The Journal of Animal & Plant Sciences, 21(4): 2011, Page: 781-786
ISSN: 1018-7081

IN VITRO SCREENING OF *ZIZIPHUS MAURITIANA* AND *TERMINALIA ARJUNA* FOR THEIR ANTHELMINTIC ACTIVITY

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ABSTRACT

The present studies have been planned to evaluate ovicidal efficacy of *Ziziphus mauritiana* and *Terminalia arjuna* leaves. For this purpose, egg hatch test (EHT) was conducted on nematode ova to investigate the *in vitro* ovicidal effects of crude aqueous extract (CAE) and crude aqueous methanolic extracts (CAME) of the leaves of the plants. Lethal concentration 50 (LC_{50}) values of CAE and CAME of *Ziziphus mauritiana* leaves were 0.1773 and 0.6778 while of *Terminalia arjuna* leaves were 1.502 and 3.002 respectively. This study shows that *Ziziphus mauritiana* and *Terminalia arjuna* leaves possess *in vitro* anthelmintic activity. The study also suggests further large scale pharmacological and toxicological studies for their use in veterinary medicine.

Key words: *Ziziphus mauritiana; Terminalia arjuna;* egg hatch test; Anthelmintic activity; Leaves; Pakistan.

INTRODUCTION

Helminths are recognized as a major constrain to livestock production throughout the tropics and elsewhere (Githiori *et al.,* 2004)**.** They cause retarded growth (Kochapakdee *et al.,* 1995), lowered productivity (Perry and Randolph, 1999), mortality (Sykes, 1994) and high economic losses (Iqbal *et al.,* 1993). The prevalence of helminths in different species of animals has been reported and ranged from 25.1 to 92% in Pakistan (Khan *et al.,* 1989).

Most of the parasite control programs are based upon a combination of chemotherapeutic control, grazing management, dietary management, biological control, vaccination and ethnoveterinary treatment (FAO, 2002). Chemotherapeutic control practices have led to a number of problems including resistance of helminths to various anthelmintic groups (Chartier *et al.,* 2001), chemical residues, increased cost of treatment and non-availability of the medicine in remote areas of Pakistan. These emerging issues diverted the researchers' attentions towards the development of alternate strategies for the treatment of helminthiasis (Iqbal *et al.,* 2003). Herbal medicine have gained much importance in recent years due to the good efficacy and cost effectiveness (Dahiru *et al.,* 2006). Plants constitute a huge part of traditional veterinary practices and are a rich source of herbal anthelmintics of veterinary importance for centuries (Iqbal *et al.,* 2003; Iqbal *et al.,* 2004).

Ziziphus mauritiana Lam. (Family: Ramnaceae) is commonly known as "beri" in Pakistan. The different parts of the plant are used as cuts and ulcers healer, pulmonary ailments, fevers, laxative, sedative, anti-nausea, anti-rheumatic areas, anti-diarrhoeal, wounds and abscesses healer, swelling, gonorrhoea curer (Michel, 2002) and also used as anthelmintic in ethnoveterinary medicinal system in Pakistan (Hussain *et al.,* 2008). The plant is also used in liver diseases, asthma and fever, gingivitis, febrifuge and epilepsy (Msonthi and Magombo, 1983; Morton, 1987). *Terminalia arjuna* (Family: Combretaceae) is a plants that holds a reputable position in both Ayurvedic and Unani Systems of medicine. Common name of the plant in Pakistan is "Arjun". Different parts of the plant are useful as cardiovascular tonic, alexteric, styptic and anthelmintic. The plant is also used in diuresis, ulcers, asthma, heart disease, biliousness, fractures, tumours, leucoderma, anemia, excessive perspiration, internal and external problems of urinary discharge, endocarditis, mitral regurgitation, pericarditis, angina and heart tonic (Bharani *et al.,* 1995; Bharani *et al.,* 2002; Karthikeyan *et al.,* 2003; Sarwat *et al.,* 2006).

Keeping in view of the traditional uses of these plants, the present project was designed to study the ovicidal effects of crude aqueous and crude aqueous methanolic extracts of the leaves of *Ziziphus mauritiana* and *Terminalia arjuna* against the eggs of nematodes.

