IN VITRO EFFECTS OF *TRICHOSANTHES DIOICA* LEAVES ON ANNELIDS AND NEMATODES

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Summary

The *in vitro* activities of defatted methanol (MeOH) extract of the leaves from *Trichosanthes dioica* Roxb. (Cucurbitaceae), and its ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH) fractions was evaluated against *Pheretima posthuma* (Annelida) and *Ascaridia galli* (Nematoda). All the extracts demonstrated concentration dependent paralytic and lethal effects on *P. posthuma* and lethal effects on *A. galli*. The EtOAc fraction was found to be the most potent followed by the defatted MeOH extract and its *n*-BuOH fraction. *A. galli* was found to be more sensitive than *P. posthuma* against all test extracts indicating *T. dioica* as an effective nematocide. The present study establishes promising *in vitro* anthelmintic property of *T. dioica* leaves, substantiating its traditional uses in India.

Key words: *Trichosanthes dioica*, anthelmintic, *Pheretima posthuma*, *Ascaridia galli*.

Introduction

Helminths are parasitic worms and helminth infections are prevalent globally, one third of world's population harbours them, but is more common in developing tropical and subtropical countries with poorer personal and environmental hygiene (1). In developing countries they pose a great threat to public health and contribute to the prevalence of malnutrition, anaemia, stunted growth, cognitive impairment and increased susceptibility to other diseases, particularly in children. Although majority of incidences of helminth infections are generally limited to tropical regions, they can also occur to travelers who have visited those areas and some of them can develop in temperate climates (2). In addition to human, domestic animals are very susceptible to helminth infections which adds to the economic burden of developing countries and also a problem for agriculture in many developed countries (3).

The helminths which infect the intestine are cestodes e.g. tapeworms (*Taenia solium*), nematodes e.g. hookworm (*Ancylostoma duodenala*), roundworm (*Ascaris lumbricoids*) and trematodes or flukes (*Schistosoma mansoni* and *Schistosoma haematobolium*) (3). Increasing problems of resistance development in intestinal helminths against synthetic anthelmintics have led to the proposal of screening of medicinal plants for their anthelmintic activity. The resiatance against synthetic anthelmintics for gastrointestinal worms is a worldwide problem of sheep, goat and pig breeding resulting in considerable economic losses (4). Diseases due to nematode infections continue to be the greatest constraint in sustainable livestock production worldwide, primarily due to rapid evolution of drug resistance in these parasites to all classes of synthetic anthelmintics. In addition, global appreciation and general endorsement of organic farming pose serious restriction to the prophylactic use of synthetic drugs (5, 6). Hence, there is an increasing need towards natural anthelmintics. A number of medicinal plants have been traditionally used in Indian subcontinent to treat different helminth infections in man and animals (7, 8).

Trichosanthes dioica Roxb. (Cucurbitaceae), called pointed gourd in English, *Potol* in Bengali, *Palval* in Hindi and *Patola* in Sanskrit is a dioecious climber found wild throughout the plains of North and North-East India from Punjab to Assam and Tripura. It is cultivated for its fruits, a common culinary vegetable in India. In India all parts of this plant has been traditionally used for various medicinal purposes. According to Ayurveda, the leaves are antipyretic, anthelmintic, aphrodisiac, cholagogue, aperient, tonic, digestive and antibilious and used as expectorant, bitter tonic, laxative, alternative and in the cases of bilious fever, in sub-acute cases of enlarged liver and spleen, haemorrhage, fistula-in-ano, fevers, leprosy, intrinsic haemorrhage, erysipelas, alopecia, diseases of mouth, inflammations and wounds (9-12). The leaves and tender shoots are used as culinary spinach in West Bengal and Assam, called as *Palta* in Bengali.

Previous workers reported different phytochemical and pharmacological studies on *T. dioica* fruits and seeds in experimental animals. As there are no reports on anthelmintic activity of *T. dioica* leaves, we found it necessary to evaluate defatted methanol extract of *T. dioica* and its different fractions for their *in vitro* effects against *Pheretima posthuma* and *Ascaridia galli* to justify the traditional and folkloric beliefs.

Materials and methods

Plant material: The young leaves and tender shoots of *T. dioica* were collected during August 2008 from Majdia, Nadia district, West Bengal, India. The species was identified at the Central National Herbarium, Botanical Survey of India, Howrah, West Bengal, India, and a voucher specimen (SB-02) was deposited at Pharmacognosy Reasearch Laboratory, Bengal School of Technology (A College of Pharmacy), Delhi Rd, West Bengal 712102, India. Just after collection the plant material was washed thoroughly with running tap water and shade dried at room temperature (24-26 ºC) and ground mechanically into a coarse powder.

Drugs and chemicals: All the chemicals used were of analytical grades, obtained from Ranbaxy Fine Chemicals Ltd., New Delhi, India. The reference drug Albendazole was obtained as gift sample from Mepro Pharmaceuticals Pvt. Ltd., Surendranagar, Gujrat, India. Doubled distilled water from all-glass still was employed throughout the study.

