Effects of *Acacia oxyphylla* and *Securinega virosa* on functional characteristics of *Raillietina echinobothrida* (Phylum: Platyhelminthes; Class: Cestoidea), a poultry cestode parasite

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Abstract The histochemical distribution and quantification of alkaline phosphatase, acid phosphatase, and adenosine triphosphatase have been investigated in the adult cyclophyllidean cestode, *Raillietina echinobothrida* after treatment with crude ethanol extract of traditionally used medicinal plants *Acacia oxyphylla* and *Securinega virosa*. Estimation of free amino acid pool and vital trace elements like calcium and magnesium were also performed to study the effect of the plant extracts on the parasite. A decrease in the levels of phosphatases and trace elements was noticed in the treated parasites when compared to control groups. At the same time, a lot of disparity was noted in the free amino acid pool as the levels of many amino acids were seen to increase or decrease in relation to the levels observed in control parasites. Role of phyto-products in the changed physiology of cestode and their possible chemotherapeutic targets are discussed.

Keywords *Raillietina echinobothrida* ·
Acacia oxyphylla · *Securinega virosa* · Chemotherapeutic

Introduction

Protein metabolism, though less well known in parasites than carbohydrate metabolism, is considered very important since all animals require nitrogen in their metabolism,

thus making protein metabolism a vital factor for energy provision. Emergency energy requirements in cestodes are believed to be met through an interconnected network of tegumental phosphatases, free amino acid pool and certain trace elements within the parasite tissue (Pal and Tandon 1998; Kar et al. 2004; Das et al. 2004). The maintenance of harmony between the three systems is a fundamental homeostatic mechanism that must have arisen very early in the evolution of helminth physiology. Therefore, disruption of this harmony can cause a setback to helminth adaptation during physiological stress. Phosphatases are important tegument proteins (Giménez-Pardo et al. 2004), whose association with membrane transport have been implied by their ubiquitous presence in tissue of secretory or absorptive function. They play a major role in active transport, energy metabolism, lipid synthesis, and also possess digestive capability. ATPase activity in the isolated apical brush border of cestodes is osmotically sensitive (Rahman et al. 1981a, b). The free amino acid pool is known to function in osmotic regulation since similar pools of amino acids are present in the lumen of the host gut and within the cestode parasite. The movement of amino acids in and out of the parasite maintains equilibrium between the two amino acid pools. Apart from contributing to the regulation of the internal osmotic pressure, the constant influx and efflux of amino acids also ensures that there is a sufficient supply of each amino acid, especially those that are rare in the host tissue, for protein synthesis. Trace elements like calcium and magnesium, on the other hand, act as the mediators that help in the error-free functioning of the phosphatases and the free amino acid pool as they function as second messengers or as enzyme cofactors. The ATPase in the apical membrane of cestodes is Ca and Mg dependent and helminths are known to require ions coupled to the amino acid transport system (Wani and Srivastava

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1995; Chernin 2000; Burenina 2007). However, little information is available about cestode reactions in relation to the three systems when subjected to induced stress caused by anthelmintics. With this view we attempt to broaden this important yet neglected area of helminth physiology by studying the effect of traditionally used (by tribes of Mizoram, India) anthelmintic plants like *Acacia oxyphylla* (local name: Khang) and *Securinega virosa* (local name: Saisiak) on cestode test parasite *Raillietina echinobothrida*.

Materials and methods

Preparation of extracts, collection of parasites and reference cestocidal drug

Both the plants were collected from different villages around Aizawl, Mizoram. Crude ethanol extracts were made following standard protocol as described earlier (Roy et al. 2008). Adult *R. echinobothrida* were collected from the intestine of domestic fowl, *Gallus domesticus* and maintained in 0.9 % PBS at 39 ± 1 °C. Two sets of parasites (having five replicates) were exposed to 10 and 25 mg of *A. oxyphylla* and *S. virosa*, respectively, per ml of PBS having 0.1 % dimethylsulfoxide (DMSO). Praziquantel (PZQ) was used as standard reference cestocide (World Health Organization 2011) along with simultaneous maintenance of controls in PBS having 0.1 % DMSO. Time taken for complete inactivation of the parasites was recorded and the death was confirmed by dipping such worms in slightly warm water. Immediately after paralysis, the parasites were fixed and proceeded for further biochemical studies.