MATERIALS AND METHODS

1. Collection of Plant Material: Leaves of the *Ziziphus mauritiana* and *Terminalia arjuna* were collected from

various districts of Punjab, Pakistan with the assistance of local healers through transect walking along the culverts and identified from a botanist using preserved germplasm, Department of Botany, University of Agriculture Faisalabad. Voucher specimens were kept at the Herbarium, Ethnoveterinary Research and Development Center, Department of Parasitology, University of Agriculture, Faisalabad. Leaves were dried under the shade and dried leaves were ground to the powder in an electric mill and stored in cellophane bags at 4°C until use.

2. Preparation of aqueous extract: Crude aqueous extract (CAE) of powdered plants was prepared according to the standard methods (Fenado *et al.,* 1989). Briefly, 100 g of the powdered leaves were mixed with 500 mL of distilled water in a 1 L flask and boiled for 1.5 hours. Brew was filtered using Whatman No.1 filter paper after cooling it to 40°C. The filtrate was concentrated in a rotary evaporator under vacuum and the extract was stored at 4°C until use.

3. Preparation of crude aqueous methanolic extract: Crude aqueous methanolic extract of powered plants was prepared according to the standard methods (Gilani *et al.,* 2004). Briefly, 1 kg of ground plant material was soaked in sufficient quantity of 70% aqueous-methanol by cold maceration at room temperature for three days after which the filtrate was collected through a piece of muslin cloth and then filter paper and the plant material was re-soaked twice. The filtrate was concentrated in a rotary evaporator at 40°C under reduced pressure to yield crude extract. This extract was stored at 4°C until use. The crude extract (as much as needed) was dissolved in distilled water on the day of the experiment to prepare stock solution and different dilutions for the purpose of evaluating anthelmintic activity.

4. In vitro ovicidal activity

4.1. Nematode egg recovery technique: Eggs of the nematodes were recovered using the technique described by Le Jambere (1976). Briefly, approximately 50 gm of faeces obtained from the sheep, suspended in approximately 50 mL of water using electric mixer. This suspension was washed through a sieve with saturated NaCl solution. The mixture was poured into a shallow tray having the depth of 4 cm and a sheet of plastic cut to the shape of the tray was floated on top. As the specific gravity of the mixture is greater than some nematode eggs, so they float to the top and adhere to the plastic sheet. After about 15 min. the plastic sheet was removed and the eggs were washed off with a stream of water from a wash bottle into a beaker. Number of eggs was estimated by McMaster technique (Soulsby, 1982).

4.2. Egg suspension: The concentration of eggs was estimated in 50 µl samples and adjusted to 500 eggs mL^{-1}. The egg suspension was diluted with filtrate from the first step of egg extraction that had been centrifuged for 5 min at 100 × *g* to eliminate organic debris to provide bacteria for larval development. To avoid the proliferation of fungi 5 µg of amphotericin B was added per mL of suspension.

Test Procedure: The egg hatch assay was carried out using the World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) guidelines for determination of anthelmintic resistance (Coles *et al.,* 1992) with modifications that allowed the testing of the natural compounds (Alawa *et al.,* 2003). A suspension of 0.2 ml was distributed in a 24-well flat-bottomed microtitre plate containing approximately 100 fresh eggs and mixed with the same volume of plant extract having different concentrations (1, 2, 4 & 8 $mgmL^{-1}$). The control plates contained the diluent water and dimethyl sulfoxide (DMSO). The plates were incubated for 48 hours at room temperature and after incubation 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 five replicates for each concentration and control. Data was expressed as percentage of unhatched eggs.

Statistical analysis: Typical dose response curve (sigmoid) was transformed to linear function through probit transformation. The concentration of the extract required to inhibit 50% of eggs from hatching also called as lethal concentration 50 (LC_{50}), was calculated by the linear regression (Hubert and Kerboeuf, 1992).

RESULTS AND DISCUSSION

Aqueous extract as well as methanolic extracts of *Ziziphus mauritiana* and *Terminalia arjuna* exhibited anthelmintic activity by inhibiting hatching of nematode eggs. The LC_{50} was calculated graphically by the regression equation as shown in the Figs 1, 2, 3 and 4. The values of LC_{50} of aqueous and methanolic extracts of *Ziziphus mauritiana* were 0.1773 and 0.6778 $mgmL^{-1}$, respectively. Values of regression and correlation of regression of the aqueous extract were $y = -1.0727x + 5.1902$ and $R^2 = 0.9514$, respectively, while of methanolic extract were $-1.1833x + 5.1145$ and R^2 0.9572, respectively. Aqueous extract showed stronger anthelmintic activity against egg hatch than that of the methanolic extract (Figs 1 and 2).

