Therapeutic efficacy of the leaf extract of *Croton joufra* Roxb. against experimental cestodiasis in rats

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Received: 20 May 2015 / Accepted: 1 August 2016 / Published online: 16 August 2016
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Abstract The leaves of *Croton joufra* have been used as a traditional medicine in Assam (Northeast India) to treat the intestinal helminthic infections. The aim of this study was to establish the in vitro and in vivo anthelmintic efficacy of the leaf extract of this plant on *Hymenolepis diminuta*, a zoonotic tapeworm. The plant extract was also screened for its chemical constituents. The in vitro anthelmintic efficacy of the plant was evaluated by physical motility test on adult cestodes, following their exposure to 10, 20, 30 mg/ml concentrations of the crude leaf extract, while the in vivo effect was determined based on the eggs per gram (EPG) counts and worm recovery rates, using an experimentally induced model of *H. diminuta* infections in Wistar rats. The in vitro study results showed a dose-dependent effect on the parasite and the highest concentration of the extract (30 mg/ml) caused mortality of the worms in 1.53 ± 0.12 h as compared to 3.46 ± 0.10 h shown by 1 mg/ml concentration of the reference drug, praziquantel (PZQ). The in vivo results showed that a single 800 mg/kg dose of the extract, given for 3 days, was highly efficacious against adult parasites as it reduced the EPG count and worm burdens of rats by 94.74 and 75 %, respectively. The extract also showed almost similar degree of effects against the larval stage of the parasite. As ascertained by the qualitative screening tests, the leaf extract of *C. joufra* contains terpenoids, tannins, alkaloids, flavonoids, glycosides and reducing sugar. The overall findings of this study suggest that *C. joufra* leaf extract possesses significant

anthelmintic efficacy and support its traditional anthelmintic claims.

Keywords *Croton joufra* · *Hymenolepis diminuta* · Anthelmintic · Anticestodal · Pharmacology · Intestinal helminths · Soil-transmitted helminths

Introduction

Medicinal plants have been used as primary source of treatments against various diseases and ailments, including helminthic infections since time immemorial, yet it is only in the past 20–30 years that they have received attention as a promising source for developing new drugs (Gurib-Fakim 2006). A literature review reveals that there exists an enormous variety of plant-based traditional remedies to treat parasitic infections (Githiori et al. 2005; Tandon et al. 2011). Several workers have attempted to scientifically validate the putative anthelmintic effects of such herbal remedies, using various in vitro and in vivo models (Nisha et al. 2007; Zahir et al. 2009; Yadav and Temjenmongla 2012).

Croton joufra Roxb., a member of Euphorbiaceae family, is a small tree and is found distributed at altitudes below 1000 m ASL in India, Bangladesh, Bhutan, Myanmar and Vietnam. The Mishnging tribe in the state of Assam (Northeast India) uses the leaves of this plant, locally called as ‘*Gochmahudi*’, as a traditional medicine to treat the intestinal worm infections. To the best of our knowledge, no scientific evidence is available in the literature about the anthelmintic properties of this plant. Therefore, the present study was undertaken to investigate the therapeutic effects, if any, of *C. joufra* leaf extract using against *Hymenolepis diminuta*, a zoonotic cestode parasite. In

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addition, the leaves of this plant were also subjected to analysis of their chemical constituents.

Materials and methods

Plant material and extraction

The fresh leaves of *C. joufra* (Fig. 1) were collected in October 2011 from Lakhimpur district of Assam and duly identified. The leaves were dried under shade and powdered for extraction with methanol using Soxhlet extractor at 40 °C as per the procedure described by Redfern et al. (2014), with slight modifications. The final yield of the extract was 0.74 % from the fresh plant material.

Phytochemical studies

The qualitative phytochemical analysis of the leaf extract of plant was carried out to confirm the presence of various chemical constituents, following the procedures described by Harborne (1973), Trease and Evans (1989), and Sofowra (1993).

Experimental animals

Hymenolepis diminuta (Cestoda: Hymenolepididae) was selected as model parasite to induce the experimental cestodiasis in Wistar rats. Animals of about 200–250 g of both sexes were utilized as host for this purpose. All the animals were deparasitised with 5 mg/kg dose of praziquantel and their faecal samples were examined to confirm that they are free from any intestinal cestode infections. Due approval was obtained from the Institutional Animal

Fig. 1 The leaves of *Croton joufra* Roxb.

Ethics Committee (Animal model) of North-Eastern Hill University, Shillong prior to experiments on animals.

