Antiparasitic activity of methanolic extract of *Acacia oxyphylla* (Leguminosae) against *Raillietina echinobothrida*

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Abstract *Acacia oxyphylla* has been used traditionally by the natives of Mizoram against intestinal worm infections. In the present study, the crude methanolic extract of the plant was tested in vitro on the cestode parasite *Raillietina echinobothrida* to evaluate its potential anthelmintic efficacy. The test parasites were exposed to varying concentrations of the plant extract in phosphate buffered saline and they all slipped into a flaccid paralytic state at varying periods of time post incubation. Ultrastructural observations on the paralysed worms revealed wide scale destruction of the parasite tegument with intense vacuolization of the syncytium and swellings of the basal lamina accompanied by deformities in the cell organelles. To determine the exact mode of action of the botanicals on the parasite body surface, the tegumental enzymes viz. acid phosphatase (AcPase), alkaline phosphatase (AlkPase) and adenosine triphosphatase (ATPase) were investigated. A prominent decrease in the phosphatase activity, in comparison to the untreated control parasites was noticeable. In view of the alterations in the structural and functional integrity of the tegument, occurring in the treated parasites, it may be assumed that the changes transpire because of a compromise in the permeability of the tegument under the influence of the test plant-derived active chemical principles.

Keywords Anthelmintic · Cestode · Transmission electron microscopy · Tegumental enzymes

Introduction

The tegument of cestodes is known to play an important role in nutrition. It has also been proven that since the cestodes lack an alimentary canal, the microthrix layer serves in the uptake of nutrients and thus has absorptive function (Smyth and McManus 1989). Any disruption of the tegumental architecture is likely to have serious consequences on the worm. Synthetic anthelmintics have remained the drug of choice against cestodes but considering the side-effects and resistance that the helminths build against these drugs, more attention is being given to naturally-occurring anthelmintic phytoproducts (Goto et al. 1990; Roy and Tandon 1996; Tandon et al. 1997; Roy and Tandon 1999; Singh and Nagaich 1999). *Acacia* spp. are known to be effectual as anthelmintic, anti-bacterial and antifungal agent. The most active components of this genus are supposed to be the condensed tannins (CTs), a class of phenolic secondary metabolites that rarely have toxic effects. The genus also contains saponins like Acaciaside A and B which have been proven to have nematocidal and cestocidal activity (Ghosh et al. 1993, 1996). *Acacia oxyphylla*, used widely against gastrointestinal worms in the rural traditional medicine practised among the Mizo natives in North-East India, has recently been reported as responsible for alteration of surface ultrastructure in cestode (Roy et al. 2007; Dasgupta et al. 2010). So, the aim of the present study was to examine any alterations brought about by this plant in the tegumental enzymes of *Raillietina echinobothrida*, a cestode parasite of domestic fowl.

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Materials and methods

Plant

Acacia oxyphylla is a leguminous plant highly abundant in the forests and streams of Aizawl and adjoining towns in Mizoram. The plant was authenticated by Dr. H. S. Thapa, Plant Taxonomist of Pachhunga University College, Aizawl, India and voucher specimen of the same was deposited under dry condition at room temperature at the Department of Botany, Pachhunga University College, Aizawl, India with the number PUC-BOT-A012.

Collection of plant and preparation of extract

The stems of *A. oxyphylla* were collected from Aizawl, Mizoram and the stem barks were peeled off and washed thoroughly with deionized water. They were chopped into small pieces and shade-dried for a week after which the dried parts were pulverized into powder and refluxed in methanol (100 g/l) for 10 h at 50°C. The cooled suspension was filtered through Whatman filter paper (No. 1) and distilled for removal of the solvent. The process was repeated three times and in all, 2 g crude extract was obtained from 100 g dried material, which was stored at 4°C until further use.

