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Evaluation of Anthelmintic Activity of *Butea frondosa* (Koeing ex Roxb.) Seeds Extracts Against Benzimidazole Resistant Caprine Gastrointestinal Nematodes

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ABSTRACT

The present investigation was carried out to evaluate the anthelmintic efficacy of crude aqueous and methanolic extract of *Butea frondosa* seeds extracts against benzimidazole resistant gastrointestinal nematodes of goats through *in vitro* and *in vivo* methods. *In vitro* investigation was carried out by egg hatch assay (EHA), larval paralysis test (LPT) and adult mortality test (AMT) against different stages of gastrointestinal nematodes whereas, *in vivo* by faecal egg count reduction (FECR) test in goats naturally infected with benzimidazole resistant GI nematodes. In *in vitro* trial, methanolic extract showed better ED_{50} in egg hatch assay and larval paralysis test as compared to aqueous extract. Moreover, in adult mortality tests, the methanolic extract gave better average corrected mortality as compared to aqueous extract. *In vivo* results revealed that the group treated with methanolic extracts showed the significant reduction ($p<0.05$) on 21st day whereas, the group treated with aqueous extract showed the highest and significant reduction ($p<0.01$) on 14th day. The results of both *in vitro* and *in vivo* trials suggest that *B. frondosa* possess anthelmintic activity and could be considered as one of the alternatives to the chemical anthelmintic.

HIGHLIGHTS

- The methanolic and aqueous extracts of *B. frondosa* possess anthelmintic activity against the benzimidazole resistant GI nematodes of goats.
- Methanolic extract showed better efficacy as compare to aqueous extract.

Keywords: *Butea frondosa,* Benzimidazole resistance, Anthelmintic activity, Gastrointestinal nematodes, Goat

The era of modern anthelmintics began in the mid-20th century, with the introduction of phenothiazine and piperazine. These anthelmintics have been extensively used by veterinarians and livestock producers to control parasites either by drenching or injecting them into cattle, buffalo, sheep and goats (Soulsby, 1982). However, due to indiscriminate uses, the worms started developing resistance against anthelmintics. Anthelmintic resistance in GI nematodes, particularly high prevalence of multidrug resistance, has been reported among the three broad-spectrum and narrow-spectrum anthelmintic drugs from several countries across the world (Verma *et al.*, 2018). Although the matter is more rampant in small ruminants, reports have also been documented globally from cattle, horses and pigs (Temesgen and Tone, 2019; Vohra *et al.*, 2019). This has triggered the evaluation of conventionally used plants for their anthelmintic properties (Tandon *et al.*, 2011). *B. frondosa* popularly referred to as 'flame of

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the forest', widely found in tropical and sub-tropical parts of the Indian Subcontinents and Southeast Asia (Patil *et al.*, 2006). The seed of *B. frondosa* has exhibited excellent anthelmintic property especially for roundworms (Iqbal *et al.*, 2006; Sant *et al.*, 2014; Singh *et al.*, 2015). Keeping in view, the study was planned to evaluate the anthelmintic activity of *B. frondosa* seeds extracts against benzimidazole resistant GI nematodes of goats in order to counter the anthelmintic resistance in livestock.

MATERIALS AND METHODS

Location of work

The study was carried out in the Department of Veterinary Parasitology and institutional goat farm maintained in semi intensive conditions at Amanala, College of Veterinary Science & Animal Husbandry, Nanaji Deshmukh Veterinary Science University (NDVSU), Jabalpur district which is located in agro-climatic zone III (23 ° 10 79 N, 79 ° 56 and E and 411 m MSL) of the state of Madhya Pradesh, India. The proposed work was carried on the goat farm, which already has been reported to have benzimidazole resistance in gastrointestinal nematodes (Das *et al.*, 2015; Dixit *et al.*, 2017).

