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**Full Length Article**

## ***In Vitro* and *In Vivo* Anthelmintic Activity of *Terminalia arjuna* Bark**

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### **ABSTRACT**

The present study was carried out to evaluate the anthelmintic activity of *Terminalia arjuna* (Roxb.) bark locally used as an anthelmintic. Lethal median concentration (LC<sub>50</sub> values) of methanolic extract of *T. arjuna* bark in egg hatch and larval development tests against *Haemonchus contortus* ova and larva were found to be 645.65 and 467.74 µg mL<sup>-1</sup>, respectively. In adult motility assay, efficacy of the extract was evident by the mortality of *H. contortus* at different hours post exposure. *In vivo* results revealed maximum (87.3%) egg count percent reduction (ECR) in sheep treated with crude methanolic extract @ 3 g kg<sup>-1</sup> body weight on day 11 post-treatment (PT). The data revealed dose-dependent anthelmintic activity both in the *in vitro* and *in vivo* studies, thus justifying its use in the traditional medicine system of Pakistan.

**Key Words:** *Terminalia arjuna*; Bark; Anthelmintic; Sheep

### **INTRODUCTION**

In recent years, several reports of apparent failures in the treatment of human nematodes have been published (e.g., De Clercq *et al.*, 1997; Reynoldson *et al.*, 1997). Although the interpretation and the implications of these studies are still being debated, they have led to an increased awareness of the potential problem of anthelmintic resistance (AR) in the treatment and control of human helminths. The concerns about AR are not superfluous in the context of serious issues of development of drug resistance in majority of the nematodes infesting animals (Waller *et al.*, 1995, 1996; Van Wyk *et al.*, 1997). It would, therefore, be imperative to explore possibilities of developing new anthelmintic compounds. This has drawn attention of researchers to the validation of traditionally used botanical anthelmintics (Hammond *et al.*, 1997; Akhtar *et al.*, 2000; Waller *et al.*, 2001; Iqbal *et al.*, 2003).

Arjun tree (*Terminalia arjuna* (Roxb.) Wight and Arn. (Combretaceae)), known for its ethnomedicinal significance (Kumar & Prabhakar, 1987) is frequently used in cardiovascular disorders (Singh *et al.*, 1982a; Dwivedi & Jauhari, 1997; Jain *et al.*, 1992; Ram *et al.*, 1997) as an antimicrobial (Ray & Majumdar, 1976; Samy *et al.*, 1998; Shukla *et al.*, 2000) and antiviral agent (Kusumoto *et al.*, 1995). Bark of the some species of genus *Terminalia* like *T. macroptera*, *T. superba* and *T. vorensi* have also been reported for their use as antidiarrheal, antidysenteric (Alawa

*et al.*, 2002) and trypanocidal (Adewunmi, *et al.*, 2001). Decoction prepared from the bark of *T. arjuna* is used as an anthelmintic both in man and animals in Pakistan (personal communication). There is, however, no scientific evidence for the anthelmintic effects of *T. arjuna* bark. The present work was therefore, carried out to validate the anthelmintic activity of *T. arjuna* bark in the light of its use by the traditional healers.

### **MATERIAL AND METHODS**

**Plant material.** Bark of *T. arjuna* was collected from the trees alongside the roads of University of Agriculture, Faisalabad, Pakistan. A voucher specimen (# 09/2006) of the plant material has been preserved in the Ethnoveterinary Research and Development Centre, Department of Veterinary Parasitology, University of Agriculture, Faisalabad, Pakistan. The bark was dried in shade, ground finely to a powder in an electric mill and stored in cellophane bags at 4°C until use.

**Extraction.** Powdered *T. arjuna* bark was exhaustively extracted with methanol in a Soxhlet's apparatus (Asuzu & Onu, 1994). The extract was concentrated in a rotary evaporator at 40°C under reduced pressure and dried in a vacuum oven. The w/w yield of *T. arjuna* bark was 38%. The extract was stored at 4°C until use.

***In vitro* anthelmintic activity.** *In vitro* anthelmintic activity was assessed through egg hatch test, larval development test and adult motility assay.

