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Original Article

Antidiarrheal activity of aqueous extract of leaves of *Euphorbia hirta* L.

[Actividad antidiarreica del extracto acuoso de hojas de *Euphorbia hirta* L.]

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Abstract

Context: *Euphorbia hirta* L. is widely distributed in the Indian subcontinent and has many ethnomedicinal uses. Traditionally, the leaves of *E. hirta* have been used as antidiarrheal agents, for bronchial and respiratory diseases and against conjunctivitis.

Aims: To evaluate the antidiarrheal activity of the aqueous extract of leaves of *Euphorbia hirta*.

Methods: Steam and aqueous extracts of leaves of *E. hirta* were evaluated for their antidiarrheal activity by *in vitro* antiprotozoal and antimicrobial activity testing, *in vivo* evaluation of % protection against diarrhea in castor-oil induced rat diarrhea model, gastrointestinal transit and enteropooling assays in rat model and *ex vivo* tissue contractility studies.

Results: Steam and aqueous extracts of leaves of *E. hirta* demonstrated antiprotozoal activity against *Entamoeba histolytica* and *Giardia lamblia* with MICs ranging from 31.25-62.5 µg/mL to 125-250 µg/mL. Both the extracts showed antibacterial activity against *Salmonella abony*, *Escherichia coli* and *Shigella boydii*. *In vivo* antidiarrheal activity evaluation in castor-oil induced diarrhea model in rats revealed that the steam extract at a dose of 1621 mg/kg body weight (b.w.) produced % inhibition in defecation comparable to loperamide (4 mg/kg b.w.). Aqueous extract produced significant reduction in fecal output when compared to the untreated control group ($p<0.05$). However, the extracts failed to have any significant effect on the gastrointestinal transit of charcoal meal in rats. Also, gastric fluid accumulation was significantly reduced by steam extract at 2162 mg/kg b.w. and aqueous extract at 100 mg/kg b.w. ($p<0.05$) when compared to the untreated group. The smooth muscle relaxation produced by the steam extract on the isolated rat ileum was comparable to that produced by atropine (0.05-0.4 µg/mL).

Conclusions: The results of this study provide support for the traditional use of *Euphorbia hirta* leaves as an antidiarrheal agent.

Keywords: castor-oil induced diarrhea; enteropooling; *Euphorbia hirta;* gastrointestinal transit; isolated rat ileum; protozoa.

Resumen

Contexto: *Euphorbia hirta* L. se distribuye ampliamente en el subcontinente indio y tiene muchos usos etnomedicinales. Tradicionalmente, las hojas de *E. hirta* se han utilizado como agentes antidiarreicos, para enfermedades bronquiales, respiratorias y contra conjuntivitis.

Objetivos: Evaluar la actividad antidiarreica del extracto acuoso de hojas de *Euphorbia hirta*.

Métodos: Los extractos de vapor y acuosos de hojas de *E. hirta* se evaluaron para determinar su actividad antidiarreica mediante pruebas *in vitro* de actividad antiprotozoaria y antimicrobiana, evaluación in vivo del% de protección contra la diarrea en el modelo de diarrea en ratas inducida por aceite de ricino, tránsito gastrointestinal y ensayos de enteropooling en modelo de rata y estudios de contractilidad tisular *ex vivo*.

Resultados: Los extractos de vapor y acuosos de hojas de *E. hirta* demostraron actividad antiprotozoaria contra *Entamoeba histolytica* y *Giardia lamblia* con MICs que varían de 31,25-62,5 µg/mL a 125-250 µg/mL. Ambos extractos mostraron actividad antibacteriana contra *Salmonella abony*, *Escherichia coli* y *Shigella boydii*. La evaluación de la actividad antidiarreica *in vivo* en un modelo de diarrea inducida por aceite de ricino en ratas reveló que el extracto de vapor a una dosis de 1621 mg/kg de peso corporal (p.c.) produjo un % de inhibición en la defecación comparable a la loperamida (4 mg/kg p.c.). El extracto acuoso produjo una reducción significativa en la producción de heces en comparación con el grupo de control no tratado ($p<0,05$). Sin embargo, los extractos no tuvieron ningún efecto significativo sobre el tránsito gastrointestinal de la harina de carbón en ratas. Además, la acumulación de líquido gástrico se redujo significativamente con el extracto de vapor a 2162 mg/kg p.c. y extracto acuoso a 100 mg/kg p.c. ($p<0,05$) en comparación con el grupo no tratado. La relajación del músculo liso producida por el extracto de vapor en el íleon de rata aislado fue comparable a la producida por la atropina (0,05-0,4 µg/mL).

Conclusiones: Los resultados de este estudio apoyan el uso tradicional de hojas de *Euphorbia hirta* como agente antidiarreico.

Palabras Clave: agrupación enteral; diarrea inducida por aceite de ricino; *Euphorbia hirta*; íleon de rata aislado; protozoos; tránsito gastrointestinal.

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INTRODUCTION

According to 2017 WHO report, diarrhea is the second leading cause of death in children below five years of age, and is responsible for 5 25 000 child mortalities every year (WHO Antidiarrheal Factsheet, 2017). Diarrhea is reported to be caused by a number of bacterial, viral and parasitic organisms, often spread through feces-contaminated water. *Escherichia coli*, *Shigella* and *Entamoeba* species are some of the common causative agents of diarrhea in low-income developing countries (WHO Antidiarrheal Factsheet, 2017).

Euphorbia hirta L. belongs to the family *Euphorbiaceae* and is traditionally used for the treatment of various diseases like female disorders, gastrointestinal disorders, respiratory ailments, inflammations of the skin and mucous membranes, in kidney and liver disorders, tumors and in conjunctivitis. The plant is found mainly in India, China, Philippines, Australia, Africa and Malaysia (Kumar et al., 2010; Kausar et al., 2016).

