In vitro and in vivo anthelmintic activity of *Murraya koenigii* against gastro-intestinal nematodes of sheep

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Abstract The present study have been conducted to evaluate the anthelmintic activity of crude aqueous and crude methanolic leaf extracts of *Murraya koenigii*. Infection of ruminants with gastro-intestinal (GI) parasite has become a worldwide problem. The parasite causes economic losses in a variety of ways. Previously sheep producers relied heavily on anti-parasitic drugs to control gastro-intestinal parasites of the flocks. But due to misuse of these drugs the parasites become resistant to drugs. Thus created interest in studying medicinal plants as an alternative source of controlling the GI parasites. Adult motility assay (AMA) and egg hatch assay (EHA) have been done for in vitro study, and faecal egg count reduction (FECR) assay have been done for in vivo study. The in vitro study revealed anthelmintic effects of *M. koenigii* on *Haemonchus contortus* as evident from their paralytic condition and/or death at eight hour post exposure in different concentrations (12.5, 25, 50 mg/ml) of aqueous and methanolic extracts which exhibit to be dose-dependent. Aqueous and methanolic extracts of *M. koenigii* were found to have low percent inhibitory effect on egg hatching. It may be concluded that *M. koenigii* showed significant anthelmintic activity.

Keywords Anthelmintics · *Murraya koenigii* · Ruminants · *Haemonchus contortus*

Introduction

Gastrointestinal parasites cause a major constraint for the breeding of small ruminants throughout the world. This

problem explains the current interest in search of alternative solutions in order to reduce the parasites by chemotherapy. In particular, there is renewed interest in veterinary drugs and plants with anthelmintic properties in both temperate and tropical countries (Hammond et al. 1997; Waller et al. 2001).

Compared with other GI nematode parasites, *H. contortus* is a highly pathogenic parasite of live stock and is capable of causing acute disease and high mortality in small ruminants. Commercial anthelmintic drugs have been used for some decades throughout the world to minimize the losses caused by helminth infections. However, the threats of anthelmintic resistance, availability, and high cost especially to farmers of low income group have led to the need of other alternative control methods (Baker et al. 1992).

Tandon et al. (1997) studied in vitro anthelmintic activity of root tuber extract of *Flemingia vestita* against *Paramphistomum* sp. The treatment of gastro-intestinal parasites with the crude extract (50 mg/ml) in PBS revealed complete immobilization of the trematodes in about 43 min. Akhtar et al. (1999) studied the anthelmintic activity of *Chenopodium album* against sheep nematodes. The aqueous and ethanolic extracts of *C. album* showed significant percentage of egg reduction.

Athanasiadou et al. (2000) studied the possible direct anthelmintic effect of a condensed tannins (CTs) extract (Quebracho extracted from *Schinopsis*) on the population and fecundity of the *T. colubriformis*. In the treated sheep, the worm burdens and EPG of faeces per worm had been reduced by 30 % in comparison to control sheep. In 2001 the same authors studied the possible direct anthelmintic effects of CT from Quebracho towards different ovine GI nematode larvae (*H. contortus*, *T. circumcinata* and *T. vitrinus*). Brelin (2002) evaluated the Neem leaves

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(*Azadirachta indica*) as an alternative anthelmintic for helminth control. They observed that fresh neem leaves significantly reduced the numbers of *H. contortus* in the abomassum of the treated sheep.

Alawa et al. (2003) examined the in vitro anthelmintic activity of *Vernonia amygdalina* and *Annona senegalensis* extracts against *H. contortus* eggs and reported that the extract of *V. amygdalina* did not show any significant activity at concentrations up to 11.2 mg/ml. However, the extract of *A. senegalensis* showed significant ($P < 0.001$) reduction in egg hatch at a concentration of 7.1 mg/ml. Lateef et al. (2003) explored in vitro and in vivo anthelmintic activity of *Adhatoda vasica* in comparison to levamisole. The in vitro studies revealed anthelmintic effects ($P \geq 0.05$) of crude aqueous extracts (CAE) and crude methanolic extracts (CME) of *A. vasica* on *H. contortus* as evident from their mortality.

