EVALUATION OF ANTHELMINTIC ACTIVITY OF *LAGENARIA SICERARIA* (MOLINA) STANDL AND *ALBIZIA LEBBECK L.* AGAINST GASTROINTESTINAL HELMINTHS OF SHEEP

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ABSTRACT

The present study was designed to determine *in vitro* and *in vivo* anthelmintic activity of *Lagenaria (L.) siceraria* and *Albizia (A.) lebbeck* against gastrointestinal helminths of sheep. *In vitro* anthelmintic activity of crude aqueous methanolic extract (CAME) of both plants was evaluated against *Haemonchus (H.) contortus* and their eggs through adult motility assay (AMA) and egg hatch test (EHT), respectively. *In vivo* anthelmintic activity of different concentrations (1.0-8.0g/kg^{-1}) of crude powder (CP) and CAME of both plants was determined using faecal egg count reduction test (FECRT) in sheep naturally infected with gastrointestinal helminths. CAME of both plants exhibited strong *in vitro* anthelmintic activity and distinct inhibitory effects on hatching of *H. contortus* eggs as determined through AMA and EHT. In AMA, the efficacy of *A. lebbeck* (3.75 μg/ mL^{-1}) was higher ($P \leq 0.05$) as compared to *L. siceraria* (4.21 μg/mL^{-1}), while in EHT, *L. siceraria* (2.53 μg/mL^{-1}) was found more potent ($P \leq 0.05$) than *A. lebbeck* (2.75 μg/mL^{-1}). However, *in vivo*, maximum reduction in egg per gram of faeces was observed as 46.7% and 45.9% with CP and CAME of *L. siceraria* and as 39.0% and 47% with those of *A. lebbeck* at 8g/ kg^{-1} on 15 days post-treatment, respectively. The present data may indicate that *L. siceraria* and *A. lebbeck* contain strong anthelmintic agent that act either *in vivo* or *in vitro* which may justify their traditional use as ethnoveterinary medicine.

Keywords: *Anthelmintic activity; Lagenaria siceraria; Albizia lebbeck; Gastrointestinal helminths; sheep*

INTRODUCTION

Hehminthiasis is considered as a major health constraint lowering livestock productivity round the globe (**Githiori *et al.*, 2004**). Most of the parasite control programs are based upon a combination of chemotherapeutic control, grazing management, dietary management, biological control, vaccination and ethnoveterinary treatment (**Waller, 1999; FAO, 2002**). Various problems have been evolved with chemotherapeutic control practices such as development of resistance to various commercially available drugs (**Vermunt *et al.*, 1995; Chandrathani *et al.*, 1999; Chartier *et al.*, 2001; Leathwick *et al.*, 2001**), chemical residues and toxicity problems (**Kaemmerer and Buttenkotter, 1973; Muhammad *et al.*, 2004**), un-economical, non-adaptability and non-availability of drugs in remote areas. However, in developing countries like Pakistan, a huge proportion of farmers are dependant on alternative control strategies like ethnoveterinary medicine (EVM) as evident from the latest survey conducted by **Hussain *et al.* (2008)**. A variety of plants have been scientifically validated for their anthelmintic properties *in vitro* and *in vivo* (**Akhtar *et al.*, 2000; Iqbal *et al.*, 2003; 2004; 2005; 2006a, b, c, d; 2007**).

Lagenaria (L.) siceraria, commonly known as bottleground, is a common weed in Pakistan. *L. siceraria* is straggling herb which produces bottle shaped fruits. It has been used traditionally for the treatment of helminths (**Hussain *et al.*, 2008**), ulcer, jaundice, fever, pectoral cough, asthma and bronchial disorders (**Sivarajan and Balachandran, 1981; Kirtikar and Basu, 1987; Chopra, 1992; Ng, 1993**). Moreover, *L. siceraria* fruits have been reported to have antioxidant activity (**Jiwjinda *et al.*, 2002**), hypolipidemic, antihyperlipidemic effects (**Ghule *et al.*, 2006**) and anticancer activity (**Wang and Ng, 2000**).