Collection and extract preparation of plant material

Seeds of *B. frondosa* (Koeing ex Roxb.) were collected from in and around Jabalpur (M.P). The plant specimen was identified and authenticated by Forest Botany & Eco Division, State Forest Research Institute, Jabalpur with collection number 16342 and registration number 8052. Seeds were cleaned, washed and air dried followed by drying in an incubator at 40°C. The dried seeds were ground in an electrical mixer grinder to make powder and stored in airtight containers for the preparation of extracts. Both aqueous and methanolic extracts were prepared as per the procedure as describe by Kanojiya *et al.* (2015a).

In-vitro evaluation of anthelmintic activity of *B. frondosa* extracts

Egg hatch assay

The egg hatch assay was performed as per the method described by Coles *et al.* (1992) with slight modifications. The number of egg were estimated per ml and adjusted to 100-150 eggs/200 µl. 200 µl of fresh egg suspension were placed in each well containing 1 ml extract of different concentration ranging from 0.156% (1.56 mg/ml) - 10% (100 mg/ml) in a multi-well plate while the control well-received PBS. The volume of each well was made up to 2 ml by adding 800 µl of distilled water. The plates were incubated under humidified conditions at 27°C for 48 hrs and later, a drop of Lugol's iodine solution was added to every well to prevent further hatching. Hatched larvae and un-hatched eggs were then counted under a light microscope.

Larval paralysis test

Larvae were obtained by culturing the per-rectally collected faecal samples from the goat of the farm (Sloss *et al.,* 1997). 100 µl containing 100-150 live larvae were exposed to different concentration of extracts ranging from 0.156% (1.56 mg/ml) - 10% (100 mg/ml) in the multi-well plates. After 24 hrs, live (motile) and dead larvae were counted.

Adult mortality test

Adult *Haemonchus contortus* worms were procured from the abomasii of goats after slaughter or during postmortem. The motile adult worms were cleaned with lukewarm normal saline and transferred in a beaker containing phosphate buffer saline (PBS) and were kept in an incubator at 37°C until required for the experiment on the same day. For AMT, ten adult *H. contortus* were taken in small Petri dishes having different dilutions of extracts in PBS ranging from 0.156% (1.56 mg/ml) – 10% (100 mg/ml), while control Petri dish received only PBS. The total volume of each Petri dish was kept at 5 ml. It was then incubated at 37±1°C for 24 hrs and the number of live and dead adult worms were counted at 1, 2, 4, 6, 12, 18 and 24 hrs of exposure as described by Sujon *et al.* (2008).

% corrected mortality =

$$\frac{\text{Total mortality} - \text{mortality in the control group}}{\text{Total mortality}}$$

In-vivo evaluation of anthelmintic efficacy of *B. frondosa*

Ethical approval

This study was approved by Institutional Animal Ethical Committee, College of Veterinary Science and Animal Husbandry (NDVSU), Jabalpur (M.P) keeping in mind the guidelines of CPCSEA (Certificate No: 115/IAEC/VETY/2017).

Selection and treatment of animals

The goats having EPG ≥ 300 were selected and divided into 3 groups with 8 goats in each. Group-I and II were treated with aqueous and methanolic extract of *B. frondosa*, respectively at the rate of 100 mg/kg b. wt. orally on day 0, 3rd and 7th whereas, Group III was kept as an untreated control.

Faecal egg count reduction test (FECRT)

As per the standard protocol, faecal samples were collected from the rectum of goats of treated and control groups individually on the day 0 (pre-treatment), the day 7, 14, 21 and 28 (post-treatment). The intensity of infection was determined by the modified McMaster technique (Sloss *et al.*, 1997). The faecal egg count data were analyzed by faecal egg count reduction test (FECRT %) for the anthelmintic efficacy of the extracts on a particular day using the following formula given by Dash *et al.*, 1988.

$$\% \text{ efficacy} = \frac{\text{EPG of control group} - \text{EPG of treated group}}{\text{EPG of control group}}$$

Statistical analysis

The data of *in-vitro* test *viz.* Egg Hatch Assay and Larval Paralysis Test were analyzed by log probit analysis for calculation of ED_{50}Value (Finney, 1971). However, *in-vivo* anthelmintic efficacy was determined by FECRT% and significant effect was determined by students't' test (Snedecor and Cochran, 1994).