Ademola et al. (2004) studied the in vitro and in vivo anthelmintic activity of ethanolic as well as aqueous extracts of *Khaya senegalensis* against GI nematodes of sheep and reported that nematode larvae decreased after treatment. Maphosa et al. (2010) evaluated the crude aqueous extracts of leaves of *Aloe ferox* and *Leonotis leonurus* and roots of *Elephantorrhiza elephantina* for anthelmintic activity. They reported that inhibition of egg hatching and larval development increased significantly ($P < 0.05$) with increasing concentrations of the crude extracts. *E. elephantina* and *L. leonurus* extracts had 100 % egg hatch inhibition at concentration as low as 2.5 and 1.25 mg/ml respectively, whereas extracts of *A. ferox* had 100 % inhibition at concentrations of 20 mg/ml. At the lowest concentration tested (0.625 mg/ml), *E. elephantina* inhibited egg hatching is greater than 96 % and this was comparable to albendazole at the same concentration. However, *E. elephantina* and *L. leonurus* inhibited totally the larval development at concentrations of 1.25 mg/ml. Jaliwala et al. (2011) conducted a study to evaluate the anthelmintic activity of *Argemone mexicana* aqueous and alcoholic extracts. Both alcoholic and aqueous extracts has shown significant anthelmintic activity in comparison to standard drug albendazole.

The present study was designed to evaluate the in vitro and in vivo anthelmintic efficacy of crude aqueous and crude methanolic leaf extracts of *M. koenigii* upon gastrointestinal nematode parasites.

Materials and methods

Collection of plant materials

Fresh leaves of locally available medicinal plant namely, *M. koenigii* L. (Curry) (Fig. 1) were collected from the garden of

the Department of Botany, University of Kalyani and Faculty of Agriculture, Bidhan Chandra Krishi Viswavidyalaya, Kalyani campus, West Bengal. The plants were identified based on their physical characteristics by the plant taxonomists. *M. koenigii* belonging to family Rutaceae is used as a spice for its characteristic flavour and aroma. It is reported to have anti-oxidant, anti-diabetic, anticarcinogenic, antidiysenteric, stimulant, hypoglycaemic and antimicrobial activities (Khanu et al. 2000; Ningappa et al. 2008). Phytochemical investigations on this plant revealed the presence of carbazole alkaloid and coumarin (Adebajo and Reisch 2000).

Preparation of plant extracts

Aqueous extract

The crude aqueous extracts of selected plant were prepared according to the method as described by Iqbal et al. (2006a). The powdered plant parts (500 g) were extracted with distilled water (2,000 ml) at 90–100 °C in a Soxhlet extractor for 8 h. The extracts were filtered, evaporated under reduced pressure of 22–26 mmHg in a vacuum rotary evaporator at 55 °C and the aqueous extract were stored at 4 °C for further use.

Methanolic extract

The powdered plant parts (500 g) were extracted with methanol (2,800 ml, Qualigens) at 60 °C in a Soxhlet extractor using for 8 h. The extracts were filtered, evaporated under reduced pressure of 22–26 mmHg in a vacuum rotary evaporator at 55 °C and methanolic extract were stored at 4 °C until used.

Fig. 1 Photograph of *M. koenigii*

In vitro study

Parasite collection

Adult female parasites of *H. contortus* were collected from the abomasum of infected sheep from local abattoir. Then the worms were washed and crushed to liberate eggs. The eggs were then cultured in a plastic jar filled with sterilised sheep faeces for 8 days at room temperature. At the end of the eighth day, the infective (L₃) larvae were harvested by rinsing the side of the culture jar with a drop of water. After overnight withdrawal of feed and water two sheep were administered orally with the third stage infective larvae (L₃) at the dose rate of seven hundred L₃/kg body weight using a 50-ml plastic syringe. These sheep served as *H. contortus* egg donors for the egg hatch assay trial.