Albizia (A.) lebbeck, commonly called as Siris, is moderate to large deciduous tree which has been used as a fodder tree during dry season in Pakistan. It has been reported as folk remedy for many ailments including treatment of cough, abdominal tumor, flu, lung problems, gingivitis and inflammation (**Lowry *et al.*, 1994; Duke, 2008**). Additionally, the bark of *A. lebbeck* has also been reported to possess psychoactivity (**Rätsch, 2004**).

The above mentioned reports of medicinal uses of *L. siceraria* and *A. lebbeck* accompanied with the claims of traditional healers for their anthehmintic activity (**Hussain *et al.*, 2008**) made the basis of designing this project for *in vitro* and *in vivo* scientific validation of these priceless novel herbs in Pakistan. The present study may be helpful for the veterinarians and local farmers to control nematode infections.

MATERIALS AND METHODS

Collection and processing of plant materials

Leaves of *L. siceraria* and *A. lebbeck* were collected from the local fields of District Sahiwal (Punjab, Pakistan) during a survey (previous part of author's research), identified and authenticated by a botanist by comparing with the specimens stored in the herbarium of Department of Botany, University of Agriculture, Faisalabad, Pakistan. The voucher specimen numbers 0125 (*Lagenaria siceraria* (Molina) Standl. leaves) and 0132 *(Albizia lebbeck* (L.) Benth. leaves) described in previous report of **Hussain *et al.* (2008)** were stored in the Ethnoveterinary Research and Development Centre (ERDC), Faculty of Veterinary Science, University of Agriculture, Faisalabad, Pakistan. Plant materials were processed through heat drying in an oven at 40°C, ground to a fine powder and stored in polythene bags at 4°C until use **(Jabbar *et al.*, 2007)**.

Extract preparation

Plant materials (in varying amount depending upon availability of plant) were dried under shade at a well ventilated place and cleaned of adulterants, ground to powdered form and were soaked in sufficient amount of 70% aqueous-methanol by cold maceration at room temperature for a total of 3 days. The filtrate was collected through a piece of porous cloth and filter paper and re-soaked twice. The combined filtrate was concentrated in a rotary evaporator at 40 °C under reduced pressure to yield a thick and dark colored crude extract. This extract was stored at -4°C until use. On the day of experiments, stored extract was dissolved in distilled water to prepare stock solution and different dilutions for the purpose of evaluating pharmacological activity. Percentage yield of extract was calculated through the formula as under **(Jabbar *et al.*, 2007)**:

Extract yield = weight of dry matter before dipping – weight of dry matter after dipping

Percentage yield = (extract yield/weight of dry matter before dipping) × 100

In vitro anthelmintic activity

Collection of worms recovery of eggs

Briefly, live worms were collected by giving the longitudinal incision along the greater curvature of abomasums of freshly slaughtered sheep in the local abattoir. The worms present in ingesta or attached to the surface of guts were picked manually using forceps. The worms were washed and finally suspended in a bottle containing cooled (4°C) phosphate buffer saline (PBS). For egg hatch test (EHT), some of the collected female worms were triturated in pestle and mortar and the suspension was filtered using sieve 100-mesh (150μm pore size). The material left on sieve was washed again and transferred to Clayton Lane tubes. Filtrate was centrifuged in for 2 min at about 300 x *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 x *g*. Cover slip 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 x *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 mL^{-1} using the McMaster technique **(Soulsby, 1982)**.

Adult motility assay

Mature live *Haemonchus (H.) contortus* from sheep were used to determine the effect of crude aqueous methanolic extract by method described previously by **Singh *et al.* (1985)**. A minimum of ten worms were exposed in three replicates to each of the following treatments in separate Petri dishes at room temperature (25-30 °C):

1. Crude aqueous methanol extract at the rate of 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39 and 0.19 mg ml^{-1}
2. Levamisole 0.5 mg ml^{-1}
3. Phosphate buffer saline (PBS)

The inhibition of motility and/or mortality of the worms kept in the above treatments was used as the criterion for anthelmintic activity. The motility was observed after 0, 2, 4, 6, 8 and 12 hr intervals. Finally, the treated worms were kept for 30 min in the lukewarm fresh PBS to observe the revival of motility. The number of dead and survived worms was recorded for each treatment.