Study of tegumental enzymes

AcPase and AlkPase activities were assayed by estimating the *p*-nitrophenol product following the method described by Plummer (1988). A 10 % tissue homogenate of the parasite was prepared in sodium acetate buffer (for AcPase) and sodium glycine buffer (for AlkPase). The homogenate was centrifuged at 5,000 rpm at 0 ± 2 °C for 20 min and the supernatant was used for enzyme assay with *p*-nitrophenyl phosphate as the substrate. The decrease or increase in the optical density was measured at 405 and 420 nm for AcPase and AlkPase, respectively, using a UV–Vis spectrophotometer (Varian 100). The amount of *p*-nitrophenol produced was calculated from a linear standard curve prepared using different concentrations of *p*-nitrophenol (0.01–0.1 mM). ATPase activity was assayed by the method of Kaplan (Pearse 1968). A 10 % tissue homogenate (w/v) was prepared in glycine buffer (0.2 M,

pH 9.1), sonicated for 30 s and then used for enzyme estimation with Na-ATP as the substrate. The liberated inorganic phosphate was estimated as per the method of Fiske and Subbarow (1925). The change in optical density was measured at 700 nm for ATPase activity. Protein content for all enzyme assays was measured according to the method of Lowry et al. (1951).

Free amino acid analysis (FAA)

The experimental samples were dissolved in milli Q water (200 mg/ml) and centrifuged. The supernatant was taken and passed through C18 column (1 cm. length) to remove the peptides and proteins from the sample. The eluate was collected. Following this, the column was also eluted with 1 ml of 40 % acetonitrile and this eluate was combined with the earlier one. The pooled eluate was then lyophilized to a dry powder and stored at -70 °C for further experimentation. The samples were then isobarically labelled with iTRAQ reagents and introduced to QSTAR-XL MS/MS mass spectrometer using nano ES spray capillaries. The inlet voltage was fixed at 1,100 kV. In IDA (Information dependent acquisition) method ions (1+, 2+, and 3+) were scanned from 200 to 450 m/z. The three most intense ions which exceed at least 10 counts were allowed for MS/MS fragmentation and data was acquired. Ion tolerance was fixed at 50 atomic mass unit (amu). The duration of data acquisition was 10 min with a cycle time of 4.002 s. From the IDA method the total ions were selected and compared with the standard amino acid mass values from the list. For the estimation of % amino acid composition the peak area for each ion was taken for analysis.

Trace elements analyses

Fresh worms in control group were directly taken for analyses of their trace metal contents. Cestodes in different treatment groups were harvested immediately after they succumbed to paralytic state, and thoroughly washed with deionised double distilled water. Whole worms were dried in an incubator at 50 °C. The dried worms were finely pulverized to powder. The powdered tissue (2 g) was digested in 10 ml of concentrated HNO₃ in an airtight corked conical flask for overnight at 50 °C. The fully digested solution was transferred to and kept on a hot plate at 70 °C for ~2 h to allow complete evaporation of the acid. After cooling down, 10 ml of deionised water was then added, vigorously mixed with magnetic stirrer, and filtered through Whatman filter paper (110 mm Φ). The volume was finally made to 100 ml by adding more deionised water to the filtrate. The final solution was directly used for quantitative measurement of the trace

elements using a single beam atomic absorption spectrophotometer (Perkin Elmer 3110) at the absorbance wavelengths of 422.6 nm for calcium and 285.2 nm for magnesium.

