphylococcus epidermidis and Proteus spp.* We found that the sample at 250 and 500 µg/mL had varying degrees of antimicrobial activity (zone of inhibition = 6-19 mm, Figure 3) against all the bacterial strains (Table 04). Interestingly, we observed the highest zone of inhibition against *Vibrio cholerae* (19 mm).

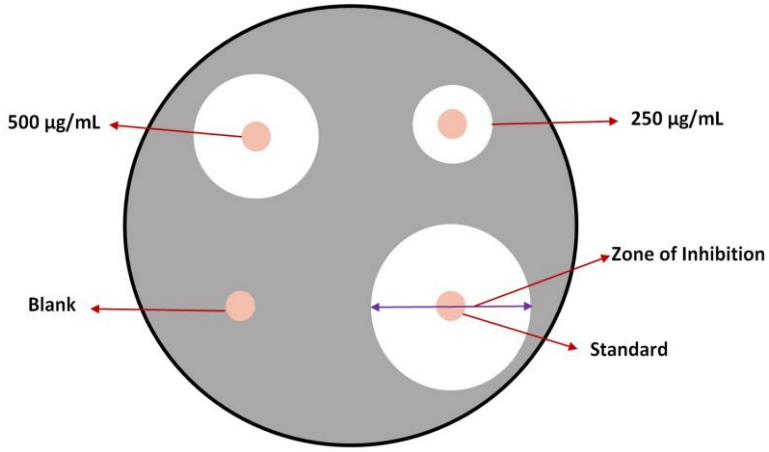

Figure 03. A representative image showing zone of inhibition (Clear area) in the agar media impregnated with bacterial strain.

Table 04. *In vitro* antibacterial activity of the ethanolic extract of leaves of *T. cannabina* Lour

Serial no	Bacterial strains	Type of bacterial strains	Diameter of Zone of Inhibition (mm)			
			Blank	Kanamycin (30 µg/disc)	Extract of (250µg/disc)	Extract (500µg/disc)
1	*Escherichia coli*	Gram(-)	-	26	-	-
2	*Enterococcus faecalis*	Gram(+)	-	32	6	6
3	*Streptococcus pyogenes*	Gram(+)	-	33	6	8
4	*Shigella boydii*	Gram(-)	-	17	-	-
5	*Shigella dysenteriae*	Gram(-)	-	22	11	10
7	*Staphylococcus aureus*	Gram(+)	-	20	-	-
7	*Staphylococcus epidermidis*	Gram(+)	-	17	-	-
8	*Proteus spp.*	Gram(-)	-	22	-	-
9	*Vibrio cholerae*	Gram(-)	-	51	-	19

Gram (-):-Gram Negative Bacteria; Gram (+):-Gram Positive Bacteria; (-):- No inhibition

IV. Discussion

The results presented here explore the potential evaluation of phytochemical and pharmacological properties of *T. cannabina* Lour. Previously, it was reported that its ethanolic leaf extract possesses analgesic activity suggested by the presence of phytochemicals (such as tannins, alkaloids, and others) in it (Hossain et al. 2013). Therefore, this study investigated the pharmacological properties that were not carried out before. Herein, we tested pharmacological properties like analgesic, antidiarrhoeal, antioxidant, and antimicrobial properties.

Phytochemical screening is critical for the identification of chemical compounds such as alkaloids, phenols, flavonoids, tannins, glycosides, steroids and others that characterizes their pharmacological properties. These important steps are thus essential for evaluating them as a therapeutic medicine (Shah et al. 2014; Na et al. 2015; Jahan et al. 2022). Herein, we found the presence of carbohydrates, alkaloids, steroids, tannin, and the absence of gum, saponins, glycosides as presented in Table 01. This

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coincided with the previous report (Hossain et al. 2013). Next, we focused on our findings for the evaluation of the pharmacological properties of the extract.

Phytochemicals like flavonoids and polyphenols have been suggested to be critical secondary metabolites and major bioactive compounds of medicinal herbs/plants, which are essential antioxidants (Mohan and Priya, 2009; Bhatt et al. 2012; Xue et al. 2017). In our study, both qualitative and quantitative assay was performed to confirm the antioxidant activity of the sample. DPPH, a free radical assay is often used to evaluate the antioxidant activity of a test sample (Kedare and Singh, 2011). This is quantified in terms of the half maximal inhibitory concentration (IC50) (Sánchez-Moreno et al. 1998; Hossain et al. 2017). Our finding showed a strong indication of the presence of antioxidants in the ethanolic extract of *T. cannabina* Lour (Figure 01). The sample showed moderate free radical scavenging activity in the DPPH assay where the obtained value for IC_{50} was ≈201 µg/mL (Figure 02) comparable to the ascorbic acid IC_{50} value (≈13 µg/mL). Interestingly, we found this to be comparatively more than earlier findings which were 110.25 µg/mL (Uddin et al. 2008). This suggests that the plant could be beneficial for identifying the significant antioxidants that require further analysis.