Egg hatch test

Egg hatch test was conducted by the method described by **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 (0.25 to 8 mg mL^{-1}) of plant extract i.e., CME. The positive control wells received different concentrations (0.09 to 3.0 μg mL^{-1}) of oxfendazole (Systamex®, ICI Pakistan, Ltd.; 2.265%, w/v) while negative control wells contained the diluent and the egg solution. The plates were incubated at 27°C for 48 h and then 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 in three replicates for each treatment and control plates.

In vivo anthelmintic activity

In vivo anthelmintic activity of study plants was determined using fecal egg count reduction test (FECRT) as described by **Iqbal *et al.*, (2006e)**.

Study animals

Sixty four (64) Lohi sheep of both sexes (≤1 year of age) weighing 18–25 kg naturally infected with mixed parasitic worms having eggs per gram (EPG) of >500 i.e. 1300-1600 were selected from the Allah Dad cattle farm, District Khanewal, Punjab, Pakistan. The selected animals were routinely vaccinated against different bacterial/viral diseases and dipped with ectoparasiticide. The selected sheep were segregated into eight groups comprising of eight animals in each group and completely randomized block design was applied **(Petrie and Watson, 1999)**. The sheep were kept on wood shaving and fed with fresh green fodder, concentrate and water *ad libitum*.

Treatment and follow-up procedures

The pre-treatment worm burden was determined through examination of faeces, directly collected from rectum using modified floatation technique **(MAFF, 1986)**. Taxonomic identification of eggs in the faeces was carried out using keys **(Soulsby, 1982)** and confirmed through identification of morphological characterization of larvae recovered through coproculture as described by **Thienpont *et al.* (1979)**. The nematodes identified from selected animals *Haemonchus contortus*, *Trichostrongylus spp.*, *Strongyloides papillosus* and *Trichuris ovis*. The selected groups were subjected to following treatments:

Group 1: Untreated control.

Group 2: Levamisole HCl (Nilverm® 1.5%, w/v; ICI Pakistan Limited, Animal Health Division) at 7.5 mgkg−1 body weight (b.w.), PO.

Group 3: Crude powder (CP) at 1 gkg−1 b.w, PO.

Group 4: CP at 4 g kg−1 b.w, PO.

Group 5: CP at 8 g kg−1 b.w, PO.

Group 6: crude aqueous methanolic extract (CAME) at equivalent dose rate 1 gkg−1 b.w. of CP, PO.

Group 7: CAME at the equivalent dose rate 4 g kg−1 b.w. of CP, PO.

Group 8: CAME at the equivalent dose rate 8 g kg−1 b.w. of CP, PO.

The post-treatment faecal egg counts per gram of feces (EPG) were performed through on days 0, 3, 6, 9, 12 and 15 using Whitlock universal egg counting chamber **(Whitlock, 1960)**. Percentage of egg count reduction was determined by using the following formula as described by **Jabbar et al. (2007)**.

ECR (%) = {(pre-treatment EPG – post-treatment EPG)/pre-treatment EPG} × 100

Statistical analyses

For egg hatch test, probit transformation was performed to transform a typical sigmoid dose-response curve to linear function **(Hubert and Kerboeuf, 1992)**. The extract concentration required to prevent 50%, i.e., lethal concentration 50 (LC50) of

hatching of eggs was 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

Lagenaria siceraria (Molina) Standl.

Adult motility assay

Adult motility assay of *Lagenaria siceraria* (Molina) Standl. showed that mean number of live worms treated with CAME at dose rate of 100 mg mL^{-1} became zero at 2nd hour PT and remained zero until the end of experiment. The results of above mentioned dose rate were comparable with that of positive control as there was a non significant difference ($p \geq 0.05$) between levamisole (0.5 mg ml^{-1}) and CAME (100 mg ml^{-1}). Other concentrations also showed time and dose dependent response and significant difference with negative control ($p \leq 0.05$). The mean number of untreated control worms remained unaltered until the end of experiment (Fig. 1).