The values of LC_{50} of aqueous and methanolic extracts of *Terminalia arjuna* were 1.502 and 3.002 $mgmL^{-1}$, respectively. Values of regression and correlation of regression of the aqueous extract were $y = -0.715x + 4.6495$ and $R^2 = 0.9075$, respectively, while of methanolic extract were $-0.575x + 4.6015$ and R^2 0.8514, respectively. In case of *Terminalia arjuna* also aqueous

extract showed stronger anthelmintic activity against egg hatch than that of the methanolic extract (Figs 3 and 4).

These *in vitro* tests determine the effects of anthelmintic drugs on physiological processes like hatch, development, mortality and motility of the parasites (Varady and Corba, 1999). The *in vitro* assays provide cheaper, economical and rapid turn over in contrast to *in vivo* assays as for as anti-parasitic properties of plants and plant extracts are concerned (Githori et al., 2006). Higher levels of anthelmintic activity of CAE of *Ziziphus mauritiana* revealed that active ingredient; responsible for the anthelmintic activity is relatively a polar compound (Iqbal *et al.,* 2010). There is no report available on an anthelmintic activity of leaves of *Ziziphus mauritiana* and this is the first scientific evidence on anthelmintic activity of the plant, however it is used in indigenous system of medicine as anthelmintic (Hussain *et al.,* 2008). Phytochemical reports on Ziziphus species has revealed the presence of polysaccharides (Yamada *et al.,* 1985; Zhao *et al.,* 2006a), a pectin composed of D-galacturonic acid, L-rhamnose, D-galacturonic acid as methyl ester and O-acetyl groups (Shimizu and Tomoda, 1983), cyclopeptides (Barboni *et al.,* 1994; Gournelis *et al.,* 1998; Singh *et al.,* 2002), peptide alkaloids (Tschesche *et al.,* 1974), flavonoides (Nawar *et al.,* 1984; Cheng *et al.,* 2000), dodecaacetylprodelphinidin B3 (Weinges and Schick, 1995), Ziziphine N, O, P and Q (Suksamrarn *et al,* 2005), saponins and fatty acids (Zhao *et al.,* 2006b). However, those responsible for its anthelmintic activity have not yet been explored. Anyhow, anthelmintic activity of various phytochemicals including flavonoides, saponins, alkaloids (Lateef *et al.,* 2003; Hussain *et al.,* 2010) and tannins (Molan *et al.,* 2000a,b; Iqbal *et al,* 2007; Hussain *et al.,* 2010) strongly support this speculation.

Crude aqueous extract of *Terminalia arjuna* leaves proved to be more efficacious than crude aqueous methanolic extract as in case of *Ziziphus mauritiana* depicting that the active principal responsible for the anthelmintic activity is a relatively polar compound. Phytochemical studies of the plant reveals that it contains the constituents lilke arjunic acid, terminic acid, glycosides, plant flavones, tannins, oligomeric proanthocyanidins, pyrocatechol tannins and glucotannic acid, together with sodium, calcium salts, magnesium salts, and phytosterols (Kandil and Nassar, 1998; Bharani *et al.,* 2002; Ali *et al.,* 2003).

The active principal responsible for the anthelmintic activity of leaves of *Ziziphus mauritiana* and *Terminalia arjuna* and their mechanisms of action have not so far been elucidated. However, phytochemical screening of the plant extract revealed the probable presence of tannins, flavonoids and saponins (Dahiru et al., 2005) which are good anthelmintics (Athanasiadou et al., 2000; Molan *et al.,* 2000a,b; Athanasiadou *et al.,* 2001; Iqbal *et al.,* 2002; Hussain *et al.,* 2010).