After, testing the antioxidant activity of the extract, we checked for its analgesic activity. By virtue of the nature of pain sensation, we respond to external stimuli. The stimuli are responsible for releasing arachidonic acid intracellularly from the phospholipids of the affected tissues. Also releasing substances like prostaglandins, prostacyclin (PGI2), PGE2, PGF2α, cytokines, and leukotrienes. Our study with the extract revealed that the ethanolic extract of leaves of *T. cannabina* has potent analgesic activity. This was confirmed by observing a significant inhibition of the writhing reflex at both doses. This was confirmed using diclofenac as a standard drug (Table 02). Interestingly, this was in agreement with the previous work (Hossain et al. 2013). The authors found that at 250 mg/kg and 500 mg/kg, the extract had 34.15% and 47.56 % writhing inhibition in mice, respectively. In agreement with this, our study showed that the extract at 250 mg/kg and 500 mg/kg had 28.95% and 40.79% % writhing inhibition in animals, respectively. For analgesic activity, it was hypothesized that PGE2 and PGF2a peripherally may have acted to mediate acetic acid-induced writhing effects in mice (Adzu et al. 2003).

The other pharmacological property of the extract we tested for include antidiarrhoeal activity. Diarrhoea is one of the most common gastrointestinal disorders contributing to the number of deaths worldwide. This elevates frequency of bowel movements, abdominal pains, and wet stool (Wang et al. 2015; Shang et al. 2018). In our work, we assessed the antidiarrhoeal activity for the extract in response to the castor oil. Several studies on medicinal herbs/plants have been conducted on animals to test this activity because of its popularity (Wang et al. 2015). Ricinoleic acid, being a major castor oil constituent, has been suggested to induce irritation of the gastrointestinal mucosa and also the inflammation that mediates increased motility (Schmeltz and Metzger, 2007). We found that the ethanolic leaf extract reduced the number of stools at both doses. Further, we also observed a moderate increase in the latent period (Table 03). These findings suffice to conclude that the extract has antidiarrhoeal activity and pointed out that further analysis would be necessary to understand the underlying mechanism for this activity.

We were then interested in observing if the extract has any antimicrobial effects other than analgesic, antidiarrhoeal and antioxidant. It has been challenging to develop antibacterial agents to combat infectious diseases to avoid their resistance mechanism to antibiotics. The disk diffusion assay was used to determine the antibacterial activity against selected bacterial strains. This assay was used since it is common and less expensive to perform (Balouiri et al. 2016). One of the studies suggested that the ethanolic leaf extract of *T. cannabina* Lour. have antimicrobial effects against *Staphylococcus aureus, Staphylococcus epidermidis, Plesiomonas shigelloides, Shigella dysenteriae and Vibrio cholerae* and null activity for *Salmonella typhi and Shigella boydii.* (Uddin et al. 2008). However, in this work we found the ethanolic extract at 250 and 500 µg/mL doses to be effective against *Enterococcus faecalis, Streptococcus pyogenes, Shigella dysenteriae, and Vibrio cholerae* (500 µg/mL only). On the other hand, we did not find any doses to be effective against *Escherichia coli, Shigella boydii, Staphylococcus aureus, Staphylococcus epidermidis, and Proteus spp* (Table 4). Interestingly, *Vibrio cholerae* at 500 µg/mL had highest zone of inhibition (19 mm). This suggests that *T. cannabina Lour.* have significant antibacterial activity against some of the gram-positive and gram-negative bacterial strains that confirms its antibacterial effects.

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However, further investigations at a molecular level are required to explore this plant as a source of active antimicrobial agents.

V. Conclusion

Collectively, the present study provided evidence that the ethanolic extract of *T. cannabina* Lour. leaves exhibit potential antioxidant, analgesic, antidiarrhoeal, and antimicrobial properties. This study emphasizes the importance of medicinal plants that could serve as potential therapeutic agents. However, this study deserves further investigation that should be conducted to identify the major components and underlying mechanisms for pharmacological activities.

Conflict of interest

The authors declare no conflict of interest.

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HOW TO CITE THIS ARTICLE?

MLA
Shah, V. K. and Rahman, M. A. "Antioxidative and animal model-based pharmacological properties evaluation of leaves of *Trema cannabina Lour*". Bioscience and Bioengineering Communications, 04(01), (2022): 152-161.

APA
Shah, V. K. and Rahman, M. A. (2022). Antioxidative and animal model-based pharmacological properties evaluation of leaves of *Trema cannabina Lour*. *Bioscience and Bioengineering Communications*, 04(01), 152-161.

Chicago
Shah, V. K. and Rahman, M. A. "Antioxidative and animal model-based pharmacological properties evaluation of leaves of *Trema cannabina Lour*". Bioscience and Bioengineering Communications, 04(01), (2022): 152-161.

Harvard
Shah, V. K. and Rahman, M. A. 2022. Antioxidative and animal model-based pharmacological properties evaluation of leaves of *Trema cannabina Lour*. Bioscience and Bioengineering Communications, 04(01), pp. 152-161.

Vancouver
Shah, VK and Rahman, MA. Antioxidative and animal model-based pharmacological properties evaluation of leaves of *Trema cannabina Lour*. Bioscience and Bioengineering Communications, 2022 September, 04(01): 152-161.

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