Results

Enzymes analysis

Table 1 shows the effect of *A. oxyphylla*, *S. virosa*, and PZQ on the tissue activities of the tegumental enzymes in *R. echinobothrida*. At the physiological level, the activity of ATPase in the parasite was found to be highest, averaging about 155.28 units/g wet weight, followed by AlkPase (31.14 units/g wet wt) and AcPase (6.06 units/g wet wt). The activity of ATPase in the parasite decreased significantly by 72, 91, and 96 %, when treated with crude stem bark extract of *A. oxyphylla*, leaf extract of *S. virosa* and PZQ, respectively. A significant decrease in the activity of AlkPase (of about 96 and 98 %, respectively) was observed when the parasite was treated with *S. virosa* leaf extract and PZQ, while a relatively less decrease in activity (of about 90 %) was observed when treated with *A. oxyphylla*. The tissue activity of AcPase decreased significantly in parasites treated with both *A. oxyphylla* and PZQ. The percentage decrease of AcPase activity was recorded as 97, 34.5, and 96 % in parasites treated with crude extract of *A. oxyphylla*, *S. virosa*, and PZQ, respectively, compared to the controls.

Free amino acid pool

As depicted in Table 2, out of the amino acids detected, Gly was found to be at the maximum level, followed by

Ala, Ser, Pro, Val, Thr, Cys, Ile/Leu, Asn, Asp, Gln/Lys, Glu, Meth, His, and Phe, respectively, in a decreasing order. Other amino acids detected, namely, Arg, Tyr, and Trp were at comparatively lower levels. Quantitatively, the level of the various FAAs in the parasite was significantly affected by the treatment with the two test materials, and also with the reference drug PZQ. The treated parasites showed a marked decrease in the levels of Meth, Ile/Leu, Phe, Gly, Tyr, Asn, and Glu, though an increase in the levels of Thr (except PZQ treated parasite which remained unchanged in Thr level). *S. virosa* treated parasites showed an increase of Ala (40 %), Pro (6.3 %), Cys (100 %), and Trp (50 %). Further, in contrast to a 150 % increase in PZQ treated parasites, Ser did not show any change in level following treatment with *S. virosa* and *A. oxyphylla*. *A. oxyphylla* treatment failed to cause any alterations in Pro and Val while PZQ treated parasite showed a decrease in the same. PZQ treatment resulted in the increase of Ser, Gln/Lys and Trp levels within the parasite and consistently portrayed a decrease or no change (Ala, Thr, Cys, Glu, Arg, His) in the levels of all other amino acids. While the Gln/Lys levels showed a decrease in *A. oxyphylla*-treated parasites and a 25 % increase in PZQ-treated parasites, its level was observed to remain unchanged with *S. virosa* treatment.

Trace element studies

The quantitative observations on two vital trace elements in *R. echinobothrida*, namely, Ca and Mg, post treatment in the extracts of *A. oxyphylla*, *S. virosa*, and PZQ are presented in Table 3. The concentrations of Ca and Mg in the control worms maintained in 0.9 % PBS was about 45.2 and 6.2 ppm. Cestodes exposed to 10 mg/ml of ethanol extract of *A. oxyphylla* showed a significant reduction in

Table 1 Effect of ethanol extracts of *A. oxyphylla* (10 mg) and *S. virosa* (25 mg), and reference drug praziquantel (0.001 mg) on the activity of different tegumental enzymes of *R. echinobothrida*

Control/treated cestode with	Enzyme activity (total ^a /specific activity ^b)			% change after treatment		
	ATPase	AlkPase	AcPase	ATPase	AlkPase	AcPase
Control	155.28 ± 0.003	31.14 ± 0.001	6.06 ± 0.01	–	–	–
	3.19 ± 0.0	0.68 ± 0.11	0.43 ± 0.17			
<i>A. oxyphylla</i> (10 mg/ml)	42.98 ± 0.17	2.98 ± 0.8	0.99 ± 0.01	72	90	97
	1.36 ± 0.01	0.12 ± 0.01	0.42 ± 0.19			
<i>S. virosa</i> (25 mg/ml)	13.98 ± 0.17	1.19 ± 0.8	3.97 ± 0.01	91	96	34.5
	0.76 ± 0.01	0.18 ± 0.01	0.84 ± 0.19			
PZQ (0.001 mg/ml)	5.62 ± 0.8	0.55 ± 0.05	0.25 ± 0.01	96	98	96
	2.37 ± 1.3	0.36 ± 0.4	0.007 ± 0.1			