Preparation of extracts: The powdered plant material (200 g) was macerated with MeOH at room temperature (24-26 ºC) with frequent shaking for 4 days followed by re-maceration with methanol, similarly for 3 days. The methanol extract was filtered, concentrated and appropriate water was added to make 95 % methanol extract which was extracted with equal volume of *n*-hexane. The methanol layer was collected and evaporated to dryness *in vacuo* at 40 ºC. The residue (yield: 9.27 % w/w) was suspended in water and extracted successively with EtOAc and water saturated *n*-BuOH, and the extracts were evaporated to dryness *in vacuo* at 40 ºC to get EtOAc and *n*-BuOH fractions respectively. All dry extracts were kept in a refrigerator until use. Preliminary phytochemical screening revealed the presence of flavonoids, triterpenoids and steroids, saponins, amino acids and reducing sugars in the defatted MeOH extract. The EtOAc fraction revealed the presence of flavonoids, triterpenoids and steroids whereas the *n*-BuOH fraction indicated the presence of saponins, flavonoids and reducing sugars.

Test worms: Adult Indian earthworm *Pheretima posthuma* L. Vaill. (Annelida) were obtained from the waterlogged areas of State Paddy Research Centre, Chinsurah, West Bengal, India. Live local fowls (*Gallus domesticus* Linn.) obtained from the local abattoir at Chandernagore, West Bengal, India, were sacrificed and immediately, live *Ascaridia galli* Schrank (Nematoda) were recovered from the small intestines. Only the live adult worms with more or less the same length were selected and then collected in phosphate buffered saline (PBS, pH 7.2, 0.15 M). All nematodes were immediately maintained at 39 ± 2 ºC in an incubator. Both types of worms were identified at State Paddy Research Centre, Chinsurah, West Bengal, India.

Test samples: Test samples for *in vitro* bioassay were prepared freshly. Varying concentrations of all the test extracts viz. 50, 25, 12.5, 6.25, 3.125 mg/ml were prepared by dissolving or suspending in distilled water for annelids. Similar dilutions were made in PBS (pH 7.2, 0.15 M), supplemented with 2 % dimethyl sulfoxide (DMSO) for nematodes.

The *in vitro* bioassay (13, 14): The fresh worms of nearly equal size were selected for the study. Each types of worms were divided into 17 groups (n = 6). The first group served as positive control and kept in 9 cm Petri dishes (one worm in each) containing 20 ml of albendazole (10 mg/ml) in distilled water (for annelids) and in 2 % DMSO in PBS (for nematodes). The second group served as negative control and kept in distilled water, and in 2 % DMSO in PBS (pH 7.2, 0.15 M) for annelids and nematodes respectively. Five groups were kept in Petri dishes containing 20 ml of MeOH extract at five different concentrations (50 to 3.125 mg/ml). Similarly, rest ten groups were kept in varying concentrations of EtOAc and *n*-BuOH extracts as prepared for annelids and nematodes. The Petri dishes were maintained at 25 ± 2 ºC for annelids and at 39 ± 2 ºC for nematodes. Physical activity of the worms was observed and the time taken for complete paralysis and death were recorded. The mean paralysis time and mean lethal time for each group were determined. Time for paralysis were noted when no movement of any sort could be observed except when the worms were stimulated gently by a blunt pin or probe to activate the worms. Death was ascertained when complete immobility was noted upon poking and shaking, and dipping the parasite in tepid water (~50 ºC) that induced movement in living sentient worms along with observation of fading away of their body colour. Albendazole served as reference vermicide drug in the positive control group.

Statistical analysis: The data were presented as Mean ± Standard Error of Mean (SEM).

Results

The results of *in vitro* evaluation of different test extracts from *T. dioica* leaves in *P. posthuma* and *A. galli* are summarized in Table 1 and 2 respectively. Against *P. posthuma* all the test extracts exhibited significant paralytic and lethal actions in a concentration dependent manner (Table 1). The EtOAc fraction was the most potent showing the shortest paralysis and lethal time, followed by defatted MeOH extract and its *n*-BuOH fraction which was least active, only at higher concentrations, exhibited most prolonged paralytic and lethal time. In one case (at the lowest concentration of defatted MeOH extract) lethal time was not detected till 24 h of observation. Neither paralysis nor death of test worms was found in cases of lower concentrations of the *n*-BuOH fraction till 24 h of observation.

In case of *A. galli*, here also all the test extract demonstrated concentration dependent lethal effects (Table 2). Once the movement of worms ceased i.e. paralyzed, death occurred instantaneously, hence the paralysis times were not detected in this case. The EtOAc fraction was the most active, followed by defatted MeOH extract and its *n*-BuOH fraction which was least active. The EtOAc fraction showed the shortest lethal time (17.06 min) as compared against *P. posthuma* at the same concentration (50 mg/ml). At all the concentrations of all test extracts mortality of worms were detected.