Fourteen triterpenoids, seven coumarins, four lignans and six diterpenes have been isolated from the aerial parts of *E. hirta* (Li et al., 2015). 2-Butanone, 3, 3-dimethyl-1-(methylsulfonyl)-O-[(methylamino) carbonyl] oxime, hexadecanoic acid, 1, 3, 4, 5-tetrahydroxycyclohexanecarboxylic acid and 9, 12, 15-octadecatrienoic acid were identified in the methanolic extract of leaves of *E. hirta* (Ping et al., 2012). Wang et al. in 2012 isolated diisobutyl-O-phthalate, diethylhexyl phthalate, hispidulin, acetyl peroxide, quercetin and gallic acid from leaves of *E. hirta.* Glycoalkaloids and acids such as melissic, gallic and tannic were isolated from *E. hirta* by Rizk (1987).

Galvez et al. in 1993 were the first researchers to report the antidiarrheal activity of aqueous extract of whole plant of *E. hirta* in mice. Quercitrin, a flavonoid possessing antidiarrheal activity was also isolated from *E. hirta*. Hore et al. (2006) demonstrated the antidiarrheal activity of aqueous extract of leaves of *E. hirta* in castor-oil induced diarrhea model of mice. However, no details of temperature conditions and other procedural details for the extraction of leaves of *E. hirta* have been described by the authors. Aqueous-methanol extracts of whole herb of *E. hirta* and its fractions have been used by Ali et al. (2020) to study castor oil-induced diarrhea in mice. The authors have reported gut-stimulatory effects of *E. hirta* in rat ileum.

Gupta and Gupta (2019) have investigated the antimicrobial activity of *E. hirta* leaves against *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus* and *Saccharomyces cerevisiae*. Antiprotozoal activity of the aqueous extract and various fractions of aerial parts (leaves and stems) of *E. hirta* has been evaluated against clinical isolates of *Entamoeba histolytica.* The IC_{50} values of *E. hirta* aqueous extract and its methanol fraction and methylene chloride fraction against *E. histolytica* were reported to be 145.95 µg/mL, 67.18 µg/mL and 194.04 µg/mL, respectively after 72 h of incubation (Pechangou et al., 2014). Abubakar (2009) evaluated the efficacy of methanolic, hexane and aqueous extracts of the entire plant of *E. hirta* in treating enteric infections caused by *Escherichia coli*, *Klebsiella pneumoniae*, *Shigella dysenteriae*, *Salmonella typhi* and *Proteus mirabilis*. The aqueous extract was found to possess maximum activity against these enteric infection causing organisms. No reports on antimicrobial and antiprotozoal activity of *E. hirta* leaves against microorganisms and protozoa commonly implicated in infectious diarrhea viz. *Salmonella abony, Shigella boydii, Entamoeba histolytica* and *Giardia lamblia,* have been reported.

The steamed leaves of *E. hirta* have been used traditionally in Gujrat for management of diarrhea by Ajabai Pethabhai Harijan from Mudhan, Lakhapat, Kachch. The present study provides evidence-based scientific validity to the folklore anti-diarrheal use of the steamed preparation of *E. hirta* leaves by a host of *in vitro, ex vivo* and *in vivo* methods, including castor oil-induced diarrhea model, enteropooling effect, effect on gastrointestinal motility and effect on isolated ileum; a battery of evaluation parameters that have hitherto not been tested for steamed preparation of *E. hirta* leaves. Their possible role in treating infectious forms of the disease is evaluated by estimating

activity against *Escherichia coli, Salmonella abony, Shigella boydii, Entamoeba histolytica* and *Giardia lamblia*.

MATERIAL AND METHODS

Plant material and extraction

Leaves of *E. hirta* were obtained from Biotechnology Industry Research Assistance Council-Society for Research and Initiatives for Sustainable Technologies and Institutions (BIRAC–SRISTI), Ahmedabad (geographic coordinates: 23°1'18"N, 72°34'47"E). The plant was authenticated by a botanist at Mumbai and a voucher specimen (rsp181286416) was deposited at his herbarium. The dried leaves of *E. hirta* were extracted by two methods- steam and aqueous extraction using water as the solvent.

Steam method

Leaves weighing 500 g were steamed for 2 h. The steamed leaves were then ground in a mixer and the mass was filtered through a muslin cloth. The extract was lyophilized to obtain a yield of 7.68% w/w.

Aqueous extraction

Leaves weighing 500 g were boiled in water for 2 h. The mass was kept for maceration for 24 h. The extract was filtered through a muslin cloth and lyophilized to get a yield of 11.86% w/w.

Materials and chemicals

Loperamide was procured from Sigma-Aldrich (St. Louis, USA). Vitamin D_2 and ferric ammonium citrate were gifts from Sun Pharmaceutical Industries Ltd. (India) and Cipla Ltd. (India), respectively. Biotin and calcium pantothenate were obtained as gift samples from Merck & Co. (India). Folic acid, cysteine, α-tocopherol, niacin, niacinamide, pyridoxal, pyridoxine hydrochloride, riboflavin, thiamine hydrochloride, vitamin A and metronidazole were obtained as gift samples from Celogen Lifescience and Technologists Pvt. Ltd. (India). All the other chemicals were obtained either from Hi Media or SDFCL (India).

Shigella boydii (ATCC 9207), *Salmonella abony* (ATCC 6017) and *Escherichia coli* (ATCC 25922) cultures were obtained from M.K. Rangnekar laboratories, Mumbai (India). *Entamoeba histolytica* (ATCC 30459) and *Giardia lamblia* (ATCC 30957) were kindly provided by Bose Institute, Kolkata (India).

Phytochemical screening of powdered leaves of *Euphorbia hirta*

Aqueous, methanolic and ethanolic extracts of leaves of *E. hirta* were prepared by hot maceration and screened for the presence of carbohydrates, alkaloids, steroids, tannins, flavonoids and phenolic compounds (Galvez et al., 1993; Li et al., 2015).