RESULTS AND DISCUSSION

In-vitro evaluation of anthelmintic activity

Egg hatch Assay

The results revealed that egg hatch percentage at the concentration of 10% (100 mg/ml) for aqueous and methanolic extract was 1.29 and 1.00% with corrected egg hatch percentage 2.61 and 1.33%, respectively. Whereas, egg hatch percentage of aqueous extract at concentration 5% (50 mg/ml), 2.5% (25 mg/ml), 0.625% (6.25 mg/ml), 0.3125% (3.125 mg/ml) and 0.156% (1.56%) were 8.91, 15.77, 33.11, 47.10, 45.19, 77.74 and 98.68%, respectively with corrected egg hatch percentage 10.23, 17.09, 34.43, 48.41, 46.51 and 79.06%, respectively. Whereas, egg hatch percentage of methanolic extract at concentration 5% (50 mg/ml), 2.5% (25 mg/ml), 1.25% (12.50 mg/ml), 0.625% (6.25 mg/ml), 0.3125% (3.125 mg/ml) and 0.156% (1.56%) were 1.84, 3.95, 16.94, 23.78, 71.10 and 88.29%, respectively with corrected hatching percentage 2.17, 4.28, 17.28, 24.11, 71.43 and 88.63%, respectively. *B. frondosa* aqueous extract gave ED_{50} value 0.463 mg/ml with lower and upper confidence limit 0.276 mg/ml and 0.683 mg/ml, respectively, and methanolic extract gave ED_{50} value 0.453 mg/ml with lower and upper limit 0.299 mg/ml and 0.638 mg/ml, respectively.

Larval paralysis test

In larval paralysis test, aqueous extract at 10% (100 mg/ml) concentration showed 86.75% efficacy with corrected percentage efficacy 84.44%, however, at 5% (50 mg/ml), 2.5% (25 mg/ml), 1.25% (12.5 mg/ml), 0.625% (6.25 mg/ml), 0.312% (3.12 mg/ml) and 0.156% (1.56 mg/kg) concentration, percentages efficacy were 74.33%, 70.67%, 53.0%, 19.0%, 14.29% and 9.36%, respectively with corrected percentage efficacy 72.00, 68.33, 50.67, 16.67, 11.96 and 7.02%, respectively. Whereas, at 10% (100 mg/ml) concentration, methanolic extract showed 97.33% effectiveness with 95% corrected efficacy. However, at 5% (50 mg/ml), 2.5% (25 mg/ml), 1.25% (12.5 mg/ml), 0.625% (6.25 mg/ml), 0.3125% (3.125 mg/ml) and 0.156% (1.56 mg/ml) concentration, efficacy percentages were 89.00, 78.00, 69.67, 38.08, 18.71 and 17.1%, respectively with corrected efficacy 95.00, 86.60,

75.67, 67.33, 35.76, 16.45 and 14.84%, respectively. *B. frondosa* aqueous extract gave ED_{50} 1.607 mg/ml with lower and upper limit 1.139 mg/ml and 2.282 mg/ml, respectively, and methanolic extract gave ED_{50} 0.854 mg/ml with lower and upper limit 0.652 mg/ml and 1.098 mg/ml, respectively.