Acute toxicity study

The acute oral toxicity study was conducted on female swiss albino mice using the limit doses of 2000 and 5000 mg/kg body weight of extract given orally (OECD 2008). In this study, five animals were dosed individually, with each dose given in a sequential manner. Following extract treatments, the animals were observed carefully for adverse clinical signs, such as tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma, gait and posture or mortality for 14 days.

Anthelmintic testing of extract

In vitro testing

Adult *H. diminuta* worms were collected from the laboratory-maintained infection in Wistar rats. After washing, the worms were transferred to petri dishes containing phosphate buffered saline (PBS) and maintained at 37 ± 1 °C in an incubator. Three concentrations, i.e., 10, 20 and 30 mg/ml, of the extract were selected as test doses praziquantel (1 mg/ml) was used as the reference drug to compare the efficacy of the extract. For all treatments, a set of five worms (three replicates) was used controls were maintained in PBS at 37 °C. The *in vitro* anthelmintic efficacy of extract was determined in terms of physical motility of worms, as evidenced by either paralysis or mortality of the test worms. In order to confirm the mortality of test parasites, two types of PBS solutions, i.e. one warm (37 ± 1 °C) and the other slightly warmer (40 ± 1 °C), were employed. The non-motile worms were first put inside the warm PBS solution and when they showed no movements during their exposure to PBS solution, they were considered as paralyzed. When the worms showed no movements in the warm PBS after keeping for about 5 min, they were immediately transferred to the more warm PBS solution so as to finally confirm their mortality (Vijaya and Yadav 2014). The mortality of parasites was assumed to have occurred when all the signs of movements had ceased even after exposing to slightly warmer PBS solution for 30 min (Vijaya and Yadav 2014). Three sets of replica were undertaken for each experiment.

In vivo testing

H. diminuta infection was maintained in the laboratory by alternating the hosts, Wistar rats/Flour beetles (Yadav and Tangpu 2012). In brief, the gravid segments of *H. diminuta* were obtained from laboratory infected rats and scratched

smoothly on to the filter papers inside the petri dishes, and the beetles were allowed to feed on flour for 72 h. These beetles were then maintained at room temperature for at least 12–14 days for the cysticeroid larva to develop. On dissecting upon the beetles, cysticeroids were collected and suspended in PBS. Using a blunt feeding needle, four cysticeroid larvae of *H. diminuta* were orally given to each Wistar rat to induce experimental cestodiasis (Yadav and Tangpu 2012). Efficacy of the crude extract was evaluated against the larval and adult stages of *H. diminuta*. For each stage, rats were divided into five groups, each comprising five animals. Against the larval stage of the parasite, the first group of animals was used as control; the second, third and fourth group of animals were orally given 200, 400 and 800 mg/kg dose of the extract, respectively on day 2, 3 and 4 post inoculation (p.i.) of cysticeroids. The fifth group of animals received 5 mg/kg of PZQ for the same duration. From day 18 p.i., faecal samples of rats were collected from each cage and the eggs per gram of faeces (EPG) counts were done for three consecutive days, i.e., days 18, 19 and 20 p.i. (Anonymous 1977). Finally, on day 31 p.i. rats were sacrificed and the percentage reduction in worm count was determined (Tangpu et al. 2004).

For the adult stages of parasite, almost similar methods were followed, except that there were minor differences treatments days and EPG counts of animals. Herein, the extract treatments were given on day 21–23 p.i. of cysticeroids and the EPG counts were done on day 18–20 p.i. (pretreatment period) and on 34–36 days (post treatment period).

Statistical analysis

All results are represented as mean $\pm$ standard error of the mean (SEM). All data were analyzed and compared by one-way ANOVA and Student's *t* test using Graph Pad Prism 5. *P* values < 0.05 were considered as statistically significant.

Results

Phytochemical studies

The qualitative phytochemical tests revealed that the leaf extract of this plant contains terpenoids, tannins, alkaloids, flavonoids, glycosides and reducing sugars as major classes of phytoconstituents.

Acute toxicity

None of the animals showed any signs of toxicity and mortality when dosed with 2000 or 5000 mg/kg limit doses

during the observation period of 14 days. LD50 for the plant extracts was found to be more than 5000 mg/kg body weight. Therefore, as per the Organization for Economic Cooperation and Development (OECD) guidelines, the oral LD50 of plant extract was estimated to be greater than 5000 mg/kg in mice.

Anthelmintic efficacy

In vitro testing of the leaf extract on adult *H. diminuta* showed dose-dependent effects (Fig. 2). At 30 mg/ml concentrations of extract, the tested worms got paralysed in 0.45 ± 0.10 h and then showed mortality in 1.53 ± 0.12 h. In comparison, the standard drug, PZQ showed onset of paralysis at 0.46 ± 0.10 h and mortality of worms, at 3.46 ± 0.10 h, respectively at 1 mg/ml concentration. In the control medium the physical activity of worms was observed till 32.44 ± 0.17 h (Fig. 2).