In vitro antimicrobial activity evaluation of the steam and aqueous extracts of leaves of *E. hirta*

In vitro *antiprotozoal activity evaluation by Resazurin Microtitre plate Assay (REMA)*

E. histolytica and *G. lamblia* were cultured in Keister's modified TYI-S-33 medium, supplemented with 10% adult bovine serum. The antiprotozoal activity was evaluated by the high throughput technique of REMA as described by Mirza et al. (2011) with minor modifications. To each well of a sterile U-bottom 96-well plate, 100 µL of TYI-S-33 medium was added. The steam and aqueous extracts of leaves of *E. hirta* were dissolved in dimethyl sulfoxide (DMSO) and further diluted with TYI-S-33 medium to maintain the concentration of DMSO below 1% in the final drug solution. Drug solutions of 100 µL were added to the wells and serial dilutions were made to achieve concentrations ranging from 15.125 to 500 µg/mL. Inoculum of 100 µL with parasite numbers between 10^3 and 10^6 cells was added to each well. The 96-well plates were then incubated at 37°C under anaerobic conditions for 24 h. After 24 h of incubation, 50 µL of 0.01% resazurin dye prepared in sterile water was added. At the end of incubation, color change in each well from blue to pink indicating protozoal growth was visually observed and recorded. Minimum inhibitory concentration (MIC) is defined as the lowest concentration of drug that prevents this change in color. The activity was

compared with standard antiprotozoal drug metronidazole. Growth control, DMSO growth control, sterile water control, sterile media control and a sterile control for each extract were also included in the plate.

In vitro *antimicrobial activity evaluation by agar well diffusion and turbidimetry techniques*

Preparation of standard bacterial suspension

The strains of *Shigella boydii*, *Salmonella abony* and *Escherichia coli* were inoculated in nutrient broth and maintained for growth in a microbiological incubator at a temperature of 37 ± 2°C for 24 h before performing the experiment.

Agar well diffusion assay

Standardized bacterial culture (1.0×10^7 cfu/mL) of 1.0 mL was seeded into 15 mL of nutrient agar and spread gently to ensure uniform distribution of the microorganisms in a Petri dish. The agar was allowed to solidify and about 5.0 mm diameter wells were bored in the plates using a sterile cork borer. Drug solution concentrations ranging from 500, 1000, 2000 and 5000 µg/mL were transferred into the wells. Amoxicillin 100 µg/mL was used as the positive control. The plates were incubated at 37°C for 24 h. Zone of inhibition was estimated by measuring the diameter after completion of incubation period (Kumar et al., 2014).

Turbidimetric assay/ macro broth dilution test

Drug solution concentrations ranging from 100, 500, 700 to 1000 µg/mL were added to sterile nutrient broth dispensed in the test tubes. Amoxicillin (100 µg/ml) was used as the positive control. A bacterial suspension of 1×10^5 CFU/mL was inoculated in the drug containing test tubes. These tubes were incubated for 16-20 h at 37°C and visually observed for bacterial growth. As per Bagul and Sivakumar (2016), minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial that inhibits the visible growth of microorganisms after incubation for 20 h. Absorbance of the tubes were recorded at 590 nm and % growth inhibition was calculated by comparing with the growth control tube (Li et al., 1993).

Animals

Adult female Wistar rats of 6-8 weeks age weighing 180-200 g were obtained from Bombay Veterinary College, Parel, Mumbai. The rats were kept in polypropylene cages at an ambient temperature of 25 ± 1°C, 12 h light/dark cycle and 55 ± 5% controlled relative humidity. The animals were fed with a standard pellet diet and water *ad libitum*. The experimental protocol followed the guidelines and regulations of the Animal Ethics Committee, Bombay Veterinary College, Parel, Mumbai, India (Reference No. BVC/IAEC/17/2017). All the standard and test drugs were dissolved in distilled water before administration to animals.

Acute toxicity test

An acute toxicity study of the steam and aqueous extracts of leaves of *E. hirta* was performed as per the OECD 423 Guidelines (2005). The animals (n = 6) were kept under fasting for 8-10 h before initiation of the experiment. The required quantity of the extract was dissolved in 0.5 mL water and administered to the animals. The animals were observed for any signs of toxicity or mortality for 14 days. They were then euthanized using carbon dioxide inhalation and organs including intestine, heart, liver and brain were evaluated to identify changes in histopathology (if any).

Evaluation of antidiarrheal activity of steam and aqueous extracts of leaves of *E. hirta* in castor oil-induced diarrhea in Wistar rats

The rats were fasted for 12 h and divided into seven groups of three rats each. Water was supplied *ad libitum*. Each rat was placed individually in a single cage lined with clean filter paper. Group I acted as the negative control and was neither induced with diarrhea nor treated with any drug. Group II received loperamide (4 mg/kg; peroral [p.o.]) and served as the positive control. Groups III and IV received steam extract of *E. hirta* (1621 mg/kg and 2162 mg/kg body weight [b.w.]) whereas groups V and VI received aqueous ex-

tracts of *E. hirta* (100 mg/kg b.w. and 200 mg/kg b.w.). Thirty minutes after the treatment with the above-mentioned doses, groups II to VII were administered 0.5 mL of castor oil, p.o. Group VII served as the untreated control in which diarrhea was induced by administration of 0.5 mL castor oil, p.o. but, no test or standard substance was administered. The fecal output was observed for a period of 4 h and % inhibition in diarrhea was calculated (Sisay et al., 2017). The percentage protection against diarrhea was calculated with respect to the number of wet feces using the formula [1].

$$\text{Inhibition (\%)} = \frac{\text{WFC average number} - \text{WFT average number}}{\text{WFC average number}} \times 100 \quad [1]$$

Where, WFC: wet feces in untreated group and WFT: wet feces in test group (Sisay et al., 2017).

The animals were euthanized using carbon dioxide inhalation and a small part of the colon was carefully dissected and stained using hematoxylin and eosin to observe for any morphological changes.

Preliminary study results using 485 mg/kg b.w. and 1041 mg/kg b.w. of steam extract of *E. hirta* showed a concentration-dependent decrease in fecal output in castor-oil induced diarrhea model. However, at the same doses, the steam extract of *E. hirta* failed to inhibit the transit of charcoal meal in gastrointestinal rat motility model. Hence, higher doses of 1621 mg/kg b.w. and 2162 mg/kg b.w. were used for further studies based on dose calculations from a previously reported study using whole herb of *E. hirta* (Galvez et al., 1993).