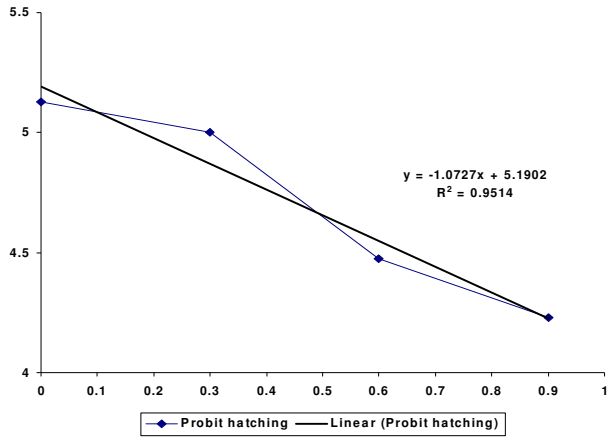

Figure 1. Probit hatching of namatode eggs against various log doses of crude acquous extract (CAE) of *Ziziphus mauritiana* (Beri).

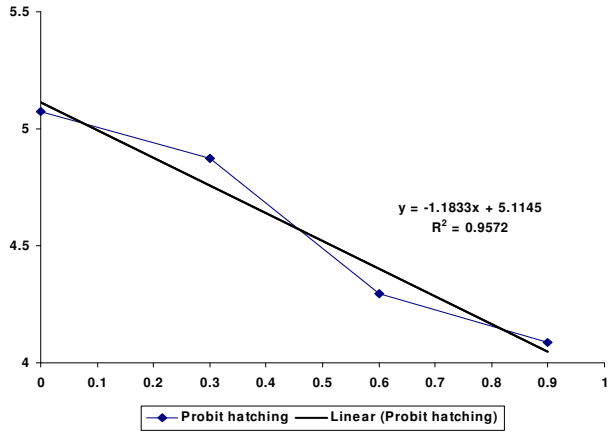

Figure 2. Probit hatching of namatode eggs against various log doses of crude aqueous methanolic extract (CAME) of *Ziziphus mauritiana* (Beri)..

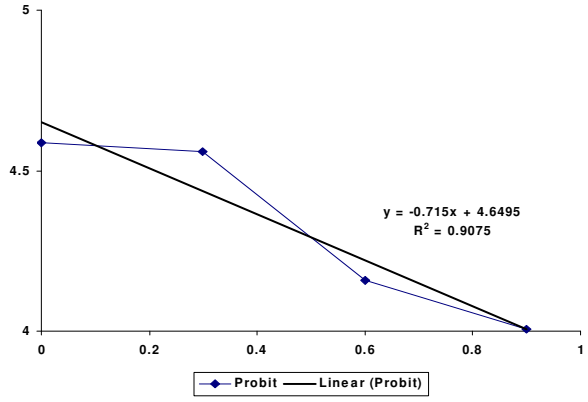

Figure 3. Probit hatching of namatode eggs against various log doses of crude acquous extract (CAE) of *Terminalia arjuna* (Arjun)

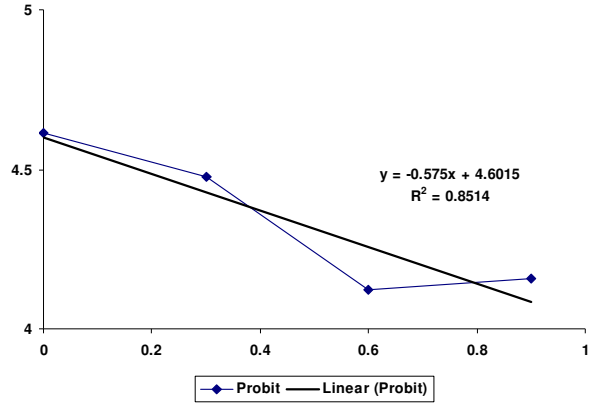

Figure 4. Probit hatching of namatode eggs against various log doses of crude aqueous methanolic extract (CAME) of *Terminalia arjuna* (Arjun).

Conclusions: The plants considered in this study and used in ethnoveterinary system of Pakistan have a potential to be used as anthelmintics. It is recommended that further research be carried out on large number of animals, identification of active ingredients of plants with proven anthelmintic activity and study of pharmacodynamics and pharmacokinetics of proven anthelmintic active ingredients of plants. Furthermore, plants from different geographic areas should be evaluated using standard parasitological procedures as same plants grow in different soils may have different chemical compositions.

Acknowledgments: The current research was sponsored by The University of Agriculture, Faisalabad, Pakistan under the Research Promotion Scheme.

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