For egg recovery, adult female *H. contortus* were collected from abomasums of naturally infected sheep, placed in a bottle containing cool (4°C) PBS (pH 7.2) and triturated in pestle and mortar. The eggs were recovered from suspension by the method described by Coles *et al.* (1992). The suspension was filtered and the filtrate was centrifuged in Clayton Lane tubes for 2 min at about 300 × *g* and supernatant was discarded. Tubes were agitated to loosen the sediment and then saturated sodium chloride solution was added until a meniscus formed above the tube. A cover slip was placed and sample re-centrifuged for 2 min at about 130 × *g*. Coverslip was plucked off carefully from tubes and eggs were washed off into a conical glass centrifuge tube. Tube was filled with water and centrifuged for 2 min at about 300 × *g*. Supernatant was decanted and eggs were re-suspended in water. The eggs were then washed thrice in distilled water and adjusted to a 500 eggs per milliliter using the McMaster technique (Soulsby, 1982).

Egg hatch test was conducted following Coles *et al.* (1992). Eggs suspension of (0.2 mL; 100 eggs) was distributed in a 24-flat-bottomed microtitre plate and mixed with the same volume of different concentrations (0.062–4 mg mL<sup>-1</sup>) of each plant extract. The positive control wells received different concentrations (3.0–0.0058 µg mL<sup>-1</sup>) of oxfendazole (Systemex—ICI Pakistan, Ltd.; 2.265%, w/v) in place of plant extracts, while negative control plate contained the diluent and the egg solution. The eggs were incubated in this mixture at 27°C. After 48 h, a drop of Lugol's iodine solution was added to stop the eggs from hatching. All the eggs and first-stage larvae (L1) in each plate were counted. There were three replicates for each treatment and control.

For larval development test, nutritive medium was prepared as described by Hubert and Kerboeuf (1992) and composed of Earle's balance salt solution plus yeast extract diluted in saline solution (1 g of yeast extract 90 mL<sup>-1</sup> of saline solution) in the proportion 1:9 volume to volume. Larval development test was conducted following Ademola *et al.* (2004). Eggs suspension of (500 µL; 100 eggs) was distributed in 5 mL test tubes each with 150 µL of nutritive medium. The tubes were covered and placed in an incubator at 27°C for hatching of the eggs to first stage larvae in 48 h. Plant extract (CME) at different concentrations (0.031–2 mg mL<sup>-1</sup>) was added to the tubes containing first stage larvae. The positive control wells received different concentrations (0.01–2.58 µg mL<sup>-1</sup>) of levamisole HCl (Nilverm (1.5% w/v)—ICI Pakistan Limited (Animal Health Division) in place of plant extracts, while negative control plate contained the diluent and the larval suspension. The larvae were incubated in this mixture at 27°C. After 7 days, larvae were counted as living third stage larvae (L3) and dead larvae. There were three replicates for each treatment and control.

Adult motility assay was conducted on mature live *Haemonchus (H.) contortus* following Sharma *et al.* (1971).

Briefly, the female mature worms were collected from the abomasums of freshly slaughtered sheep in the local abattoir. The worms were washed and finally suspended in phosphate buffer saline (PBS). Five worms were exposed in triplicate to each of the following treatments in separate Petri dishes at room temperature (25–30°C):

1. Levamisole 0.55 mg mL<sup>-1</sup>
2. CME of *T. arjuna* bark at 0.125, 0.25, 0.5 and 1.0 mg mL<sup>-1</sup>
3. PBS (control).

The inhibition of motility and/or mortality of the worms subjected to the above treatments were used as the criteria for anthelmintic activity. The motility was recorded after 0, 1, 3, 6 and 12 h intervals. Finally, the treated worms were kept for 30 min in the lukewarm fresh PBS to observe the revival of motility.

***In vivo* anthelmintic activity.** A total of 25 sheep (local Lohi breed) ≤1 year, having almost homogeneous characteristics viz., weight, eggs per gram of faeces (EPG) and composition of infecting nematode species were selected from a herd of more than 1000 animals being maintained at Livestock Experiment Station, Rakh Kherwala (Punjab, Pakistan). The animals were vaccinated against enterotoxemia and pleuropneumonia vaccines, supplied by the Veterinary Research Institute, Lahore (Punjab, Pakistan). The sheep had naturally acquired mixed parasitic infection of gastrointestinal nematodes. Infections were confirmed before the beginning of study by collecting faecal samples from the animals, by rectum and the number of nematode egg therein determined by the floatation method (Soulsby, 1982). For nematode species composition, coproculture was done for identification of larvae using standard description of MAFF (1979) and Thienpont *et al.* (1979). The nematode eggs recovered from the experimental sheep were identified by larval cultures as *Haemonchus contortus*, *Trichostrongylus* spp., *Oesophagostomum columbianum* and *Trichuris ovis*. The experimental animals were housed at the Department of Parasitology, University of Agriculture, Faisalabad, Pakistan for one month before study initiation for acclimatization. After treatment they were penned singly by treatment until the end of the study. No physical contact was possible between sheep from different treatment groups. The sheep were kept on plastered floor and fed with grass and water *ad libitum*.