In case of aqueous decoction, studies using 138 mg/kg b.w. and 297 mg/kg b.w. of aqueous extract of *E. hirta* showed an increase in fecal output at higher doses. Hence, lower doses of 100 mg/kg b.w. and 200 mg/kg b.w. of aqueous extract were used for further studies.

Effect of steam and aqueous extracts of leaves of *E. hirta* on gastrointestinal (GI) motility of charcoal meal in Wistar rats

Wistar rats were fasted for 8-10 h and divided into five groups of three animals each. Group I received water p.o. and served as the negative control, group II received the standard drug loperamide (4 mg/kg b.w., p.o.) which served as the positive control while groups III and IV received steam extract of leaves of *E. hirta* at the doses of 1621 mg/kg b.w. and 2162 mg/kg b.w., p.o., respectively. Group V received 100 mg/kg b.w. of aqueous extract of leaves of *E. hirta*. Each of the animals in Groups I-V was given 0.7 mL charcoal meal (10% charcoal, 20% gum acacia, 30% starch uniformly dispersed in water) as a marker by the oral route 15 min after drug treatments. All rats were euthanized using carbon dioxide inhalation after 30 min of administering charcoal meal. The stomach and small intestine were then removed and laid out on a clean surface. The distance traversed by the charcoal meal from the pylorus was measured (Sisay et al., 2017). Also, the length of the entire small intestine was measured. % Transit of the charcoal meal was calculated using the formula [2, 3].

$$\text{Peristaltic index (PI)} = \frac{\text{Distance from pylorus to charcoal meal}}{\text{Total length of small intestine}} \times 100 \quad [2]$$

$$\text{GI motility reduction (\%)} = \frac{\text{PIC} - \text{PIT}}{\text{PIC}} \times 100 \quad [3]$$

Where, PIC: peristaltic index of group, which was administered water alone and PIT: peristaltic index of test group (Sisay et al., 2017).

Evaluation of effect of steam and aqueous extracts of leaves of *E. hirta* on enteropooling in Wistar rats

The animals were randomly divided into 6 groups (n = 3) and were fasted for 8-10 h before initiation of the experiment. The control group I received water. Animals in group II received 4 mg/kg b.w. loperamide. Rats in groups III and IV received steam extract of leaves of *E. hirta* at the doses 1621 mg/kg b.w. and 2162 mg/kg b.w. respectively. Group V received 100 mg/kg b.w. of aqueous extract of leaves of *E. hirta*. One hour post treatment, 1 mL of castor oil was administered to all rats in groups II-VI. Group VI was the untreated group. The animals were euthanized using carbon dioxide inhalation 1 h post administration of castor oil. The small intestine of each rat was carefully excised out and weighed. The contents of the

small intestine were milked out into a measuring cylinder and the intestine was then reweighed. The difference in weight between the full and empty intestines was recorded as the weight of intestinal content. The volume and texture of the intestinal content was also noted (Sisay et al., 2017). Percentage reduction of intestinal secretion (volume and weight) was calculated relative to the negative control using the formula [4].

$$\text{Intestinal secretion reduction (\%)} = \frac{\text{MWICC} - \text{MWICT}}{\text{MWICC}} \times 100 \quad [4]$$

Where, MWICC: mean weight of intestinal content of untreated control group and MWICT: mean weight of intestinal content of test group.

$$\text{Intestinal secretion reduction (\%)} = \frac{\text{MVICC} - \text{MVICT}}{\text{MVICC}} \times 100 \quad [5]$$

Where, MVICC: mean volume of intestinal content of untreated group and MVICT: mean volume of intestinal content of test group (Sisay et al., 2017).

Ex vivo evaluation of contractile responses of steam and aqueous extracts of leaves of *E. hirta* on isolated rat ileum

Freshly excised portions of ileum from healthy untreated Wistar rats were used in the study. Excised ileum, about 2-3 cm in length was mounted in 10 mL tissue bath containing Tyrode solution of the following composition (mmol/L): 140 NaCl, 2.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 **t**rishydroxymethylaminomethane, 10 **g**lucose with pH 7.4, aerated with 95% O_2 and 5% CO_2 (carbogen) and maintained at a temperature of 37°C. A tension of 1 g was applied to the tissue and the contractile responses were recorded using kymograph. The tissue was allowed to equilibrate for a period of 30 min before addition of any drug or test material and then stabilized with a sub-maximal concentration of acetylcholine (0.15 µM). Atropine, a competitive blocker of acetylcholine, which finds usage in the treatment of spasms in the stomach, intestines, and reduction in fluid secretions was used as a positive control at concentrations of 0.05-0.4 µg/mL. The tissue was presumed stable only after the reproducibility of the said responses. The steam and aqueous extracts of leaves of *E. hirta* were examined for any spasmodic activity on the ileum preparation of rat at concentrations ranging from 0.5 to 4 µg/mL (Bello et al., 2016).

Statistical analysis

All experiments were carried out in triplicate and results were expressed as mean ± standard deviation (SD). Statistical analyses were performed by Graphpad Prism 6.0 software to determine the statistical significance of differences ($p<0.05$) in antiprotozoal activity using the one-way analysis of variance (ANOVA) with one post-hoc analysis. The post-hoc Tukey's test was performed for the comparison of MICs between the control group (metronidazole) and the test groups (steam and aqueous extracts of leaves of *E. hirta*) and for the comparison of MICs between the test groups (steam and aqueous extracts of leaves of *E. hirta)* against protozoa (*E. histolytica* and *G. lamblia*). Statistical significance of differences ($p<0.05$) in antimicrobial studies was determined by two-way ANOVA followed by Tukey's test. Statistical significance ($p<0.05$) of data in the animal studies was evaluated by one-way ANOVA followed by Tukey's test.

