IN VITRO ANTHELMINTIC ACTIVITY OF *ACACIA OXYPHYLLA*: CHANGES IN THE LEVELS OF TRACE ELEMENTS AND ACTIVITIES OF THE TEGUMENTAL ENZYMES OF THE CESTODE, *RAILLIETINA ECHINOBOTHRIDA*

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Summary

The crude alcoholic extract of the stem bark of *Acacia oxyphylla*, an indigenous anthelmintic plant among the Mizo tribes of north-east India, have been demonstrated to exhibit profound anthelmintic effects on the avian gastrointestinal cestode, *Raillietina echinobothrida*. To further investigate the efficacy of the plant extract with a view to understanding the mode of action, vital trace elements and tegumental enzymes of the parasite were studied. *In vitro* treatment of the helminth parasite with the ethanol, methanol and acetone extracts of the plant at a concentration each of 20 mg ml^{-1} indicated significant reduction ($P < 0.05$) in the levels of important trace metals such as calcium, magnesium, potassium and sodium. The enzymatic activities of the predominant tegumental enzymes such as acid phosphatase (AcPase) and alkaline phosphatase (AlkPase) were also significantly diminished. The anthelmintic effects were somewhat comparable to that of the standard reference drug, albendazole. The overall results indicate that the anthelmintic potency increases in the order albendazole>ethanol extract>methanol extract>acetone extract in bringing about the biochemical alterations. It also becomes ostensibly apparent that albendazole and the plant extracts act trans-tegumentally to induce flaccid paralysis of the worms.

Keywords: *Acacia oxyphylla*; acid phosphatase (AcPase); alkaline phosphatase (AlkPase); anthelmintic; trace elements; *Raillietina echinobothrida*.

Introduction

Despite successful achievements on the evaluations of a number of medicinal plants used in various traditional practices all over the world indicating considerable anthelmintic activities against a wide range of helminth parasites, the global crisis of accelerated widespread anthelmintic resistance is far from being ameliorated. This is primarily due to

the fact that although ethnomedicinal plants exhibit potent anthelmintic properties, their chemical nature, safety and, above all, mode of action are hardly documented, thus, remain poorly understood. Particularly when applied on large-scale clinical and veterinary treatments, plant parts and their extracts can exert undesirably serious consequences [1-3]. Several lines of recent evidences have exposed that some widely used medicinal plants and their products are not without adverse side-effects in the hosts. Many of the most well known traditional anthelmintic plants are definitely highly toxic and without practicable value in clinical and veterinary applications [4-7]. Thus, there still remains a serious hindrance for phytomedicines to find their way as subtle alternatives to conventional drugs.

Moreover, like the commercial drugs themselves, majority of the anthelmintic plants are helminth specific, showing activity against a particular species or group of the parasites [8,9]. Besides plant extracts are prepared in different solvents so that the form of extraction can also reflect the anthelmintic efficacy [10]. Therefore, it is ever more crucial to comprehend the precise mode of activity of the well-established anthelmintic plants within the parasite tissue, and which particular type of extract should be sought after. This will obviously impart better understanding of the factual efficacy of the plants and their possible effects in the host tissue.

The occurrence of vital enzymes, viz., acid phosphatase (AcPase) and alkaline phosphatase (AlkPase) have been clearly demonstrated both histochemically and biochemically in a number of helminth parasites [11-16], including the common poultry tapeworm, *Raillietina echinobothrida* [17]. These enzymes have been unequivocally revealed to be intimately associated with the tegument and subtegumental regions of cestodes and trematodes, as well as the cuticle of nematodes [18-21]. It is also firmly established that between the two tegumental enzymes, AlkPase is the major active enzyme in adult cestodes, whereas AcPase predominates in trematodes [22,23]. Especially in cestodes, the two phosphatases are posited to be closely involved in the digestive and/or absorptive functions [24].

Extracts from certain medicinal plants, including *Butea monosperma*, *Embelia ribes*, and *Roltlesia tinctoria* reportedly influence drastic decrease in the activities of both AcPase and AlkPase in the trematode, *Paramphistomum cervi* [25]. The root tuber peel extract and genistein from *Flemingia vestita* similarly caused significant reduction of the enzymes in *R. echinobothrida* [17], and in the giant fluke, *Fasciolopsis buski* [23], comparable to those of the standard pharmaceuticals, praziquantel and oxyclozanide, respectively. *F. vestita* and its bioactive isoflavone, genistein was also shown to cause as much as 39-49% decline in the concentration of calcium in *R. echinobothrida* [26].