In case of in vivo testing of the extract against the cysticeroids of *H. diminuta*, the highest tested dose, i.e., 800 mg/kg, 92.73 % reduction in EPG counts and 70 % reduction in worm counts of the infected host animals was recorded post treatment. In treated animals the effects of the extract were quite comparable with those of the reference drug reference drug, PZQ at 5 mg/kg dose, wherein reductions in EPG count and worm counts were recorded to be 80.34 and 80 %, respectively (Table 1). In vivo testing of the extract against the adult stages of parasite also

Fig. 2 In vitro anthelmintic effects of *Croton joufra* on *Hymenolepis diminuta*. The physical activity of worms maintained in the control medium was observed till 32.44 h. All data are presented as mean $\pm$ SEM (n = 5). *Significant at $p \leq 0.05$ level

Table 1 Effects of the leaf extract of *Croton joufra* on larval stages of *Hymenolepis diminuta* in rats

Groups	EPG (mean $\pm$ SEM) days 18–20	Percentage reduction in EPG counts	No. of worms recovered/rat	Percentage reduction in worm counts (mean $\pm$ SEM)
Control	5866 $\pm$ 464.10		3.8 $\pm$ 0.20	5
<i>Plant extract</i> (mg/kg)				
200	2036 $\pm$ 384.35*	–65.29	2.6 $\pm$ 0.24**	35
400	1070 $\pm$ 155.44*	–81.75	1.8 $\pm$ 0.37**	55
800	426 $\pm$ 154.37*	–92.73	1.2 $\pm$ 0.20**	70
<i>Praziquantel</i> (mg/kg)				
5	1153 $\pm$ 232.09*	–80.34	0.8 $\pm$ 0.20**	80

Number of animals in each group = 5

* $p < 0.0001$ versus control, ANOVA test

** $p < 0.0001$ versus control, ANOVA test

Table 2 Effects of leaf extract of *Croton joufra* on adult stages of *Hymenolepis diminuta* in rats

Groups	EPG (mean $\pm$ SEM)		Percentage reduction in EPG count	No. of worms recovered/ rat (mean $\pm$ SEM)	Percentage reduction in worm counts
	Pre-treatment (days 18–20)	Post-treatment (days 34–36)			
Control	7100 $\pm$ 483.53	6900 $\pm$ 465.90	–2.81	3.8 $\pm$ 0.20	5
<i>Plant extract</i> (mg/kg)					
200	6596 $\pm$ 584.86	2350 $\pm$ 216.50*	–64.37	2.8 $\pm$ 0.37**	30
400	7606 $\pm$ 388.70	930 $\pm$ 258.18*	–87.78	1.6 $\pm$ 0.24**	60
800	6466 $\pm$ 640.88	340 $\pm$ 124.99*	–94.74	1.0 $\pm$ 0.31**	75
<i>Praziquantel</i> (mg/kg)					
5	6166 $\pm$ 584.86	763 $\pm$ 197.45*	–87.62	0.6 $\pm$ 0.24**	85

Number of animals in each group = 5

* $p < 0.0001$, compared with pre-treatment value, ANOVA test

** $p < 0.0001$, compared with control, ANOVA test

showed almost a similar trend as that in the cysticercoid stage of the parasite. The animals treated with 800 mg/kg dose of the extract showed 94.74 % reduction in the EPG counts and 75 % in worm burden at the post-treatment period, as compared to 87.62 and 85 % reduction, respectively caused by 5 mg/kg dose of PZQ. On the other hand, the animals in the control group showed almost negligible reductions in EPG counts and worm counts throughout the entire observation period (Table 2).

Discussion

In recent years there has been a renewed interest to discover new pharmaceuticals from medicinal plants (Patwardhan 2005). With regard to parasitic diseases, a need to develop new drugs has been emphasized more, because as compared to other infectious diseases, only a limited number of drugs are available to treat parasitic infections. In this connection, medicinal plants not only offer a good

source for developing new antiparasitic drugs (Anthony et al. 2005), but in some regions they are the only readily available source to treat parasitic infections. In the present study we tried to investigate the anthelmintic potential of *C. joufra*, because it has long been used as a traditional remedy for intestinal helminthic infections by Mishing tribes of Assam.