The experimental sheep were randomly divided into five groups of five animals each and assigned different per os treatments as single dose as given below:

Group 1 served as negative control and received no treatment, while group 2 was a positive control, which was given a single dose of levamisole HCl 7.5 mg kg<sup>-1</sup> (ICI Pakistan Limited, Animal Health Division). Groups 3, 4 and 5 received single doses of CME @ 1.0, 2.0 and 3.0 g kg<sup>-1</sup>, respectively. Faecal samples from each animal were collected in the morning, starting from day 0 pre-treatment

and at days 3, 7 and 11 post-treatment and were evaluated for the presence of worm eggs by salt floatation technique (MAFF, 1979). The eggs were counted by the McMaster method (Soulsby, 1982). Egg count percent reduction (ECR) was calculated using the following formula:

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

**Statistical analyses.** For egg hatch and larval development tests, probit transformation was performed to transform a typical sigmoid dose-response curve to linear function (Hubert & Kerboeuf, 1992). The extract concentration required to prevent 50% i.e., lethal concentration 50 (LC<sub>50</sub>) of hatching of eggs and larval development to third stage were calculated from this linear regression (for y = 0 on the probit scale). The data from adult motility assay and *in vivo* experiments were statistically analysed using SAS software (SAS, 1998). The results were expressed as mean±standard error of mean (S.E.M.).

**RESULTS**

**In vitro anthelmintic activity.** Crude methanolic extracts of *T. arjuna* exhibited inhibitory effects on eggs hatching and LC<sub>50</sub> was determined graphically from the regression equation after correcting from negative control. The calculated LC<sub>50</sub> values of *T. arjuna* and positive control (oxfendazole) were 645.65 and 1.9 µg mL<sup>-1</sup>, respectively. The regression values and correlation of regression of the *T. arjuna* were y = -0.0006x + 5.8763; R<sub>2</sub> = 0.7976 and those of positive control were y = -1.263x + 7.485; R<sub>2</sub> = 0.786, respectively.

For larval development test crude methanolic extracts of *T. arjuna* exhibited inhibitory effects on larval development and LC<sub>50</sub> was determined graphically from the regression equation after correcting from negative control. The calculated LC<sub>50</sub> values of *T. arjuna* and positive control (levamisole) were 295.12 and 0.222 µg mL<sup>-1</sup>, respectively. The regression values and correlation of regression of the *T. arjuna* were Y = - 0.0013x + 5.8181, R<sub>2</sub> = 0.8425 and those of positive control were y = -1.113x + 6.264; R<sub>2</sub> = 0.826, respectively.

Adult motility assay crude methanolic extract of *T. arjuna* resulted in paralysis and mortality of the tested worms. All the worms exposed to levamisole (a standard anthelmintic) were found dead at 12 h; whereas none of the worms was found dead or paralyzed in PBS up to 12 h post exposure. The higher doses resulted in an early onset of activity and higher number of dead worms compared with lower doses suggesting a time and dose dependent response (Fig. 1).

**In vivo studies.** Maximum ECR (87.3%) was exhibited by the crude methanolic extract of *T. arjuna* bark @ 3 g kg<sup>-1</sup> on day 11 PT followed by crude powder (50%) @ 2 g kg<sup>-1</sup> at day 7 PT (Table I). A graded dose response in ECR was recorded in animals treated with CP and CME but it was more pronounced with the CME as compared to CP. The

**Table I. Effect of the *Terminalia arjuna* bark administration on eggs per gram of faeces in sheep naturally infected with gastrointestinal nematodes**