Table 1. Effects of *T. dioica* leaves against *P. posthuma.*

Treatment	Concentration (mg/ml)	Mean paralysis time (min) ± SEM	Mean lethal time (min) ± SEM
Positive control (Albendazole)	10	35.56 ± 0.85	64.75 ± 0.43
Negative control (Vehicle¶)	-	-	-
Defatted MeOH extract	50	20.12 ± 0.90	35.28 ± 0.78
	25	31.84 ± 1.11	71.36 ± 0.95
	12.5	43.56 ± 1.36	85.23 ± 1.25
	6.25	125.18 ± 1.14	269.50 ± 1.14
	3.125	208.47 ± 1.62	-
EtOAc fraction	50	9.33 ± 0.61	23.00 ± 0.74
	25	17.77 ± 0.94	44.64 ± 1.08
	12.5	42.13 ± 1.56	87.65 ± 0.84
	6.25	91.73 ± 1.13	168.84 ± 0.91
	3.125	118.93 ± 1.44	215.12 ± 1.11
n-BuOH fraction	50	144.32 ± 1.04	189.04 ± 1.23
	25	202.68 ± 1.65	326.15 ± 1.15
	12.5	268.06 ± 1.27	383.76 ± 1.49
	6.25	-	-
	3.125	-	-

Number of worms per group (n) = 6.
SEM = Standard Error of Mean.
¶Vehicle: Distilled water.

Table 2. Effects of *T. dioica* leaves against *A. galli.*

Treatment	Concentration (mg/ml)	Mean lethal time (min) ± SEM
Positive control (Albendazole)	10	26.08 ± 0.79
Negative control (Vehicle[¶])	-	-
Defatted MeOH extract	50	30.23 ± 0.77
	25	47.17 ± 1.15
	12.5	79.13 ± 1.18
	6.25	107.37 ± 0.86
	3.125	133.45 ± 0.71
EtOAc fraction	50	17.06 ± 0.54
	25	26.28 ± 0.92
	12.5	45.61 ± 1.12
	6.25	68.54 ± 1.36
	3.125	81.84 ± 1.00
n-BuOH fraction	50	48.19 ± 0.68
	25	67.33 ± 0.95
	12.5	94.73 ± 1.08
	6.25	139.56 ± 0.88
	3.125	213.03 ± 1.03

Number of worms per group (n) = 6.
SEM = Standard Error of Mean.
[¶]Vehicle: 2 % DMSO in PBS solution (pH 7.2, 0.15 M).

Discussion

In present investigation the *in vitro* effects of the defatted MeOH extract of *T. dioica* leaves and its EtOAc and *n*-BuOH fractions were evidenced by their paralytic and lethal actions on *P. posthuma* and *A. galli*. The adult Indian earthworm *P. posthuma*, although not helminth, have extensively been employed for initial *in vitro* anthelmintic evaluation because of their easy availability and their anatomical and physiological resemblance with the intestinal roundworm parasites in human beings (15-18). *A. galli* is a roundworm parasitizing the small intestine of birds, and is by far the most prevalent of all helminths infecting poultry. *A. galli* infections continue to be the most debilitating factor impeding poultry productivity resulting in retarded growth, weight loss, diarrhoea, poor absorption of nutrients, death and even the spread of fatal bacterial infections (18, 19). This worm is also an easily available and suitable model for *in vitro* anthelmintic evaluation (21-23).

In present study, *P. posthuma* were found to be paralyzed and eventually killed by the all test extracts in a clear-cut concentration dependent manner, showing differential toxic activities which diminished with lowering concentration. In case of *A. galli*, indeed there was no definitive identifying sign of paralysis unlike *P. posthuma*, in which a considerable time always lapsed

between paralysis and actual death. Once the nematodes indicated paralysis (being motionless) they could not be revived, death was inevitably simultaneous. The EtOAc fraction of the MeOH extract exhibited maximum potency i.e. shortest paralysis and lethal times (less than the reference drug, albendazole) in both test worms. The defatted MeOH extract also exhibited significant effects. The *n*-BuOH extract was found to be least potent against the test worms showing comparatively prolonged survival. In fact, all the test extracts were active against the test worms. Against *A. galli* lethal times for all test extracts were found to be comparatively shorter than those in the case against *P. posthuma,* thereby indicating the relative sensitivity of *A. galli* to the extracts and thus confirming their marked nematocidal potential. However, at lower concentrations paralysis and/or mortality of *P. posthuma* were not observed (see under the section 'results'). Thorough histological examination, enzymatic and elemental studies of normal and treated worms may give an insight into the structural and biochemical alterations at the organ, tissue and cellular systems during paralysis and death in pursuit of the possible mechanism of anthelmintic action of *T. dioica* leaves.

The present study confirms the *in vitro* toxic and anthelmintic potential of *T. dioica* leaves. The results indicate that its defatted MeOH extract and its EtOAc fraction especially, possess remarkable *in vitro* nematocidal property but need more positive results in other groups of parasitic helminthes, especially *in vivo*, for the anthelmintic prospective. Present investigation, provides comprehensible pharmacological evidence that *T. dioica* leaves are effective wormicide supporting the feasibility of its usage as traditional anthelmintic.

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