Egg hatch test

Crude aqueous methanolic extract of *Lagenaria siceraria* (Molina) Standl. showed inhibitory effect on egg hatching and LC_{50} was calculated graphically from the regression equation after correcting from negative control. The calculated LC_{50} values of *Lagenaria siceraria* (Molina) Standl. and positive control (oxfendazole) were 0.005528 and 0.005239 $\mu g\ mL^{-1}$ respectively. The regression values and correlation of regression of *Lagenaria siceraria* (Molina) Standl. and positive control were $y = -0.0006x + 4.7245$; $R^2 = 0.9596$ and $y = -5.5087x + 6.7184$; $R^2 = 0.6531$ respectively (Fig. 2). It was observed that CAME of *Lagenaria siceraria* (Molina) Standl. was excellently effective against egg hatch and LC_{50} of CAME of *Lagenaria siceraria* (Molina) Standl. was comparable with that of positive control.

Fecal egg count reduction test (FECRT)

Maximum FECR recorded was 46.7% with CP of *Lagenaria siceraria* (Molina) Standl. leaves at the dose rate of 8 g/ kg^{-1} at 15 days of PT while it was significantly higher ($p \leq 0.05$) than other concentrations of CP of *Lagenaria siceraria* (Molina) Standl. leaves which were recorded as 28.1 and 37.2% at dose rate of 1 and 4 g/ kg^{-1} of body weight at day 15 PT (Fig. 3). A similar trend was observed with CAME of *Lagenaria siceraria* (Molina) Standl. leaves. Maximum FECR (46.0%) was observed at the dose rate of 8 g/ kg^{-1} at day 15 PT followed in order by 35.8 and 26.6% at the dose rate of 4 and 1 g/ kg^{-1} of body weight at day 15 PT in the descending order of activity (Fig. 3). There was significant difference ($p \leq 0.05$) within concentrations as well as

among the days post treatment. However, the FECR of CP of *Lagenaria siceraria* (Molina) Standl. leaves was non significantly higher ($p \geq 0.05$) than the concentrations of crude aqueous methanolic extract.

Albizia lebbeck (L.) Benth.

Adult motility assay

Mean number of live worms treated with CAME at dose rate of 100 and 50 mg/ mL^{-1} became zero at 4th hour PT and remained zero until the end of experiment. At the 10^{th} hour PT CAME of *Albizia lebbeck* (L.) Benth, killed all the worms at dose rate of 25, 12.5, 6.25 and 3.12 mg/ mL^{-1}. Other concentrations also showed time and dose dependent response (Fig. 4). The mean number of untreated control worms remained unaltered until the end of experiment.

Egg hatch test

Crude aqueous methanolic extract of *Albizia lebbeck* (L.) Benth. showed inhibitory effect on egg hatching and LC_{50} was calculated graphically from the regression equation after correcting from negative control. The calculated LC_{50} values of *Albizia lebbeck* (L.) Benth. and positive control (oxfendazole) were 0.007493 and 0.005239 $\mu g/mL^{-1}$ respectively (Fig, 5). The regression values and correlation of regression of *Albizia lebbeck* (L.) Benth. and the positive control were $y = -0.0002x + 4.6324$; $R^2 = 0.9689$ and $y = -5.5087x + 6.7184$; $R^2 = 0.6531$, respectively. It was observed that CAME of *Albizia lebbeck* (L.) Benth. was effective against egg hatch and found less effective in this regard as LC_{50} of CAME of *Albizia lebbeck* (L.) Benth., and was higher as compared to that of the positive control.

Fecal egg count reduction test (FECRT)

Maximum fecal egg count reduction recorded was 47.0% with CAME of *Albizia lebbeck* (L.) Benth. at the dose rate of 8 g kg^{-1} (Fig. 6) at day 15 PT while it was significantly higher ($p \leq 0.05$) than all the doses and forms of *Albizia lebbeck* (L.) Benth. at day 15 PT with which the FECR was recorded as 26.2 and 29.5% at dose rate of 1 and 4 g/kg^{-1} (CAME) of body weight at day 15 PT. A similar trend was observed with CP of *Albizia lebbeck* (L.) Benth. leaves. Maximum FECR (39.0%) was observed at the dose rate of 8 g/kg^{-1} at day 15 PT followed in order by 35.7 and 26.5% at the dose rate of 4 and 1 g/kg^{-1} (CP) of body weight at day 15 PT (Fig. 6) in the descending order of activity. There was a similar trend with these doses and forms at day 3, 6, 9 and 12 PT.