Day PT	Control		Crude Methanolic Extract (g kg <sup>-1</sup> )		
	Untreated <sup>1</sup>	Treated <sup>2</sup>	1.0	2.0	3.0
0	1500±289 <sup>c</sup>	5033±731 <sup>a</sup>	2633±233 <sup>a</sup>	2500±577 <sup>a</sup>	1967±88 <sup>a</sup>
3	1533±332 <sup>bc</sup>	2133±933 <sup>b</sup>	1883±169 <sup>b</sup>	817±120 <sup>b</sup>	467±33 <sup>b</sup>
	(-2.2)	(57.6)	(28.5)	(67.3)	(76.3)
7	1483±319 <sup>c</sup>	600±200 <sup>c</sup>	1700±126 <sup>bc</sup>	683±145 <sup>b</sup>	350±29 <sup>bc</sup>
	(1.1)	(88.1)	(35.4)	(72.7)	(82.2)
11	1550±236 <sup>abc</sup>	233±83 <sup>c</sup>	1567±145 <sup>c</sup>	617±136 <sup>b</sup>	250±50 <sup>c</sup>
	(-3.3)	(95.4)	(40.5)	(75.3)	(87.3)

The values represent mean±SEM with % reduction in eggs counts per gram of faeces in parenthesis

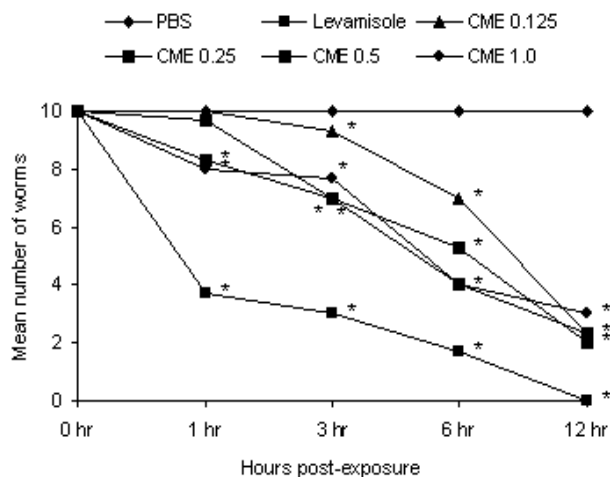
Values with similar superscripts (<sup>a-c</sup>) in a column do not differ (P ≥ 0.05)

<sup>1</sup>Untreated means infected untreated control group

<sup>2</sup>Treated means animals treated with Levamisole (a standard anthelmintic) at the dose rate of 7.5 mg/kg body weight of animals

PT= Post-treatment

**Fig. 1. Graph showing the time- and dose-dependent in vitro anthelmintic activity of *Terminalia arjuna* bark crude methanol extracts (CME) at 0.125–1.0 mg/mL concentrations in comparison with positive (levamisole; 0.55 mg/mL) and negative control (phosphate buffer saline; PBS) on mature live *Haemonchus contortus* of sheep. The inhibition of motility and/or mortality of the worms were used as the criterion for anthelmintic activity. Values shown are means, asterisk (\*) indicates significantly different from previous value at p < 0.05**



onset of activity was, however, rapid and more persistent at higher doses of CP than the lower doses. The data revealed that the levamisole was the most effective followed by the CME and CP in percent ECR.

Interestingly, the peak effect recorded with the crude powder or the methanol extract was quicker than the control drug (levamisole), as the peak effect with the test material was between 7-11 days, while levamisole exhibited peak effect from 11-15 days. The effect of crude powder was mild compared to the crude extract (maximum effect 50% as opposed to 87%). Maximum effect with crude powder was observed at 2 g kg<sup>-1</sup> and further increase in dose did not

change the effect ( $P > 0.05$ ). Levamisole, though slightly more effective than the methanol extract, but its peak effect was delayed.

## DISCUSSION

*Terminalia arjuna* bark exhibited anthelmintic activity both *in vitro* (eggs, larvae & adult of *H. contortus*) and *in vivo* studies against mixed gastrointestinal trichostrongylid nematodes of sheep. It is evident from the results that CME had higher activity compared with the CP, which may be considered as an indication for the presence of methanol soluble principles in *T. arjuna* bark for anthelmintic activity.