Treatment

Adult *R. echinobothrida* collected from the intestine of freshly sacrificed domestic fowl were maintained in 0.9% physiological buffered saline (PBS) at $37 \pm 1^\circ\text{C}$. The worms (six per replicate) were incubated in different concentrations (5 mg, 10 mg, 25 mg/ml PBS) of the crude extract with a final volume of 10 ml. Three replicates were used for each concentration. Dimethyl sulphoxide (DMSO) was added to each concentration to give a final solvent concentration of 0.1%. Controls were prepared by incubating the worms in the culture medium (PBS) containing 0.1% DMSO. Worms were also treated with the reference cestocidal drug praziquantel (PZQ) at concentrations of 0.001 and 0.01 mg/ml of PBS. Paralysis was recorded as the state in which there was no visual movement on the part of the worms even on physical stimulation. Death of the worms was ascertained by dipping them in warm PBS, which induced movements in the live worms.

Ultrastructural studies

Mature proglottides of control worms were fixed for TEM at 0 h while those of the treated worms, exposed to 10 mg/ml dosage of the plant extract, and 0.001 mg/ml dosage of PZQ were fixed after paralysis in modified Karnovsky's fixative for 4 h. After fixation, the tissue sample was washed in

three changes of 0.1 M sodium cacodylate buffer (pH 7.2) and post-fixed in 2% OsO_4 buffered in 0.2 M sodium cacodylate for 1 h and dehydrated through graded acetone. The parasite tissue was infiltrated with propylene oxide and embedded in araldite. Polymerization was done in 2 increments at 50°C (24 h) and 60°C (48 h) and ultra thin sections were cut at 60–90 nm. Sections were mounted on uncoated copper grids and stained with uranyl acetate in 50% ethanol and Reynold's lead citrate and viewed in a JEM 100CX II (Jeol) transmission electron microscope operated at 80 kV.

Histochemical studies

The enzymes viz. acid phosphatase, alkaline phosphatase and adenosine triphosphatase were investigated using fixed frozen sections cut at a thickness of 10–15 μm in a Leica CM1850 cryostat.

For the detection of AcPase activity, the modified lead nitrate method of Takeuchi and Tanoue (Pearse 1968) was employed using sodium- β -glycerophosphate as the substrate. Brownish precipitates in the tissue indicated the sites of AcPase activity. A modified coupling azo-dye method (Pearse 1968) was used for determination of AlkPase activity using Fast violet-B. The sites of AlkPase activity were coloured brown with the salts while the nuclei were stained dark blue. ATPase activity was demonstrated through the use of calcium method of Maengwyn-Davies et al. (Pearse 1968). The frozen sections were incubated in the medium (pH 9.9) for 30 min at 37°C, dehydrated and mounted in glycerine jelly.

Protein content for all enzyme assays was measured according to the method of Lowry et al. (1951).

Results

While control parasites remained alive for about 72 h in the incubation medium, treated parasites went into a paralytic state followed by death (Table 1). Paralysis set in at

Table 1 *In vitro* effect of *A. oxyphylla* extract on *R. echinobothrida*

Test substance	Concentration (mg/ml)	Paralysis (h)	Death (h)
Control	—	—	72.0 $\pm$ 0.06
Methanol	5	6.12 $\pm$ 0.07	8.06 $\pm$ 0.06
	10	3.92 $\pm$ 0.09	6.03 $\pm$ 0.06
	25	2.93 $\pm$ 0.03	4.59 $\pm$ 0.02
Praziquantel	0.01	0.50 $\pm$ 0.01	7.30 $\pm$ 0.15
	0.001	3.00 $\pm$ 0.14	9.80 $\pm$ 0.21

Values given as mean $\pm$ SE from 3 replicate assays; $n = 6$

$P < 0.05$ vs. control value, student's *t*-test

6.12 ± 0.07 h, 3.92 ± 0.09 h and 2.93 ± 0.03 h in worms treated with 5, 10 and 25 mg of *A. oxyphylla*, respectively. On treatment with 0.01 and 0.001 mg of PZQ, onset of paralysis occurred at 0.50 ± 0.01 h and 3.00 ± 0.14 h, respectively. The worms treated with various concentrations of *A. oxyphylla* attained mortality within 5–8 h of treatment while those treated with the two doses of PZQ survived for about 7–10 h.