RESULTS

Phytochemical screening of powdered leaves of *Euphorbia hirta*

Powdered leaves of *E. hirta* were extracted using various solvents to identify the presence of different classes of phytochemicals such as carbohydrates, alkaloids, steroids, tannins, flavonoids and phenolic compounds. The results of the study are as summarized in Table 1. Both aqueous and methanolic extracts showed the presence of alkaloids, steroids, tannins, flavonoids, saponins and reducing sugars. Phenolic compounds were found to be present in aqueous and ethanolic extracts. Also, ethanolic extract demonstrated the presence of alkaloids, tannins and flavonoids.

Table 1. Results of phytochemical screening of the extracts of leaves of *E. hirta.*

Phytochemical class	Extracts of *E. hirta* leaves		
	Aqueous	Methanolic	Ethanolic
Alkaloids	+	+	+
Flavonoids	+	+	+
Tannins	+	+	+
Saponins	+	+	-
Steroids	+	+	-
Reducing sugars	+	+	-
Phenolic compounds	+	-	+

Table 2. Antiprotozoal activity of steam and aqueous extracts of leaves of *E. hirta* determined by REMA.

Test samples	MIC (µg/mL)	
	Entameoba histolytica	***Giardia lamblia***
Steam extract	125-250 ± 0.00*a	62.5-125 ± 0.00*a
Aqueous extract	62.5-125 ± 0.00*a	31.25-62.5 ± 0.00*a
Metronidazole	0.325-0.75 ± 0.00	0.15-0.3 ± 0.00

Data represented as mean ± SD (n = 3). *Indicates statistically significant difference ($p<0.05$) between metronidazole control group and the test groups by means of Tukey's multiple comparison tests and 'a' denotes statistically significant difference ($p<0.05$) between the test groups (steam and aqueous extracts of *E. hirta*) by means of Tukey's multiple comparison tests.

In vitro antimicrobial activity evaluation of the steam and aqueous extracts of leaves of *E. hirta*

In vitro *antiprotozoal activity evaluation by Resazurin Microtitre plate Assay (REMA)*

The aqueous extract of leaves of *E. hirta* exhibited significantly more potent ($p<0.05$) antiprotozoal activity than the steam extract against both *E. histolytica* and *G. lamblia* (Table 2).

In vitro *antimicrobial activity evaluation by agar well diffusion and turbidimetry techniques*

Agar well diffusion assay

The steam and aqueous extracts of leaves of *E. hirta* at a concentration of 500 µg/mL showed maximum potency against *E. coli* followed by *S. abony* and least activity against *S. boydii* (Table 3). Both steam as well as aqueous extracts showed activity comparable to that of standard amoxicillin against *S. boydii* at a concentration of 2000 µg/mL. There was no statistically significant difference ($p>0.05$) in the antibacterial activity of steam and aqueous extracts of leaves of *E. hirta* against *S. abony, E. coli and S. boydii* at 500 µg/mL.

Turbidimetric assay/ Macro broth dilution test

Similar to the agar well diffusion assay, steam extract at a concentration of 500 µg/mL showed maximum activity against *E. coli*, followed by *S. abony* and least activity against *S. boydii* (Table 4). However, there was no statistically significant difference ($p>0.05$) between the two extracts at the same concentrations.

Acute toxicity test

The results of the study show that steam and aqueous extracts of leaves of *E. hirta* could be safely administered to animals at doses as high as 5000 mg/kg b.w. and the LD50 value of the drug was 0% at the dose of 5000 mg/kg b.w with no mortality being observed.

Table 3. Antibacterial activity of steam and aqueous extracts of leaves of *E. hirta* determined by agar well diffusion assay.

Test samples	Concentrations (µg/mL)	Diameter of growth inhibition zone (mm)		
		SA	*EC*	*SB*
Steam extract	500	41.1 ± 0.10* ns	47.0 ± 0.05* ns	36.0 ± 0.12* ns
	1000	46.0 ± 0.10*	49.3 ± 0.20* ns	37.3 ± 0.15*
	2000	49.0 ± 0.25*	51.9 ± 0.29*	42.3 ± 0.05*
	5000	56.0 ± 0.49*	52.9 ± 0.36*	51.9 ± 0.15*
Aqueous extract	500	42.0 ± 0.05*ns	47.4 ± 0.10* ns	36.0 ± 0.10* ns
	1000	48.1 ± 0.10*	49.3 ± 0.10* ns	36.6 ± 0.05*
	2000	59.3 ± 0.23*	55.1 ± 0.30*	43.9 ± 0.05*
	5000	61.0 ± 0.40*	63.3 ± 0.60*	50.0 ± 0.05*
Amoxicillin	100	124.3 ± 0.70	93.1 ± 0.60	45.2 ± 0.20

Data represented as mean ± SD (n = 3). *Indicates statistically significant difference ($p<0.05$) between the Amoxicillin control group and the test groups (steam and aqueous extracts of leaves of *E. hirta*) against SA, EC and SB by means of Tukey's multiple comparison tests. 'ns' indicates statistically non-significant difference ($p>0.05$) between the two test groups (steam and aqueous extracts of leaves of *E. hirta*) at the same concentrations. SA: *Salmonella abony*; EC: *Escherichia coli*; SB: *Shigella boydii*.

Table 4. Antibacterial activity of steam and aqueous extracts of leaves of *E. hirta* determined by turbidimetric assay.