DISCUSSION

According to the survey conducted by **Hussain et al. (2008)**, *L. siceraria* and *A. lebbeck* are traditionally used anthelmintics in small and large animals of district Sahiwal (Punjab, Pakistan). Regarding *A. lebbeck,* no scientific validation of anthelmintic activity has been made so far.

The present investigation is the first report on scientific validation of *A. lebbeck* showing its promising anthelmintic activity as determined through *in vivo* (AMA & EHT) and *in vitro* (FECRT) assays in view of its usage as ethnoveterinary medicine in Pakistan. Previous studies on phytochemistry of *A. lebbeck* indicated that leaves of this plants contain 3',5 Dihydroxy 4', 7 dimethoxy flavone and N- Benzoyl L phenyl alaninol **(Rashid *et al.*, 2003)**. Moreover, leaves of this plant constitutes potent chemosterilant compounds **(Shastri, 1952)** including saponins **(Pal *et al.*, 1995; Ueda *et al.*, 2003)**, macrocyclic alkaloids **(Misra *et al.*, 1995; Dixit and Misra, 1997)**, phenolic glycosides **(Maa *et al.*, 1997)** and flavonols **(El-Mousallamy, 1998)** which may be responsible for its anthelmintic activity. Previously, these compounds in various plants have also been reported to possess potent anthelmintic activity **(Akhtar, 1988; Akhtar and Aslam 1989; Akhtar and Ahmad, 1992; Asuzu and Onu, 1993; Roepke, 1996; Fakae *et al.*, 2000)**

Regarding *in vivo* anthelmintic activity of *L. siceraria*, **Akhtar and Riffat (1987)** found 89, 67, 81 and 91% reduction in EPG against seed powder, water extract and methanol extract @ 3 g/kg, and Niclosamide @ 100 mg/kg, respectively. However, in the present study, *L. siceraria* showed 46.7% and 45.9% reduction in EPG with CP and CAME, respectively as determined through FECRT. The probable reason of this lower anthelmintic activity may include difference in parts of plant used in previous study. However, the chemical constituents of *A. lebbeck* including flavonoids **(Baranoswka and Cisowski, 1994)** and cucurbitacin saponins **(Rahman, 2003)** may be responsible for its anthelmintic activity.

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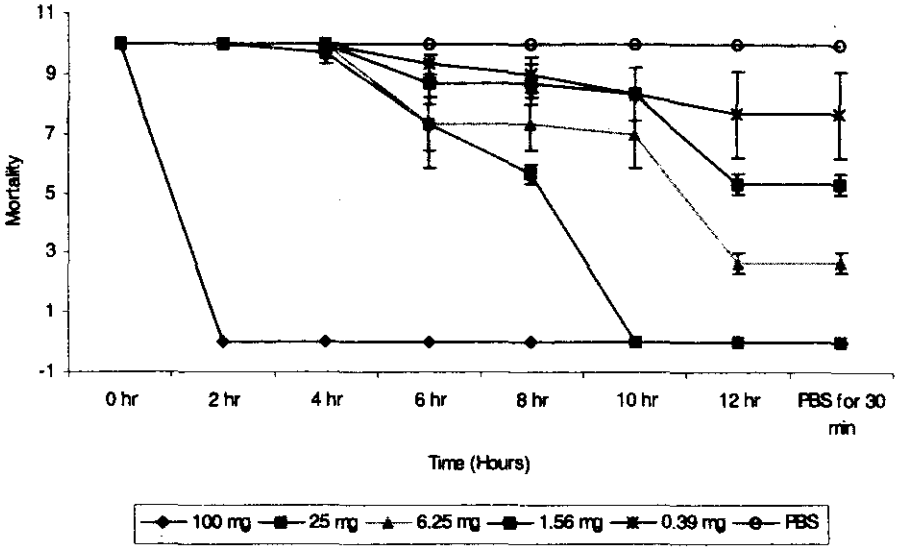

Fig. 1. Graph showing the time- and dose-dependent *in vitro* anthelmintic activity of CAME of *Lagenaria siceraria* (Molina) Standl. leaves on *Haemonchus contortus* of sheep.