Adult mortality test

The results of aqueous and methanolic extract showed 100% mortality of adult worm at 10% (100 mg/ml) and 5% (50 mg/ml) concentration with corrected mortality 100%. Whereas, aqueous extract at 2.5% (25 mg/ml), 1.25% (12.5 mg/ml), 0.625% (6.25 mg/ml), 0.3125% (3.125 mg/ml) and 0.156% (1.56 mg/ml) concentration showed 100%, 70%, 33%, 0% and 0% mortality, respectively with corrected mortality 100%, 70%, 33%, 0% and 0%, respectively. While methanolic extract at 2.5% (25 mg/ml), 1.25% (12.5 mg/ml), 0.625% (6.25 mg/ml), 0.3125% (3.125 mg/ml) and 0.156% (1.56 mg/ml) concentration showed 100%,100%, 100%, 33% and 20% mortality, respectively with corrected mortality 73%, 67%, 57%, 60% and 37%, respectively. The aqueous extract of *B. frondosa* gave 57.57% average corrected mortality and methanolic extract gave 72.00% average corrected mortality. *B. frondosa* gave 64.79% overall per cent corrected mortality.

***In-vivo* evaluation of anthelmintic activity**

The result of *in-vivo* test revealed that on 7th day post infection the mean EPG of aqueous extract treated group I increased to 912.5±363.73 from 812.5±172.62 whereas, in the methanolic extract treated group II decreased to 837.5±239.00 from 937.5±223.56, respectively. However, mean EPG on the 14th day reduced in both group I (200.0±68.14) and group II (375.0±161.19). Whereas, in group I mean EPG on 21st and 28th day was 375.0±92.10 and 525.0±170.87, respectively and in case of a group II mean EPG on 21st and 28th day was 875.0±169.82 and 762.5±162.50, respectively (Table 1). While fecal egg count reduction % in group I on 7th, 14th, 21st and 28th day were recorded as 10.98, 73.33, 20.00 and 32.79%, respectively. The highest and significant reduction ($p<0.01$) was observed on day 14th (73.33%). However, fecal egg count reduction % in group II on 7th, 14th, 21st and 28th day were recorded as 18.29, 50.00, 57.14, and 31.15%, respectively. The highest and significant reduction ($p<0.05$) was recorded on the 21st day (57.14%) (Table 2).

The results indicated that *B. frondosa* possess anthelmintic activity against benzimidazole resistant GI nematodes. The findings are in agreement with the previously published research works (Iqbal *et al.*, 2006; Sant *et al.*, 2014; Singh *et al.*, 2015). In egg hatch assay, both the aqueous and methanolic extracts of *B. frondosa* at 5% and above concentration showed higher inhibition of egg hatching compared to low concentration. Similar dose-dependent egg hatching inhibitions were evaluated using the aqueous and methanolic extract of *Allium sativum*, *Ocimum sanctum* and *Eucalyptus globulus* on naturally occurring GI nematodes of sheep (Kanojiya *et al.*, 2015a; Kanojiya *et al.*, 2015b; Kanojiya *et al.*, 2015c). Similar findings were reported by Eguale *et al.* (2007) that aqueous and hydro-alcoholic extracts of *Coriandrum sativum* extracts inhibited hatching of eggs completely at concentration of less than 0.5 mg/ml. The possible mechanism of inhibition could be due to the presence of a high concentration of phenolic, flavonoids and tannin and other secondary metabolites content in the seeds of *Butea* spp. to inhibit the hatching of nematode eggs (Carvalho *et al.*, 2012; Sant *et al.*, 2014; Singh *et al.*, 2015).

In the larval paralysis test, results indicated that aqueous extract of *B. frondosa* at 100 mg/ml concentration showed 86.75% efficacy with corrected percentage efficacy 84.44%, and methanolic extract showed 97.33% efficacy with corrected efficacy 95.0%. Similar to egg hatch tests, the larval paralysis test also followed a dose-dependent trend. Our findings were relatively in agreement with Kanojiya *et al.* (2015a) who reported that the aqueous extract and methanolic extract of *Allium sativum* on naturally occurring GI nematodes of sheep showed 100% paralysis of 3rd stage larvae at 100 mg/ml concentration. Similar efficacy has also been reported by Kamaraj *et al.* (2011) and Akhtar *et al.* (2015). This difference in efficacy might be due to variation in the active principles of these plants.