Ultrastructural studies

Control

The control worms showed normal morphology with respect to the tegument and inner parenchyma. The microtrich layer was quite intact along with its fuzzy glycocalyx coat. The thickness of the shaft of microtriches (in mature proglottides) ranged from $7.6 \times 10^{-2} \mu\text{m}$ to $1.5 \times 10^{-1} \mu\text{m}$. The distal cytoplasm was electron dense with its secretory bodies and the basal lamina and the

sub-tegumental muscles were well organized (Fig. 1a). The tegumental cells showed good connections with the surrounding parenchyma (Fig. 2a) and the inner cytoplasm had an abundance of granular endoplasmic reticulum and other cell organelles including mitochondria with prominent cristae (Fig. 3a).

Treated

Worms treated with 10 mg/ml of the methanolic extract (fixed at paralysis after about 3.92 h) showed sloughing off of the tegument with much alteration in the tegumental architecture. There was an increase in electron-lucency of the background due to both glycogen loss and vacuole formation (Fig. 1b). The subtegument showed immense vacuolization and accumulation of debris. There was release of underlying structures to the outside at the basal lamina, which was disrupted at many places. Golgi complexes were scarcely present and the nuclei showed swelling (Fig. 2b). The mitochondria had degenerated

Fig. 1 Tegument of *R. echinobothrida*. **a** Control tegument with intact microtrich layer (M); distal cytoplasm (DC) electron-dense with tegumental discs; non-disrupted basal lamina (BL) and well organized subtegumental circular and longitudinal muscle blocks

(Mc). **b** *A. oxyphylla* caused depletion of tegument (arrowhead) and intense loss of glycogen (arrows); muscle blocks (Mc). **c** Praziquantel treated section showing loss of tegument along with release of distal cytoplasm to the exterior. All bars $0.5 \mu\text{m}$

Fig. 2 Tegumental cytons of *R. echinobothrida*. **a** Control tegumental cells (Tc) with abundant mitochondria (Mt); dense granular nucleolus (Nu) within nucleus. Arrow shows the nuclear membrane. **b** *A. oxyphylla* treated parasite showing immensely swollen nuclei

within tegumental cytons. **c** Abundance of nuclei with prominent nucleolus observed in parasite treated with praziquantel. All bars $0.5 \mu\text{m}$

Fig. 3 Mitochondria of *R. echinobothrida*. **a** Electron-dense mitochondrial matrix with prominent cristae in control section. **b** Disrupted mitochondria (Mt) lying within muscle layer; *A. oxyphylla* treated section. **c** Fuzzy and ill-defined mitochondria of praziquantel treated parasite. All bars 0.5 μ m

(Fig. 3b) and the cisternae of granular endoplasmic reticulum (GER) were dilated. PZQ treated parasites were fixed at paralysis after about 3 h for electron microscopy. PZQ also brought about a loss of tegument along with release of materials to the exterior (Fig. 1c). The damage found was mostly restricted to the loss of tegument, but otherwise there was an abundance of nuclei and mitochondria within the tissue (Figs. 2c, 3c). The extent of damage revealed in the tegumental and subtegumental structure seemed to be dose dependent.

Histochemical studies

Control

Intense AcPase and AlkPase activities were observed in the tegument and subtegument areas. AlkPase activity was

higher than the AcPase activity in the parasite tissue (Table 2). Other regions like the somatic musculature displayed staining intensity at par with the subtegument while the general parenchyma showed lesser staining intensity though still retaining a good amount of enzyme activity. The parasite tissue showed less intense staining for ATPase when compared with AlkPase but the activity was more pronounced than AcPase (Figs. 4, 5, 6).