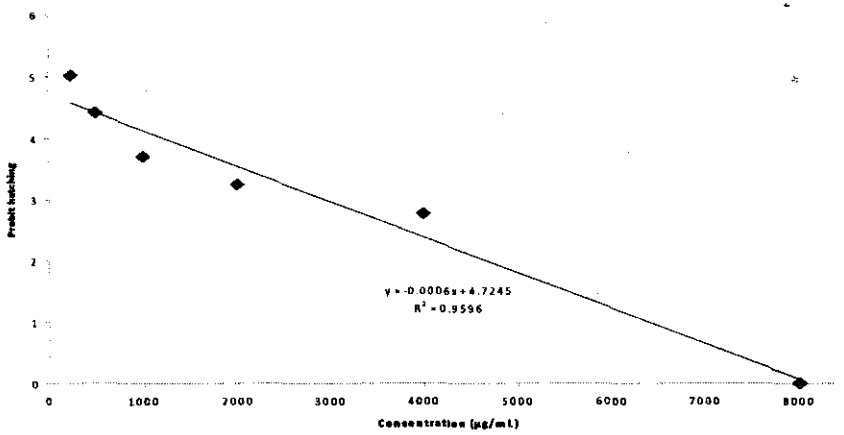

Fig. 2. Linear relationships between egg hatching % on the probit scale of *Haemonchus contortus* and CAME of *Lagenaria siceraria* (Molina) Standl. leaves concentrations ($\mu g\ mL^{-1}$).

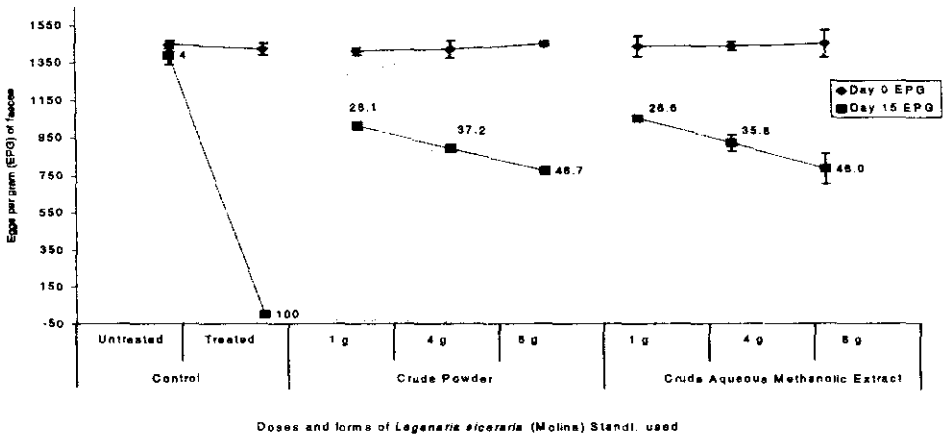

Fig. 3. Reduction in eggs per gram (EPG) of faeces in sheep treated at different doses and forms of *Lagenaria siceraria* (Molina) Standl. compared with control group.