In acute oral toxicity testing, none of the animals showed any signs of toxicity and mortality when given 2000 or 5000 mg/kg limit doses of extract. Hence, the oral LD50 of extract was estimated to be greater than 5000 mg/kg in mice as per the criterion set by OECD guidelines (OECD 2008). Regarding the anthelmintic studies on medicinal plants it emerges that various previous workers have assessed the anthelmintic potentials of different medicinal plants using either in vitro assays (Vijaya and Yadav 2014) or in vivo models (Tangpu et al. 2004; Yadav and Temjenmongla 2012). However, it is only in few cases that an attempt has been made to employ both in vitro and in vivo tests to comment upon precisely the anthelmintic

effects of a particular medicinal plant (Nath and Yadav 2015).

In the present study, *C. joufra* leaf extract showed dose-dependent in vitro and in vivo effects on *H. diminuta*. These results are in agreement with other related studies through which considering the similar dose-dependent effects, the anthelmintic credentials of various medicinal plants were established (Temjenmongla and Yadav 2005; Kundu and Lyndem 2012). In this study, at 30 mg concentration, the worms showed paralysis in about 0.45 h; however, in a related study by Kundu and Lyndem (2012) on effects of three *Cassia* spp. on fowl cestode, *Raillietina tetragona*, the plant extracts showed paralysis of worms within 1.68–4.13 h.

In the in vivo study against the larval and adult *H. diminuta* in rats, the plant extract showed significant reduction in EPG counts as well as worm counts against both the stages of the parasite. In vivo studies using *Solanum myriacanthum* fruit extract and *Clerodendrum colebrookianum* leaf extract revealed that while *S. myriacanthum* extract possesses rather more significant effect on adult stages than the larval stages of the parasite, *C. colebrookianum* extract bears significant efficacy only against the adult worms (Yadav and Tangpu 2012; Yadav and Temjenmongla 2012). It is known that activation of the larval scolex and excystation of *H. diminuta* cysticercoids takes place in the duodenum of the host. Therefore, it seems likely that the *C. joufra* extract when administered against the larval stage of parasite, induces its efficacy by affecting either the excystation or establishment of the parasite, which in turn may result in reduction of EPG counts and worm counts following treatment. In related studies on effects of papaya seeds and *Mallotus philippinensis* fruit extract on *H. diminuta* infections in rats, Sapaat et al. (2012) and Gangwar et al. (2012) also reported a similar kind of reduction in EPG counts of animals at post-treatment. In this study, for adult worms, the 800 mg/kg dose of the extract showed 75 % reduction in worm counts at post-treatment of extract. Yadav and Temjenmongla (2011) in their study on anticestodal activity of *Houttuynia cordata* leaf extract against adult *H. diminuta* infections in rats also reported almost a similar level of reduction in worm counts of treated animals.

The qualitative phytochemical study on the leaf extract of *C. joufra* revealed that it contains terpenoids, tannins, alkaloids, flavonoids, glycosides and reducing sugars. There are enough evidences available to support that phytochemicals, like flavonoids, tannins alkaloids and terpenoids, etc., possess significant anthelmintic effects against cestode parasites (Tandon et al. 1997; Parida et al. 2010; Ali et al. 2012; Semwal and Semwal 2015). For example, *Stephania glabra*, which is as an anthelmintic against intestinal worms in Meghalaya, has shown

significant effects against a cestode, *Raillietina echinobothrida* (Tandon et al. 2004). According to Semwal and Semwal (2015), as well as a study by Tandon et al. (2004) on *S. glabra*, it has been revealed that it usually contains alkaloids together with other constituents in lesser amounts. In addition, Das et al. (2009) has also identified the active principle of *S. glabra* as tetrahydropalmatine, which is an alkaloid. More recently, a study by Dhakal et al. (2015) also conclusively proved that tannins from three different plants (*Pinus* sp., *Corylus avellana* and *Trifolium repens*) bring out significant in vitro anthelmintic effects on *Hymenolepis diminuta*. Likewise, Palomares-Alonso et al. (2015) also reported that the in vitro and in vivo cysticidal activity of *Teloxys graveolens* against *Taenia crassiceps* cysts is due to its flavonoid active principle, pinocembrin. Therefore, it is assumed that either of the reported phytoconstituents of *C. joufra* may be responsible for its observed anthelmintic activity in the present study. Further studies are underway in our laboratory to isolate and identify the active principle of this plant.

In conclusion, the results of the present study indicate that *C. joufra* leaf extract possesses significant anthelmintic effects against cestode parasite and supports the traditional use of this plant as anthelmintic.

Acknowledgments Necessary facilities to carry out this work were provided by the Department of Zoology, North-Eastern Hill University, Shillong. Shyamalima Gogoi was recipient of University Grants Commission Non-NET fellowship from NEHU, Shillong. The plant material was identified by Dr. Amal Kumar Dutta, Department of Botany, Dhakuakhana College, Lakhimpur (Assam).

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