Treated

Treatment with *A. oxyphylla* caused reduction in the general staining intensity along with absence of ATPase activity in the tegument. The subtegument and parenchyma regions continued to show enzyme activity to some extent while the somatic musculature showed a higher activity. PZQ treated sections failed to show any AcPase activity in

Table 2 Histochemical localization of ATPase, AlkPase and AcPase in various structures of *Raillietina echinobothrida*

Test Substance (mg/ml)	ATPase				AlkPase				AcPase			
	T	ST	SM	P	T	ST	SM	P	T	ST	SM	P
Control	++++	++++	+++	+++	+++++	++++	+++	+++	++++	+++	+++	+++
<i>A. oxyphylla</i> (10)	–	–	+	+	++	++	++	+	+	+	+	+
PZQ (0.001)	NP	+	+	+	–	+	–	–	NP	+	–	–

T Tegument, ST Subtegument, SM Somatic musculature, P Parenchyma

NP Structure not present, +++++ extremely intense activity, ++++ very intense activity, +++ intense activity, ++ moderate activity, + mild activity, – no activity

Fig. 4 Adenosine triphosphatase activity in *R. echinobothrida*, frozen sections. **a** Control. **b** Stem bark extract of *A. oxyphylla* treated section. **c** Praziquantel treated section. All bars 50 μ m

Fig. 5 Alkaline phosphatase activity in *R. echinobothrida*, frozen sections. **a** Control. **b** Stem bark extract of *A. oxyphylla* treated section. **c** Praziquantel treated section. All bars 50 μ m

Fig. 6 Acid phosphatase activity in *R. echinobothrida*, frozen sections. **a** Control. **b** Stem bark extract of *A. oxyphylla* treated section. **c** Praziquantel treated section. All bars 50 μ m

the somatic musculature and parenchyma, and there was no trace of AlkPase staining in any part of the treated tissue except for the subtegument. Traces of ATPase activity was found in all parts of the tissue except for the tegument which was stripped off.

Discussion

In cestodes, the general body surface acts as a vital structure in terms of various functions such as attachment to the host surface, nutrient uptake, immunoprotection, osmoregulation and sensation (Meaney et al. 2001). In the present study, the tegumental surfaces of the test plant-treated parasites were found to be affected along with severe disruption of the underlying structures, as also observed earlier when treated with ethanolic crude extract of the plant by Dasgupta et al. (2010). Similar to the present observations, destruction of absorptive surface was also caused by other drugs like praziquantel, oxiclozanide, and ethanol extract of several plants in trematodes and cestodes (Mehlhorn et al. 1983; Tandon et al. 1997; Roy et al. 2008). Extensive destruction of the tegument of *R. echinobothrida* was observed on treatment with *Acacia caesia* (Lalchhandama 2009). The severe deleterious effects brought about in the tegument of the worm may account for the loss of movement, which ultimately led to death through a preceding paralytic state. The tegumental interface of *R. echinobothrida* seems to be a target organ for the active components of the plants to exert its vermifugal or vermicide effect.

In a number of helminth parasites, AcPase and AlkPase have been detected histochemically and found to be closely associated with the tegument, subtegument, somatic musculature, gut and cuticle (Pappas 1988; Fetterer and Rhoads 2000). AcPase is usually associated with lysosomes and AlkPase is regarded as indicative of membrane transport mechanisms (Barrett 1981). In the present study, alterations occurred in the enzyme activity of the parasite after treatment with the plant extracts. These alterations were observed to be at par with those seen in PZQ treated parasite tissues. The inhibition of AcPase activity by the plant botanicals observed during the present study is suggestive of the fact that absorption and intracellular digestion of drugs may involve lysosomes (Colam 1971) while inhibition of AlkPase activity may have been the result of affected membrane transport. ATPase is known to be related to energy metabolism, active transport and lipid synthesis. The enzyme was found to be located in the somatic musculature which reveals its role in ATP hydrolysis within the parasite tissue. On treatment with *A. oxyphylla*, ATPase was reduced significantly in the muscle and parenchyma and was completely absent in the tegument and subtegument layers. Similar observations have been reported for *E. multilocularis* after treatment with mebendazole and levamisole (Benediktov 1980). The decrease of the enzymes may be due to disruption of the absorptive surface (Hart et al. 1977). The ultrastructural and enzymatic alterations suggest that the tegumental enzymes may be a potential target of chemotherapy of intestinal parasites. The tegument of *R. echinobothrida*

seems to be affected most by the active components of the test plant as confirmed by the present study.

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