Test samples	Concentrations (µg/mL)	Growth inhibition (%)		
		SA	*EC*	*SB*
Steam extract	100	3.33 ± 1.79* ns	17.46 ± 9.1*ns	2 ± 1.02*ns
	500	23.93 ± 6.60* ns	25.54 ± 6.1*ns	19 ± 4.1*ns
	700	45.45 ± 10.90* ns	49.63 ± 9.4*ns	41 ± 3.9*ns
	1000	64.56 ± 5.80*ns	64.51 ± 9.6*ns	68 ± 2.9*ns
Aqueous extract	100	16.50 ± 4.07* ns	18.10 ± 11.5*ns	11 ± 3.8*ns
	500	29.22 ± 8.65* ns	32.17 ± 12.6*ns	20 ± 1.7*ns
	700	39.51 ± 9.61* ns	57.88 ± 12.5*ns	42 ± 12.9*ns
	1000	70.00 ± 9.40* ns	69.19 ± 10.8*ns	61 ± 9.8*ns
Amoxicillin	100	94.88 ± 3.37	87.87 ± 6.8	95 ± 3.7

Data represented as mean ± SD (n = 3). *Indicates statistically significant difference ($p<0.05$) between the amoxicillin control group and the test groups (steam and aqueous extracts of leaves of *E. hirta* against SA, EC and SB) by means of Tukey's multiple comparison test. 'ns' indicates statistically non-significant difference ($p>0.05$) between the two test groups (steam and aqueous extracts of leaves of *E. hirta*) at the same concentrations. SA: *Salmonella abony*; EC: *Escherichia coli*; SB: *Shigella boydii*.

Table 5. Protection of steam and aqueous extracts of leaves of *E. hirta* against diarrhea (n = 3).

Treatment	Dose (mg/kg b.w.)	Average weight of total wet feces (g)	Inhibition of defecation (%)
Steam extract	1621	0.093 ± 0.0261*ns	94.31
	2162	0.430 ± 0.6650 ns	73.71
Aqueous extract	100	1.073 ± 0.5093 ns	34.41
	200	0.853 ± 0.7870 ns	47.86
Loperamide	4	0.030 ± 0.0060* ns	98.16
Negative control	-	0.476 ± 0.1550 ns	-
Castor oil	-	1.636 ± 0.5680 ns	-

Data represents average weight of total wet feces ± SD (n=3). *Indicates statistically significant difference ($p<0.05$) in comparison to untreated castor oil control. 'ns' indicates statistically non-significant difference ($p>0.05$) between the two test groups (steam and aqueous extracts of leaves of *E. hirta*); between the two test groups (steam and aqueous extracts of leaves of *E. hirta*) and loperamide and between the two test groups (steam and aqueous extracts of leaves of *E. hirta*) and negative control.

Evaluation of antidiarrheal activity of steam and aqueous extracts of leaves of *E. hirta* in castor oil-induced diarrhea in Wistar rats

After administration of castor oil, the groups, which were treated with loperamide (4 mg/kg b.w.) and steam extract (1621 mg/kg b.w.) of leaves of *E. hirta* produced significant reduction in the fecal output when compared to the untreated group ($p<0.05$). The rats which were administered only castor oil (0.5 mL) produced watery feces. Aqueous extract at the doses of 100 mg/kg b.w. and 200 mg/kg b.w. showed concentration-dependent antidiarrheal activity whereas steam extract failed to exhibit concentration-dependent antidiarrheal activity at the evaluated doses of 1621 mg/kg b.w. and 2162 mg/kg b.w. This might be attributed to the possible irritant action due to the inflammation produced by *E. hirta* at higher doses leading to greater fecal output (Kinghorn and Evans, 1975). Steam extract of *E. hirta* at a dose of 1621 mg/kg b.w. brought about % inhibition in defecation (94.31%), which was comparable to the antidiarrheal activity of loperamide (98.16 % inhibition in defecation at 4 mg/kg b.w. dose). The results of the study are presented in Table 5.

No morphological or histological changes were observed in the colon of animals treated with the extract of *E. hirta* (Fig. 1).

Effect of steam and aqueous extracts of leaves of *E. hirta* extract on gastrointestinal motility of charcoal meal in Wistar rats

The peristaltic index of group, which was administered water alone was found to be greater than all the other groups. Groups that were administered steam extract at the doses of 1621 mg/kg b.w. and 2162 mg/kg b.w., aqueous extract at 100 mg/kg b.w. and loperamide at 4 mg/kg b.w. produced a significant reduction in the % transit of charcoal meal when compared to the group, which was administered water alone ($p<0.05$) (Table 6).

Evaluation of effect of steam and aqueous extracts of leaves of *E. hirta* on enteropooling in Wistar rats

After administration of castor oil, aqueous extract at a dose of 100 mg/kg b.w., high dose (2162 mg/kg b.w.) of steam extract of leaves of *E. hirta* and loperamide (4 mg/kg b.w.) produced significant reduction in the volume of intestinal content when compared to the untreated group ($p<0.05$) but, there was no significant reduction in the weight of the intestinal content ($p>0.05$). On the other hand, treatment of rats with low dose (1621 mg/kg b.w.) of steam extract of leaves of *E. hirta* reduced neither the volume nor the weight of the

intestinal content significantly when compared to the untreated group ($p>0.05$) (Table 7). The terpenoids and flavonoids in *E. hirta* extract maybe responsible for the reduced gut secretion by impeding NO synthesis (Sisay et al., 2017).

Ex vivo evaluation of contractile responses of steam and aqueous extracts of leaves of *E. hirta* on isolated rat ileum

Concentrations ranging from 0.5 to 4 µg/mL of steam and aqueous extracts of leaves of *E. hirta* were evaluated for their smooth muscle relaxation activity on isolated rat ileum. When compared to atropine (0.05-0.4 µg/mL), dose-dependent increase in smooth muscle relaxation was observed in steam extract (0.5-4 µg/mL) whereas aqueous extract did not exhibit any smooth muscle relaxation activity beyond the dose of 0.5 µg/mL (Table 8). The smooth muscle relaxation produced by the steam extract at doses 0.5 µg/mL and 1 µg/mL on the isolated rat ileum was comparable to the smooth muscle relaxation produced by atropine (0.05- 0.1 µg/mL).