In the adult mortality test, the aqueous and methanolic extract of seeds of *B. frondosa* showed 100% mortality at 100 mg/ml and 50 mg/ml concentrations. Singh *et al.* (2015) reported that aqueous extract of seeds of *B. monosperma* was responsible for complete mortality of *H. contortus* at same concentrations. Similar findings

Table 1: *In-vivo* anthelmintic efficacy of *Butea frondosa* extracts against gastrointestinal nematode in goat

Groups	Treatment @ 100 mg/kg b. wt. orally on 0, 3rd & 7th day	Mean EPG				
		Day 0	Day 7	Day 14	Day 21	Day 28
Group I	*B. frondosa* aqueous extract	812.5±172.62	912.5±363.73	200.0±68.14	700.0±217.12	512.5±219.12
Group II	*B. frondosa* methanolic extract	937.5±223.56	837.5±239.00	375.0±161.19	375.0±92.10	525.0±170.87
Group III	Untreated Control	962.5±230.63	1025.0±209.38	750.0±137.58	875.0±169.82	762.5±162.50

Table 2: Faecal egg count reduction per cent of different groups

Groups	Treatment	FECRT %			
		Day 7	Day 14	Day 21	Day 28
Group I	*B. frondosa* aqueous extract	10.98	73.33**	20.00	32.79
Group II	*B. frondosa* methanolic extract	18.29	50.00	57.14*	31.15

Significant **p<0.01, *p<0.05.

have also been documented by various researchers like Kalesaraj and Kurup (1962) who reported alkaloid hydrochlorides extracted from seeds of *B. frondosa* to be 100% lethal to earthworms within 24 hrs. Mansoor *et al.* (2013) reported that crude methanolic extract of *B. frondosa* seed showed significant anthelmintic activity on earthworm (*Lumbricus terrestris*), the extract exhibited 100% mortality of worms at 50 mg/ml concentration. Prashanth *et al.* (2001) reported the efficacy of methanolic extract of *B. frondosa* against adult *H. contortus*. These findings could be due to palasonin in Palash seed and having anthelmintic activity. Palasonin inhibited the glucose uptake and depleted the glycogen content in the presence of glucose indicating that palasonin affects the energy-generating mechanism of the parasite. It also significantly increased lactic acid suggesting inhibition of ATP production. Therefore, palasonin may act *via* either inhibition of energy metabolism and/ or alteration in the motor activity of the parasite (Kumar *et al.*, 1995; Mali and Mehta, 2008).

The results of *in vivo* assay revealed that aqueous extract of *B. frondosa* seeds showed the highest (73.33%) and significant ($p<0.01$) reduction of egg per gram of faeces recorded on 14th day post-treatment whereas, the methanolic extract showed the highest (57.14%) and significant ($p<0.05$) reduction recorded on 21st day post-treatment. No evidence of toxicity was recorded on the experimental doses during or after the treatment. A similar observation has been recorded by Iqbal *et al.* (2006) who reported the crude powder of *B. monosperma* seeds showed a maximum reduction of 78.4% in eggs per gram of faeces on 10th day post-treatment which was maintained till day 14th with the dose of 3 g/kg. Al-Shaibani *et al.* (2009) revealed that ethanolic and aqueous extracts of *Fumaria parviflora* showed the highest reduction of egg per gram of faeces recorded on 14th day post treatment with the dose of 200 mg/kg body weight. This difference in efficacy might be due to variation in the active principles of these plants.

CONCLUSION

The results of the present study indicated that both the aqueous and methanolic extract of *B. frondosa* possess anthelmintic activity against different stages of GI nematodes of goats as evident by the *in-vitro* and *in-vivo* studies, however, the performance of methanolic extract was better than that of aqueous extract. Therefore, *B. frondosa* could be considered as an alternative to the chemical anthelmintic.

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