Egg hatch assay (EHA)

Egg hatch assay was conducted following the procedure described by Coles et al. (2006). Approximately, fifty eggs were collected per tube; each tube contained 1 ml of

contortus were manually picked up from the mucosal surface and the contents of the abomasum have been kept in phosphate buffer saline (PBS). The experiment was conducted according to Egualé et al. (2007). Fifty actively moving worms were placed in petri-dishes containing 12.5, 25 and 50 mg/ml of aqueous and methanolic extracts of plants in PBS and PBS alone for the control group in a total volume of 4 ml. Levamisole was used as the positive control. Five replications per each treatment concentration were employed. After 24 h, the plant extracts and levamisole were washed away and the parasites suspended in PBS for 30 min for possible recovery of parasite motility. The numbers of motile and immotile worms were counted. Motility and viability of the worms were assessed by gently prodding the worms using a pointed probe or forceps. The response was recorded either as live or dead condition. Observations were made on the motility or survival of parasites at 0, 2, 4, 6 and 8 h post-exposure (PE).

Mortality index (MI): The mortality index have been calculated by adopting the following formula.

$$\text{Mortality Index} = \frac{\text{Total number of immobile worms}}{\text{Total no. of worms per well}}$$

$$\% \text{WMI} = \frac{(\text{No. of mobile worms in negative control}) - (\text{No. of mobile worms in treated group})}{\text{No. of mobile worms in negative control}} \times 100$$

phosphate buffer saline (PBS) and 1 ml of increasing concentrations of plant extracts (75, 150, 300, 600, 1,200 and 2,400 µg/ml) prepared with phosphate buffer saline (PBS). Each concentration was tested on five replicates. In addition, levamisole (0.125 mg/ml) was used as positive control and PBS as negative controls in the experiment. The tubes were covered, and the eggs were incubated for 48 h at 27 °C. Thereafter, the number of the first stage larvae (L₁) present per tube was counted using a dissecting microscope. An inhibition percentage (%) of egg hatching was calculated for each extract concentration using the following modified formula of Coles et al. (1992):

$$\text{Inhibition (\%)} = 100 \times \frac{1 - X_1}{X_2}$$

where X₁ is the number of eggs hatched in test extracts, and X₂ is the respective number in Phosphate Buffer Saline (PBS) control.

Adult motility assay (AMA)

The abomasum of sheep slaughtered at the local abattoir were brought to the laboratory. Adult stages of *H.*

In vivo study

Animals and experimental design

A total twenty four sheep of both genders (one year of age) having naturally acquired gastro-intestinal (GI) nematode infection were collected. In vivo studies were performed using the faecal egg count reduction assay. All the sheep were pre-adapted to the pen conditions for 18 days prior to commencement of the study. The water, hay and feed were provided regularly to the experimental animals. The experiment continued for a period of 15 days post-treatments. Before the commencement of the experiment, the animals were confirmed positive with an infection of mixed gastro-intestinal (GI) nematode parasites by faecal examination using the standard parasitological procedures applicable to detection of nematode eggs in sheep faeces (Soulsby 1982). The animals used for the experiment were randomly divided into six treatment groups (i.e. Gr I, Gr II, Gr III, Gr IV, Gr V and Gr VI) of four animals each on the basis of faecal egg counts (mean ± S.E. of eggs per gram of faeces) and assigned to different treatments which were administered orally using a syringe. Group I received a

single dose of crude methanolic extract (CME) at 1.0 g kg^{-1} body weight (bw). Group II received crude aqueous extract (CAE) at 1.0 g kg^{-1} body weight (bw). Group III received crude methanolic extract (CME) at 2.0 g kg^{-1} body weight (bw). Group IV received crude aqueous extract (CAE) at 2.0 g kg^{-1} body weight (bw). Group V received levamisole at 7.5 mg kg^{-1} body weight (bw) as positive control. Group VI received no treatment and served as negative control. Each group was isolated from other groups and no physical contact was possible between them from different treatment groups.

Laboratory procedure

To determine the faecal egg count reductions of gastrointestinal (GI) nematode parasites in sheep, faecal samples of each animal in the respective treatment groups were collected directly from the rectum in the morning, starting from day zero and at days five, ten and fifteen post-treatment (PT). The faecal samples were homogenized so that the eggs were uniformly distributed throughout the faeces prior to counting. The total numbers of nematode eggs (faecal egg counts) were determined using McMaster egg counting technique (Soulsby 1982); with each egg counted representing fifty eggs per gram of faeces. Faecal egg count percent reduction (FECR %) was calculated using the following formula:

$$\text{FECR}\% = \frac{(\text{Pre treatment egg count per gram}) - (\text{Post treatment egg count per gram})}{(\text{Pre treatment egg count per gram})} \times 100.$$

Phytochemical screening

Preliminary qualitative screenings for major secondary metabolites of the medicinal plant were conducted according to Debella (2002). The plant materials were screened for the presence of protein and amino acid, fatty acid, carbohydrate, tannin, saponin, alkaloid, flavonoid, steroid, triterpenoid, glycoside and phenol.