Values given as mean ± SE from five replicate assays

^a Enzyme activity as specific unit which consumes 1.0 mmol substrate/g wet wt. tissue/h

^b Specific activity as unit/mg protein/h

Table 2 Effect of crude extracts of *A. oxyphylla* (10 mg) and *S. virosa* (25 mg), and reference drug praziquantel (0.001 mg) on free amino acid pool (nmol/g wet weight) of *R. echinobothrida*

Amino acids	Test substance			
	Control	<i>A. oxyphylla</i>	<i>S. virosa</i>	Praziquantel
Ileu/Leu	76.24 ± 1.02	11.9 ± 0.06 (−84.4)	16.68 ± 0.03 (−78.12)	7.15 ± 0.32 (−90.6)
Asn	75.70 ± 1.26	28.0 ± 2.04 (−63)	67.47 ± 1.94 (−10.9)	52.6 ± 1.42 (−30.5)
Ser	95.15 ± 0.1	95.15 ± 0.05 (0)	95.15 ± 0.05 (0)	237.87 ± 0.05 (+150)
Pro	86.86 ± 0.86	86.86 ± 0.96 (0)	92.40 ± 1.02 (+6.3)	77.61 ± 1.06 (−10.6)
Val	85.36 ± 0.08	85.36 ± 0.98 (0)	39.73 ± 0.18 (−53.4)	49.80 ± 0.12 (−41.6)
Meth	67.01 ± 0.16	26.8 ± 0.01 (−60)	0.00 ± 0.4 (−100)	0.00 ± 0.01 (−100)
His	64.45 ± 0.09	32.23 ± 0.03 (−50)	64.45 ± 0.08 (0)	64.45 ± 0.06 (0)
Thr	83.89 ± 0.06	139.80 ± 0.03 (+66.6)	111.2 ± 0.06 (+32.6)	83.89 ± 0.85 (0)
Cys	82.54 ± 0.02	0.00 ± 0.21 (−100)	165.1 ± 0.03 (+100)	82.54 ± 0.37 (0)
Asp	75.13 ± 1.31	36.5 ± 1.4 (−51.4)	70.83 ± 2.1 (−5.7)	68.69 ± 0.99 (−8.57)
Gln/Lys	68.43 ± 0.18	34.21 ± 0.18 (−50)	68.43 ± 0.26 (0)	85.54 ± 0.24 (+25)
Glu	68.00 ± 0.26	18.55 ± 0.22 (−72.7)	55.64 ± 0.19 (−18.2)	68.00 ± 0.01 (0)
Tyr	55.21 ± 0.06	16.56 ± 0.03 (−70)	36.8 ± 0.08 (−33.34)	46.0 ± 0.04 (−16.7)
Trp	48.96 ± 0.02	24.48 ± 0.07 (−50)	73.44 ± 0.11 (+50)	97.92 ± 0.08 (+100)
Phe	60.53 ± 0.06	15.13 ± 0.02 (−75)	15.13 ± 0.04 (−75)	15.13 ± 0.03 (−75)
Arg	57.40 ± 0.02	28.7 ± 0.02 (−50)	57.4 ± 0.02 (0)	57.4 ± 0.02 (0)
Gly	133.21 ± 0.08	0.00 ± 0.04 (−100)	66.7 ± 0.06 (−50)	99.1 ± 0.1 (−25.6)
Ala	110.00 ± 0.1	66.00 ± 0.12 (−40)	154.00 ± 0.07 (+40)	110.0 ± 0.09 (0)
Total FAA	1,394.07	746.23 (−46.5)	1,250.55 (−10.3)	1,303.69 (−6.5)

Values given as mean ± SE from five replicate assays

Percentage increase (+) or decrease (−) of the FAA levels is given in *parentheses*