In the traditional medicine of the Mizo tribes, inhabiting the remotest region of north-east India, the stem bark of *Acacia oxyphylla* Graham ex Bentham (Family Mimosaceae) is a well-known fish stupefying agent for mass fishing and a remedy for intestinal infections. The ethanolic extract of the plant part have been shown to cause dose-dependent paralysis and mortality, associated with irrevocable damaging effects on the tegument and tissue layers of *R. echinobothrida* [27]. Therefore, the present study is an attempt to promulgate the probable primary route of action of *A. oxyphylla* extracts and to establish the possible changes in the tissue biochemical composition by assessing the activities of the vital tegumental enzymes and trace metals therein.

Methods

Preparation of plant extract

Acacia oxyphylla is a perennial climbing leguminous plant highly abundant on the banks of rivers and streams in Mizoram, India. The fresh stems of *Acacia oxyphylla* were collected in July 2005 from the nearby forest of Aizawl (between latitude 21° 58' to 24° 34' North and longitude

92° 15' to 93° 25' East), the capital city of Mizoram. The stem barks were peeled off, thoroughly washed with deionized water, chopped off into small pieces, and dried in a hot air oven at 50ºC. The dried parts were crushed to fine powder and a pre-weighed amount was refluxed with ethanol (100g/l) for 8 h at 60ºC, as described earlier [9,27]. The solution obtained was filtered through Whatman filter paper (No. 1) and then evaporated to complete dryness at 50ºC.

A portion of the alcoholic extract was treated with methanol (100g/l) in a fractionating flask, with several changes of the solvent with constant and vigorous mixing in a rotary shaker for 24 hours. The resultant solution was filtered and refluxed as before. After complete evaporation of the solvent, precipitates were obtained and collected as the methanol extracts. The total yield was 0.35%. Similarly, another portion of the alcoholic extract was treated with acetone to get the acetone extract with a net yield of 0.12%. The extracts were obtained as deep green powdered precipitates, which were immediately refrigerated at 4ºC until further use.

1 h before the actual experimental assay, 20 mg ml^{-1} of the ethanol, methanol and acetone extracts were prepared by dissolving them in 0.9% phosphate buffered saline (PBS, pH 7-7.3), supplemented with 1% dimethylsulfoxide (DMSO). The solutions were then maintained in an incubator at 37±1ºC.

Chemicals and drugs

All the chemicals used were standard analytical grades, obtained from Merck or S.D. Fine-Chemicals Limited, India, except where otherwise stated. Ethanol was supplied by Bengal Chemicals, Kolkata, India, and the reference drug albendazole is a product of GlaxoSmithKline Pharmaceutical Limited, India. Albendazole is a broad-spectrum anthelmintic drug highly effective for cestodes and nematodes.

Recovery and *in vitro* treatments of parasites

Live local fowls (*Gallus domesticus* Linnaeus) were obtained from the local abattoir in Aizawl, Mizoram, India. They were sacrificed and on immediate autopsy, live worms, *R. echinobothrida*, were recovered from the intestines. Only the live adult worms with more or less the same body size and length were selected to attain uniformity in the experimental protocol and then collected in 0.9% PBS. All specimens were immediately incubated at 37±1ºC in a glass-chambered automated incubator. The fresh worms were directly treated with the 20 mg ml^{-1} of the ethanol, methanol and acetone extracts of *A. oxyphylla* dissolved in PBS containing 1% DMSO in separate Petri dishes. Similar treatment was performed for albendazole (20 mg ml^{-1}, the commercial dosage) as a reference drug, and one group is maintained in a medium containing only PBS with 1% DMSO as control experiment. Each incubation medium consisted of 5 replicates.

Measurement of trace elements

Persistence on the motility of the worms were observed, time taken for the onset of paralysis was recorded, as previously described [9,27]. The onset of paralysis of the cestodes was defined as a complete loss of motor activity even after physical stimulation of the worms in culture. The parasites subjected to treatments were quickly garnered the moment they indicated paralysis. Worms in control medium were directly taken for comparison with the treated groups. They were washed with double distilled water and quick-dried in an incubator set at 50°C. 2 g of the powdered dry worms was digested in 10 ml of concentrated HNO_3 in a corked conical flask for overnight at 50°C. The fully digested solution was transferred to and kept on a hot plate at 70°C to allow complete evaporation of the acid. 10 ml of deionised water was then added and filtered through Whatman filter paper (110 mm Φ). The volume was finally made to 100 ml by further adding deionised water to the filtrate. The final solution was directly used for quantitative analysis of trace elements using a single beam atomic absorption spectrophotometer (model Chemito AAS-

201, India) at the absorbance wavelengths of 422.6 for calcium, 285.2 nm for magnesium, 589.0 nm for sodium, and 766.5 nm for potassium.