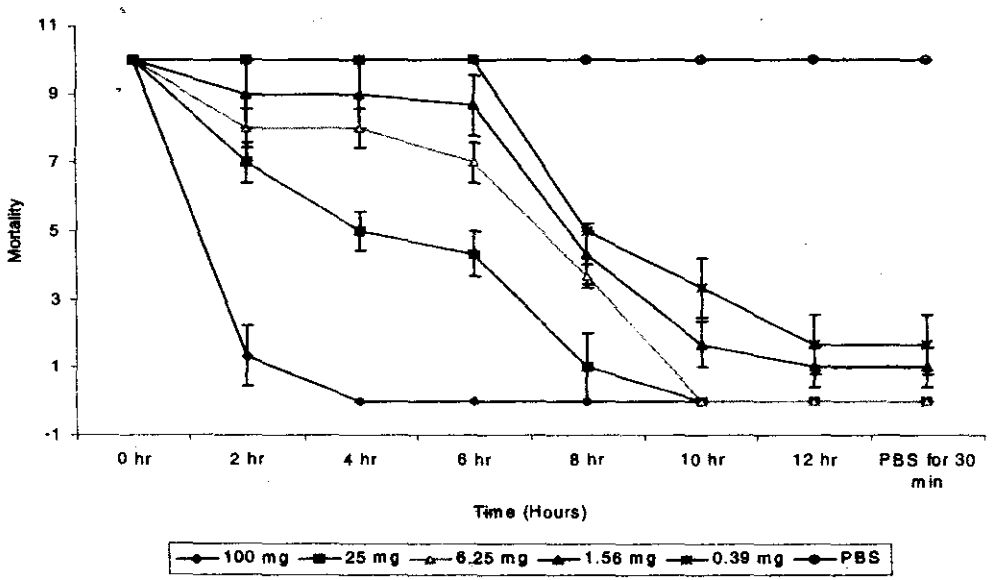

Fig. 4. Graph showing the time- and dose-dependent *in vitro* anthelmintic activity of *Albizia lebbeck* (L.) Benth. on *Haemonchus contortus* of sheep.

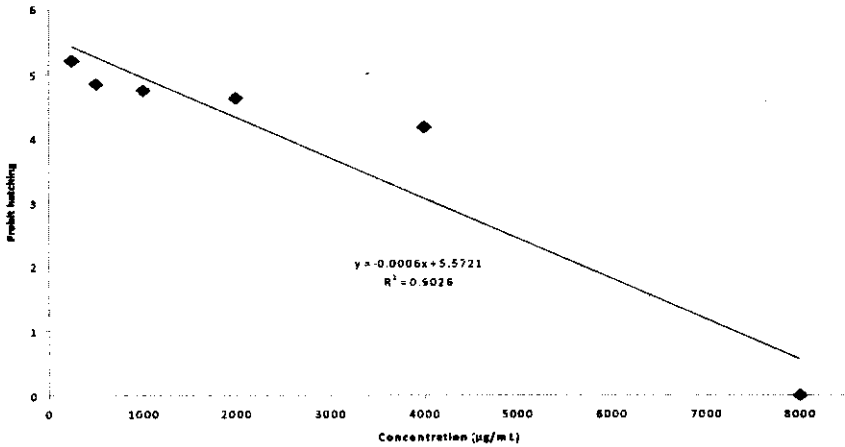

Fig. 5. Linear relationships between egg hatching % on the probit scale of *Haemonchus contortus* and *Albizia lebbeck* (L.) Benth. leaves crude aqueous methanolic extract concentrations ($\mu g\ mL^{-1}$).

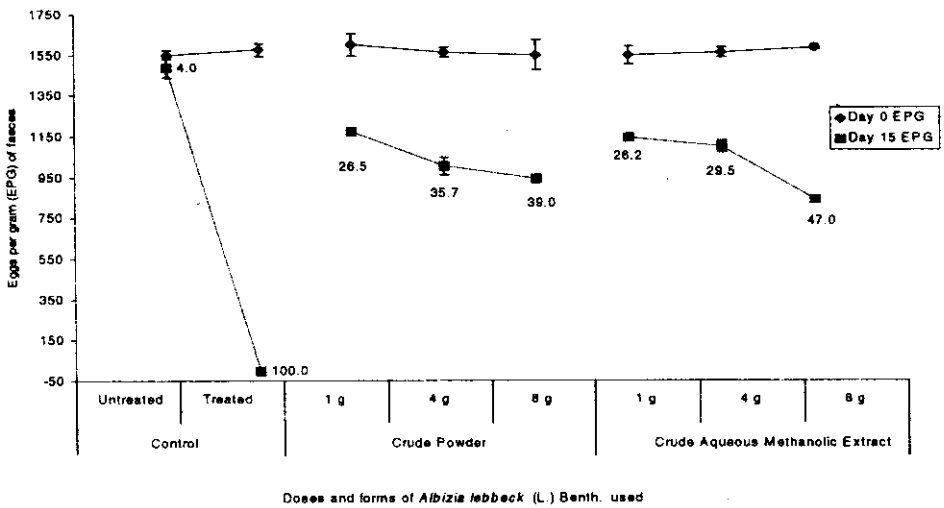

Fig. 6. Reduction in eggs per gram (EPG) of faeces in sheep treated at different doses and forms of *Albizia lebbeck* (L.) Benth. compared with control group.