Table 3 Effect of ethanol extracts of *A. oxyphylla* (10 mg) and *S. virosa* (25 mg), and reference drug praziquantel (0.001 mg) on the levels of trace elements (ppm) in *R. echinobothrida*, effluent and untested plant media

Control/treated cestode with	Test parasite/media	Ca	Mg
Control	Parasite tissue	45.2 ± 1.22	6.2 ± 0.07
<i>A. oxyphylla</i> (10 mg/ml)	Parasite tissue	9.7 ± 0.13	3.6 ± 0.4
	Effluent*	33.1 ± 0.87	5.2 ± 0.05
	Untested plant media**	9.5 ± 0.01	1.84 ± 0.02
<i>S. virosa</i> (25 mg/ml)	Parasite tissue	10.2 ± 0.46	4.6 ± 0.11
	Effluent*	35.9 ± 0.67	6.0 ± 0.37
	Untested plant media**	18.6 ± 0.02	2.79 ± 0.01
PZQ (0.001 mg/ml)	Parasite tissue	10.1 ± 0.8	5.7 ± 0.37
	Effluent	11.4 ± 0.61	5.8 ± 0.46
	Untested PZQ media***	10.7 ± 0.02	2.6 ± 0.01

Values given as mean ± SE from five replicate assays

$p < 0.05$ vs. control value, student's t test

* Trace elements in the media where parasites were incubated

** The media containing only plant extract without any parasites

*** Media containing only PZQ

the levels of the two elements (9.7 ± 0.13 and 3.6 ± 0.4), and a pronounced increase in their levels in the incubation media (33.1 ± 0.87 and 5.2 ± 0.05). The untested plant media showed a much less concentration of the two elements than the effluent. The concentrations of Ca and Mg in the cestodes treated with 25 mg/ml of ethanol extract of *S. virosa* were reduced to 10.2 ± 0.46 and 4.6 ± 0.11 , respectively, and the concentration of Ca in the effluent rose to 35.9 ± 0.67 from 18.6 ± 0.02 in the untested plant media. PZQ brought about a decrease in the level of Ca and Mg in the parasite tissue (10.1 ± 0.8 and 5.7 ± 0.37), and the incubation media showed Ca and Mg levels of 11.4 ± 0.61 and 5.8 ± 0.46 , respectively, as compared to their levels of 10.7 ± 0.02 and 2.6 ± 0.01 in the untested PZQ media.

Discussion

Intense ATPase, AlkPase, and AcPase activities were observed in control *R. echinobothrida*, an important parasite of poultry of Northeast India, while the enzymes were noted to diminish in the parasites treated with both the plant extracts. Tegumental enzymes play a very important

role in maintaining the tissue homeostasis within the cestode parasite. Phosphatase functions in regulating metabolic processes. Alkaline phosphatase and adenosine triphosphatase take part in active transport through cellular membranes and acid phosphatase deals with intracellular digestion processes (Read 1966; Lumsden et al. 1968). It is widely speculated that when there is a decrease in the tissue ATPase activity, the permeability of the glycocalyx-plasmalemma system is disrupted causing the plasmalemma parts to get damaged. This in turn causes the AcPase to diffuse out to digest the damaged membrane parts leading to a decrease in the tegumental AcPase levels. Damage of the parasite membrane also causes an increased inflow of salts from the external media which accumulates in the basal infolds (Fairweather and Boray 1999). To compensate, there is penetration of water from the surrounding tissue leading to swellings in the basal infolds and vacuolization of tissue (as observed earlier with *A. oxyphylla* treatments (Dasgupta et al. 2010)). In treatments with the two plant extracts, AcPase diminished the most followed by ATPase in case of *A. oxyphylla*-treated parasites while AlkPase declined most in case of *S. virosa*-treated parasites. This proved that there was extensive lysis of the tegument in the former (in tune with observations made by electron microscopy) by tegumental AcPase which eventually leaked out causing a drop in its level, while in the latter, the tissue pH and glucose uptake was majorly disrupted due to lowering of the AlkPase level and most of the damages were interior in the subtegumental level, as also observed through transmission electron microscopy (Roy et al. 2012). The reference drug PZQ also showed the highest decrease in AlkPase followed very closely by AcPase so that the damages observed were mostly concentrated in the tegument which was lysed.