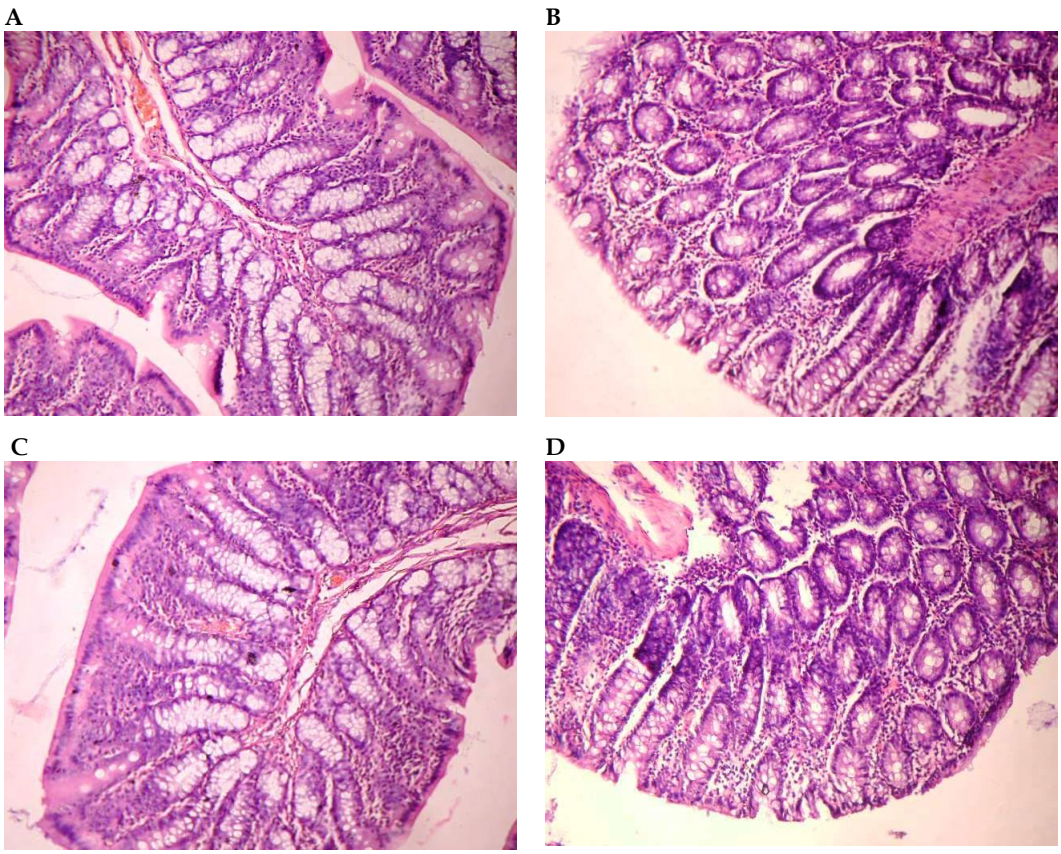

Figure 1. Rat colon histological sections for evaluation of antidiarrheal activity.
A and B: Groups treated with 1621 mg/kg b.w. steam extract and 200 mg/kg b.w. aqueous extract of leaves of *E. hirta* respectively; **C**: Negative control; **D**: Loperamide 4 mg/kg b.w.

Table 6. Effect of steam and aqueous extracts of leaves of *E. hirta* on gastrointestinal motility in Wistar rats.

Treatment	Dose (mg/kg b.w.)	Length of small intestine (cm)	Distance moved by charcoal meal (cm)	% transit/ peristaltic index	Inhibition (%)
Steam extract	1621	83.33 ± 3.65	64.16 ± 3.09	77.20	9.25*ns
	2162	87.16 ± 10.26	66.66 ± 7.09	76.81	9.71* ns
Aqueous extract	100	84.50 ± 5.01	61.00 ± 9.11	72.17	15.10* ns
Loperamide	4	92.56 ± 2.34	48.00 ± 13.04	52.09	38.76* ns
Negative control	-	77.60 ± 4.31	65.76 ± 1.22	85.07	-

Data represents length of small intestine ± SD (n = 3) and distance moved by charcoal meal ± SD (n = 3). *Indicates statistically significant difference ($p<0.05$) in comparison to negative control. 'ns' indicates statistically non-significant difference ($p>0.05$) between the two test groups (steam and aqueous extracts of leaves of *E. hirta*) and between the two test groups (steam and aqueous extracts of leaves of *E. hirta*) and loperamide.

Table 7. Effect of steam and aqueous extracts of leaves of *E. hirta* on gastrointestinal fluid accumulation in Wistar rats (n = 3).

Treatment	Dose (mg/kg b.w.)	Volume of intestinal content (mL)	Reduction (%)	Weight of intestinal content (g)	Reduction (%)
Steam extract	1621	2.16 ± 0.760	35.13	2.51 ±0.44	25.51ns
	2162	1.66 ± 0.572	50.15*	2.80 ± 0.60	16.91ns
Aqueous extract	100	1.86 ± 0.230	44.14	2.17 ± 0.30	35.60ns
Loperamide	4	1.03 ± 0.550	69.06*	1.35 ± 0.65	59.94*ns
Negative control	-	1.06 ± 0.513	-	0.96 ± 0.70	-
Castor oil	-	3.33 ± 0.098	-	3.37 ± 1.37	-

Data represents volume of intestinal content ± SD (n = 3) and weight of intestinal content ± SD (n = 3). *Indicates statistically significant difference ($p<0.05$) in comparison to untreated castor oil control. 'ns' indicates statistically non-significant difference ($p>0.05$) between the two test groups (steam and aqueous extracts of leaves of *E. hirta*); between the two test groups (steam and aqueous extracts of leaves of *E. hirta*) and loperamide; between the two test groups (steam and aqueous extracts of leaves of *E. hirta*) and negative control and between the two test groups (steam and aqueous extracts of leaves of *E. hirta*) and untreated castor oil control.

Table 8. Effect of steam and aqueous extracts of leaves of *E. hirta* on contractility of isolated rat ileum.