The statistical package-SPSS-10.0 was applied for the analysis of data and $P < 0.05$ was taken as the level of significance.

Results

The major secondary metabolites detected were tannin, saponin, phenol, triterpenoid and flavonoid (Table 1).

Adult motility assay (AMA)

Effect of crude aqueous extracts and crude methanolic extracts of *M. koenigii* (CMEMK) was dose-dependent (Fig. 2). Highest mortality (100 %) of worms have been observed in eight hours post-exposure @ 50 mg/ml of crude methanolic extract. It resulted in mean percentage worm motility inhibition (%WMI) of 93.52 %, as observed after the worms were put in PBS for thirty minutes after exposure to different treatments. The percentage of worm mortality index of the crude methanolic extract and crude aqueous extract of *M. koenigii* (CAEMK) were hundred percent after eight hours post treatment. The methanolic extract of *M. koenigii* was more potent than the aqueous extract. There was hundred percent mortality of worms in levamisole (used as a reference drug) within two hours post-exposure. The result of in vitro anthelmintic activity of crude methanolic extract of *M. koenigii* as compared with levamisole was statistically non-significant, and was statistically significant when compared with PBS.

Egg hatch assay (EHA)

The crude aqueous and crude methanolic extracts of *M. koenigii* exhibited ovicidal effects; whereas, egg hatching was not affected by the distilled water (negative control). Inhibition of egg hatching (percent egg hatch) for *H.*

contortus exposed to extracts of *M. koenigii* was low in comparison to eggs exposed to levamisole. Crude methanolic extract ($\text{LC}_{50} = 2.25 \text{ mg/ml}$) was found higher inhibitory effects in comparison to that of aqueous extract ($\text{LC}_{50} = 2.90 \text{ mg/ml}$) on egg hatching. The data of correlation of regression revealed a dose dependent response of plant extracts both for aqueous and methanolic. Lethal concentration 50 (LC_{50}) for the inhibition of egg hatching have been shown in Table 2.

Faecal egg count reduction test (FECRT)

The mean eggs per gram counts (EPG) and percentage reduction in faecal egg counts of Bonpala sheep treated with different doses of the aqueous and methanolic extracts of *M. koenigii* and levamisole have been presented in Fig. 3. The in vivo anthelmintic activity (in terms of egg count percentage reduction) of methanolic extracts of *M. koenigii* in sheep infected with mixed species of GI

Table 1 Phytochemical analysis of methanolic leaf extract of *M. koenigii*

Protein and amino acid	–
Fatty acid	–
Carbohydrate	–
Tannin	+
Saponin	+
Alkaloid	–
Flavonoid	+
Steroid	–
Triterpenoid	+
Glycoside	–
Phenol	+

+ indicates presence; – indicates absence

Fig. 2 In vitro anthelmintic efficacy of crude extracts of *M. koenigii* on *H. contortus***Table 2** Regression values and correlation of regression of the effect of *M. koenigii* different extracts on egg hatching of *H. contortus*

Treatment	LC ₅₀	Regression values and correlation of regression
Methanolic extract of <i>M. koenigii</i>	2.25	$y = -0.0002x + 4.6331$, $R^2 = 0.9685$
Aqueous extract of <i>M. koenigii</i>	2.90	$y = -0.0003x + 5.1328$, $R^2 = 0.9141$
Levamisole	1.80	$y = -0.2161x + 6.2452$, $R^2 = 0.755$

parasites demonstrated significant anthelmintic activity of extracts tested. The result revealed that a gradual reduction in faecal egg count (%FEC) of experimental animals treated with both the aqueous and methanolic plant extracts and were significant ($0 < 0.5$) on day fifteen post-treatment from day zero pre-treatment.