Free amino acid pool is considered to be the protein precursor of the amino acid pool and is thus a very important prelude to study of protein metabolism. Little information is available regarding the amino acid metabolism in helminths. The excretion of amino acids is a way of detoxifying ammonia that is known to have a neurotoxic effect and also to cause various neurological disorders (Cooper and Plum 1987; Campbell 1991), of which paralysis of the worm may be one such manifestation. There is evidence that the free amino acids may be involved in osmotic regulation (Lussier et al. 1978; Wack et al. 1983) as there is always a danger that high levels of a single osmolyte will disrupt protein structure. In treatments with *A. oxyphylla*, Gly, Ala, Cys, Arg, and Glu were seen to decrease drastically while Pro, Ser, and Val levels remained unchanged. This simultaneous accumulation of Pro with an absence of Ala could be due to competition for substrate of the enzymes in the Ala and Pro biosynthesis as they are the true metabolic end products of carbohydrate metabolism (Roberts, 1983). In case of

S. virosa treatments, in spite of a decrease in Gly (50 %) and Glu levels, there was increase in the levels of Pro, Ala, and Cys. Arg levels remained unchanged just like PZQ-treated parasites. Thus, there was an increase in the end products of carbohydrate metabolism on treatment with *S. virosa*. As Pro is the kind of amino acid that is different in helminths and mammals (Mehlhorn 2008), this point of energy metabolism can be exploited in drug design against intestinal parasites. Glutamic acid is a common substrate of glutamine, arginine and proline and the primary NH_4^+ acceptor, as well as the product of ammonia assimilation. The levels of Glu show a significant decline with *A. oxyphylla* treatment. The *S. virosa* treatment is thus mainly causing protein catabolism as is evidenced by the increase in the levels of many amino acids. As degradation products (amino acids here) are osmotically more active than the macromolecules (proteins), their breakdown perhaps generates cellular osmolarity (Lang, 2007). *A. oxyphylla* primarily shows a decrease in the levels of amino acids signifying an increase in protein synthesis. In previous studies, *A. oxyphylla* has been shown to cause swellings in the subtegumental cells of *R. echinobothrida* (Dasgupta et al. 2010). Cell swelling is known to cause glycine and alanine oxidation, glutamine breakdown, stimulate protein synthesis and inhibit proteolysis (Lang et al. 1998). A decline in the level of many amino acids also indicates towards their utilization through amino acid oxidase system for emergency energy processes during stress (Shiau et al. 2001).

Any significant alterations induced by a chemotherapeutic agent in the Ca^{2+} influx/efflux of a cestode or trematode system can be critically disturbing for the normal contraction of the parasite musculature because Ca^{2+} , as second messengers, are responsible not only for contraction but also for glycogen breakdown. The correlation between vacuolization of the parasite tegument and the concentration of Ca^{2+} has been well documented (Bricker et al. 1983). Tetas and Lowenstein (1963) have demonstrated that a number of divalent cations, including calcium, copper, magnesium, and manganese, catalyze the hydrolysis and transphosphorylation of phosphate esters in the absence of the appropriate enzymes. Reserves of phosphates and inorganic ions are used during emergency (phosphates for phosphorylation reactions and the ions as enzyme cofactors) for example, when a cestode immediately requires rapid synthesis of ATP for strong muscular attachment and active transport of nutrients (Smyth and McManus 1989). So, alterations in the levels of these elements, the tegumental phosphatases and the free amino acids perhaps had a devastating effect on the transport system of cestode parasites eventually leading to their paralysis. We thus postulate that these functional characteristics of the parasite may act as possible chemotherapeutic targets for the natural anthelmintics.

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