Treatment	Dose (µg/mL)	Response recorded (mm)	Response recorded (%)
Steam extract	0.5	20 ± 6.2ns	100
	1.0	18 ± 1.0ns	90
	2.0	11 ± 2.0ns	55
	4.0	13 ± 6.5ns	65
Aqueous extract	0.50	3 ± 1.70ns	100
Atropine	0.05	26 ± 5.20	100
	0.10	23 ± 3.64	88.46
	0.20	5 ± 2.64	19.23
	0.40	4 ± 2.00	15.38

Data represents contractile responses recorded ± SD (n = 3). 'ns' indicates statistically non-significant difference ($p>0.05$) in comparison to atropine control.

DISCUSSION

Diarrhea is the second leading cause of death in children below 5 years of age. Medicinal plants possessing antidiarrheal activity have been reported to exert their action owing to their antispasmodic effect, suppressed gut motility, increased water adsorption or decreased electrolyte secretion. Reports exist, which attribute the antidiarrheal activity of herbal medicines to the presence of phytochemicals such as tannins and flavonoids, which act by increasing colonic water and electrolyte reabsorption (Palombo et al., 2006). Flavonoids have also been reported to possess antidiarrheal activity through inhibition of intestinal motility and antimicrobial activity. Tannins reduce peristaltic index by decreasing the irritability of the bowel (Mulla et al., 2011). Inspite of cheap and effective antidiarrheal treatments available, many patients as well as physicians opt for phytomedicines owing to their safety and reduced side effects. Hence, steam and aqueous extracts of leaves of *E. hirta* were evaluated for their antidiarrheal activity.

Diarrhea is caused by a number of bacterial, viral and parasitic organisms, usually spread by feces-contaminated water. The steam as well as aqueous extracts of leaves of *E. hirta* demonstrated antiprotozoal activity by REMA with MICs of 125-250 µg/mL and 62.5-125 µg/mL against *Entamoeba histolytica* and 62.5-125 µg/mL and 31.25-62.5 µg/mL against *Giardia lamblia,* respectively with the aqueous extract demonstrating significantly better ($p<0.05$) antiprotozoal activity than the steam extract against both *Entamoeba histolytica* and *Giardia lamblia*. Both the extracts showed antibacterial activity against *Salmonella abony*, *Escherichia coli* and *Shigella boydii* when evaluated by agar well diffusion and turbidimetric techniques.

The acute toxicity profile of the extracts was determined according to OECD 423 Guidelines (2005). Both steam as well as aqueous extracts of leaves of *E. hirta* were found to be safe at doses as high as 5000 mg/ kg b.w.

Diarrhea results from the imbalance between secretory and absorptive processes of the gastrointestinal tract and disturbance in gastric motility. Castor oil has been used as diarrhea inducer due to the presence of ricinoleic acid, which acts by releasing nitric oxide and consequently increases the gastrointestinal membrane permeability for calcium. This in turn stimulates prostaglandin synthesis resulting in vasodilation, increased flow of fluid and electrolytes into the lumen of the bowel, smooth muscle contraction, mucus secretion and increased peristalsis (Sisay et al., 2017).

Castor oil-induced diarrhea model was used to evaluate the antidiarrheal potential of *E. hirta*. Total weight of wet feces and % reduction in fecal output were recorded. The steam extract at a dose of 1621 mg/kg produced % inhibition in defecation comparable to loperamide. Aqueous extract of leaves of *E. hirta* produced significant reduction in fecal output when compared to castor oil ($p<0.05$). The aqueous extract showed concentration dependent antidiarrheal activity whereas the steam extract failed to exhibit concentration dependent antidiarrheal activity. This might be attributed to the possible irritant action of *E. hirta* at higher doses. Since the extracts of *E. hirta* inhibited the ricinoleic acid-induced diarrhea, it can be assumed that its antidiarrheal action is mediated by an antisecretory mechanism.

Antidiarrheal agents can exert their activity by reducing the gastrointestinal motility. However, it was observed that both steam as well as aqueous extracts of leaves of *E. hirta* did not produce a significant reduction in % transit when compared to negative control. On the other hand, the extracts produced antispasmodic activity in isolated rat ileum preparation.

To assess the gastric fluid accumulation, extracts were evaluated in an enteropooling model. High dose of steam extract and aqueous extract of leaves of *E. hirta* and loperamide produced significant inhibition in the volume of intestinal content when compared to castor oil ($p<0.05$) whereas

they failed to significantly reduce the weight of the intestinal content ($p>0.05$). Treatment of rats with low dose of steam extract of leaves of *E. hirta* reduced neither the volume nor the weight of the intestinal content significantly when compared to castor oil ($p>0.05$). It has been reported that terpenoids and flavonoids impede NO synthesis which plays a role in gut secretion (Sisay et al., 2017).

In addition to its antimotility and antisecretory effects, *E. hirta* extract has demonstrated good antibacterial and antiprotozoal activity. Hence, *E. hirta* can possibly be a good candidate for diarrhea resulting from diverse etiologies.

CONCLUSIONS

The results of our study suggest that the leaves of *E. hirta* have significant antidiarrheal activity. *E. hirta* leaf extracts exhibited good *in vitro* antibacterial and antiprotozoal activity. The formation of soft feces in enteropooling studies also indicate that the drug may have osmotic effect, thus improving the fecal consistency without inducing diarrhea. Results of evaluation of gastrointestinal motility have shown that the drug inhibits the % transit of charcoal meal in the small intestine of the rats. The *ex vivo* studies performed to determine the effect of the extracts on contractile response of excised ileum revealed that the extracts possess smooth muscle relaxant activity. Leaves of *E. hirta* have the potential to be further developed as an effective antidiarrheal agent. Further studies need to be carried out to completely elucidate the mechanism of antidiarrheal activity of the plant.

CONFLICT OF INTEREST

The authors declare no conflicts of interests.

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Contribution	Shah R	Ravat J	Momin M	Mukne A
Concepts or ideas			x	x
Design			x	x
Definition of intellectual content	x	x	x	x
Literature search	x	x	x	x
Experimental studies	x	x	x	X
Data acquisition	x	x	x	x
Data analysis	x	x	x	x
Statistical analysis	x	x	x	x
Manuscript preparation	x	x	x	x
Manuscript editing			x	x
Manuscript review	x	x	x	x

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