Discussion

The result of the present study suggested that the plant extracts exhibited significant in vitro and in vivo anthelmintic activity against *H. contortus* of sheep, and has the potential to contribute the control of gastro-intestinal parasites of sheep. As recorded in the recent study, larvicidal and ovicidal effects of some plants have also been reported earlier against *H. contortus* eggs and larvae (Ketzi et al. 2002; Assis et al. 2003; Hordegen et al. 2003; Maciel et al. 2006; Hordegen et al. 2006; Bizimenyera et al. 2006; Al-Shaibani et al. 2009). Molla and Bandyopadhyay (2013) reported anthelmintic activity of crude aqueous and methanolic extract of *P. guajava* against *H. contortus*. The study showed highest nematode mortality in the higher concentrations of methanolic extract rather than in aqueous extracts of the same concentration. This result could be explained by the fact that the methanolic extracts possess several chemical compounds, and many of them could display ovicidal activity. The total action of the extracts is a sum of the activities of their constituents (Rates 2001). The phytochemicals obtained from *P. guajava* were flavonoid, triterpenoid, fatty acid and tannin (Table 1).

Min and Hart (2003) postulated that the condensed tannin decrease the viability of the larval stages of the nematode parasites and also interfere with the parasite egg hatching and development to infective larval stage. The faecal egg count reduction test was the first test developed for evaluating anthelmintic efficacy and remains the one most widely used for routine diagnosis in commercial flocks and herds. The test provides an estimation of anthelmintic efficacy by comparing faecal egg counts from hosts treated or not, before and after treatment and thus evaluating faecal egg count reduction (Cabaret and Berrag 2004). Iqbal et al. (2006b) revealed significant in vitro anthelmintic activity of crude aqueous extracts and crude methanolic extracts of *Swertia chirata* against *H. contortus*. The ethanolic extract of seed of *Melia azedarach* was the most active on eggs ($LC_{50} = 0.36 \text{ mg mL}^{-1}$) and the leaf ethanol extract showed the best inhibition of larval development ($LC_{50} = 9.18 \text{ mg mL}^{-1}$) of *H. contortus* (Maciel et al. 2006). Alawa et al. (2003) reported that the extract of *Annona senegalensis* showed significant ($P < 0.001$) reduction in egg hatch (*H. contortus*) at a concentration of 7.1 mL^{-1} . The less effectiveness was observed for *Spigelia anthelmia* extracts on *H. contortus* egg hatching that was effective in 50 mg mL^{-1} concentration (Assis et al. 2003).

Githiori et al. (2003) observed significant reduction in faecal egg count of *H. contortus* of sheep after treatment with *Albizia anthelmintica*, *Olea europaea*, *Annona squamosa* and *Ananas cosmosus* water extracts. The efficacy of *Myrsine africana*, *Albizia anthelmintica* and *Hilderbrandtia*

Fig. 3 Mean faecal egg counts and percentage reduction in egg counts for *M. koenigii* extracts

sepalosa against nematode parasites (*Haemonchus* spp., *Trichostrongylus* spp., *Oesophagostomum* spp. and *Moniezia* spp.) was 77, 89.9, 90 and 100 % respectively (Gathuma et al. 2004).

Beriajaya et al. (1998) recorded the in vitro anthelmintic effect of different concentrations of *Zingiber purpureum* infusion (0, 2.5, 5, 10, 20, 40 % w/v) and extract (0, 0.5, 1, 2, 4 and 8 % w/v) on adult worms of *H. contortus*. Singh et al. (2004) also reported the in vitro anthelmintic efficacy of methanolic, chloroform and aqueous extracts of *Allium sativum*, *Areca catuha*, *Azadirachta indica*, *Butea monosperma*, *Mallotus phillipensis*, *Psoralea corylifolia*, *Pygeum persica*, *Scindapsus officinalis* and *Vernonia anthelmintica* compared with fenbendazole against *H. contortus* of sheep.

Conclusion

Based on the results of the present experiment it may be concluded that *M. koenigii* possess adequate in vitro and in vivo anthelmintic activity against different parasitic stages including eggs and adults of gastro-intestinal nematode parasites of sheep. However, more detailed studies are needed to identify and evaluate the active components and the mechanisms of action of *M. koenigii* extracts and also studies on toxicity and evaluation of the effects.

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