As far as ascertained, this is the first scientific evidence of the anthelmintic activity of *Terminalia arjuna* bark, which may be mainly attributed to its tannin content. The condensed tannin (CT) content of the *T. arjuna* bark used in this study was determined as 95.1 g kg<sup>-1</sup> of the dry matter (DM) following Porter *et al.* (1986). Earlier, Molan *et al.* (2000; 2002) have suggested that the extracted CT from forages (400 µg of CT mL<sup>-1</sup>) are more potent inhibitors of egg hatching and larval development (87 to 100%) than of larval motility (21 to 39% inhibited). The condensed tannin detected in this study using butanol-HCl assay from *T. arjuna* bark was 55.1 g kg<sup>-1</sup> DM. The bark of *T. arjuna* has been reported to contain tannin and ellagic acid (Row *et al.*, 1970a, b; Kumar & Prabhakar, 1987). The tannins contained in plants have been reported to possess antiviral (Cheng *et al.*, 2002), antibacterial (Perumal *et al.*, 1998) and anthelmintic (Paolini *et al.*, 2003, 2005; Ademola *et al.*, 2004, 2005) activities. It is postulated that CT may impair vital processes such as feeding and reproduction of the parasite or may bind and disrupt the integrity of the parasites' cuticle (Niezen *et al.*, 1995). The anthelmintic effects of tannins may be attributed to its capacity to bind free protein available in the tubes for larval nutrition and thus reduced nutrient availability could have resulted in larval starvation or decrease in gastrointestinal metabolism directly through inhibition of oxidative phosphorylation (Scalbert, 1991), causing larval death (Athanasidou *et al.*, 2001). Different plant extracts have been reported previously to affect biology of the parasitic eggs (Hounzangbe-Adote *et al.*, 2005).

Tanniferous plants have been evaluated in several studies for their anthelmintic activity against gastrointestinal nematodes of small ruminants (Kahn & Diaz-Hernandez, 2000). Field studies have indicated that the consumption of tanniniferous forages by parasitized sheep reduced the egg output and the worm burdens (Niezen *et al.*, 1998 a, b, 2002). In addition, some *in vitro* assays in experimentally infected sheep, using quebracho extracts as a rich source of condensed tannins, tended to confirm the results obtained in natural infections (Athanasidou *et al.*, 2000 a, b, 2001) as it was used in the present study. *In vivo* reduction in the egg output has also been observed previously by Spanish goats grazing the tanniniferous plant *Lespedeza cuneata* (Min *et al.*,

2004) and has been largely described in sheep consuming other tanniniferous legumes such as sulla, *Lotus pedunculatus* (Niezen *et al.*, 1995, 1998a) and to a lesser extent *Lotus corniculatus* (Mareley *et al.*, 2003). The active principle in current study may be the condensed tannins which are also soluble in methanol (Butler *et al.*, 1982). Min *et al.* (2005) showed that gastrointestinal parasites were controlled when Angora does were grazed on *Lespedeza cuneata* (152 g of CT kg<sup>-1</sup> of DM) but not when goats were grazed on control forages such as crabgrass (*Digitaria ischaemum*)/tall fescue (*Festuca arundinacea*) having 3.2 g of CT kg<sup>-1</sup> of DM. Tannins may also induce physiological changes in the gut of the host resulting in secretion of mucous and chemicals harmful to the parasite (Terrill *et al.*, 1992).

Though, condensed tannins have been reported to exert direct anthelmintic effects, other phytochemicals like alkaloids, flavonoids and oleane type triterpenes (Anjaneyulu & Prasad, 1982; Gary & Kasera, 1983; Tripathi *et al.*, 1992; Irobi *et al.*, 1994; Brantner *et al.*, 1996) of *T. arjuna* may also have their independent or synergistic effect. The phytochemicals noted above are known for their antimicrobial activity (Cowan, 1999) and may have their application as an anthelmintic as well.

In conclusion, the use of *T. arjuna* bark as an anthelmintic in the form of decoction seems valid in the light of results of the current study. Therefore, quality controlled extracts of *T. arjuna* bark or possibly isolated bioactive compounds could be promising alternatives to conventional anthelmintics in the future. However, research is needed for studies on artificially induced infections of particular species of parasites, the bioactive constituents, as well as on the reproducibility, dosage, application regime, toxicity and effectiveness of *T. arjuna* bark. Moreover, chemical constituents can vary considerably between individual plants due to genetic or environmental differences, development stages of the plant at harvesting, drying process and storage technique (Croom, 1983). Thus, a quality control of the plant material, the extraction scheme and the extract itself is strongly recommended for further studies.

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