Estimation of AcPase and AlkPase activities

The AcPase activity was estimated using *p*-nitrophenol product from an enzyme source following the method as described by Plummer (1988) [28] with slight modification in the concentration of the buffer and substrate. A 10% (w/v) of the cestode tissue was homogenized in sodium acetate buffer. The homogenate was centrifuged at 5,000 rpm at 4°C for 20 minutes. The supernatant obtained was used as the enzyme source for estimation of AcPase activity. The AlkPase activity was also estimated following the same method of Plummer [28]. A 10% (w/v) of the cestode tissue was homogenized in glycine buffer. The homogenate was centrifuged at 5,000 rpm at 4°C for 20 minutes. The supernatant obtained was used as the enzyme source for estimation of AlkPase activity.

For both the AcPase and AlkPase, *p*-Nitrophenyl phosphate was used as the substrate. Incubation was carried out at 37±1°C and the reaction was stopped by adding of 0.02 N NaOH. The absorbances of both the blank and incubated solutions were measured at 405 nm in UV-VIS Spectrophotometer (Systronics model 119, India). The enzyme activity was calculated from a linear standard graph of *p*-nitrophenol. One unit of AcPase or AlkPase activity was defined as that amount which catalyzed the formation of 1 mM of *p*-nitrophenol/h at 37°C.

For all the enzymatic assays, the total protein content was estimated following the method of Lowry *et al.* [29] using bovine serum albumin as the standard protein and Folin-Ciocalteau reagent as the substrate.

Data analysis

All data are presented as means plus or minus the standard error (SE) of the mean. Comparison of the mean values between the treated and control groups was made using Student's *t*-test, and the level of significant probability considered at $P < 0.05$.

Results

Alterations in the levels of trace metals in *R. echinobothrida*

The quantitative observations of vital trace elements in *R. echinobothrida*, with the effects of albendazole as a standard reference drug, and the ethanol, methanol and acetone extracts of *A. oxyphylla* are shown in Table 1 and Figure 1. The data indicate that the cestodes exposed to 20 mg ml $^{-1}$ each of albendazole and all the three extracts of *A. oxyphylla* resulted in marked reduction of vital trace metals. The concentrations of calcium, magnesium, sodium and potassium at the basal level of the control worms maintained in 0.9% PBS with 1% DMSO were 296.2 ± 8.1, 953.0 ± 6.4, 435.7 ± 3.6 and 132.8 ± 3.6 µg/g dry tissue weight, respectively.

The most effective reduction was caused by albendazole reducing the levels to 128.6 ± 6.3, 629.5 ± 1.6, 268.3 ± 5.1 and 87.0 ± 2.6 µg/g dry tissue weight, respectively. Of the three extracts of the plant, ethanol appeared to exert the most compelling change resulting into a decrease of 231.0 ± 3.5, 687.4 ± 2.7, 368.3 ± 4.2 and 116.8 ± 5.2 µg/g dry tissue weight, respectively. It is clearly depicted in Figure 1 that the extent of alterations in the levels of the trace metals decreases in the order albendazole>ethanol extract>methanol extract>acetone extract. Therefore, all the three extracts of *A. oxyphylla* caused highly significant reduction in the levels of vital trace metals in *R. echinobothrida*, and the ethanol extract evidently is the most active, and the possible source of all or any anthelmintic component in the plant.

The enzymatic activities of AcPase and AlkPase in *R. echinobothrida* maintained as untreated control and treated with albedazole and the three extracts of *A. oxyphylla* are presented in Table 2 and Figure 2. Cestodes in control group indicated high activity of AcPase and AlkPase,

10.3 ± 2.4/1.7 ± 0.3 and 37.6 ± 1.8/3.4 ± 0.6 total activity/specific activity, respectively. The result also indicates that between the two enzymes, AlkPase is showed to exhibit higher enzymatic activity in comparison to that of AcPase in *R. echinobothrida*.

Table 1. Effects of albendazole and extracts of *A. oxyphylla* stem bark on the levels of trace elements in *R. echinobothrida*.

Incubation medium		Concentration (µg/g dry tissue weight) of			
		Calcium	Magnesium	Sodium	Potassium
Control (PBS+DMSO)		296.2 ± 8.1	953.0 ± 6.4	435.7 ± 3.6	132.8 ± 3.6
Albendazole (20 mg ml^{-1})		128.6 ± 6.3[a]	629.5 ± 1.6[a]	268.3 ± 5.1[a]	87.0 ± 2.6[a]
A. oxyphylla extract (20 mg ml^{-1})	Ethanol	231.0 ± 3.5[a]	687.4 ± 2.7[a]	368.3 ± 4.2[a]	116.8 ± 5.2[a]
	Methanol	252.3 ± 6.4[a]	703.2 ± 1.5[a]	391.8 ± 4.0[a]	104.3 ± 3.1[a]
	Acetone	268.7 ± 2.4[a]	714.6 ± 5.0[a]	372.0 ± 3.2[a]	112.6 ± 4.5[a]

Values are expressed as mean ± SE (n = 5)
[a] P value significant at < 0.05 in treatments compared to control group.

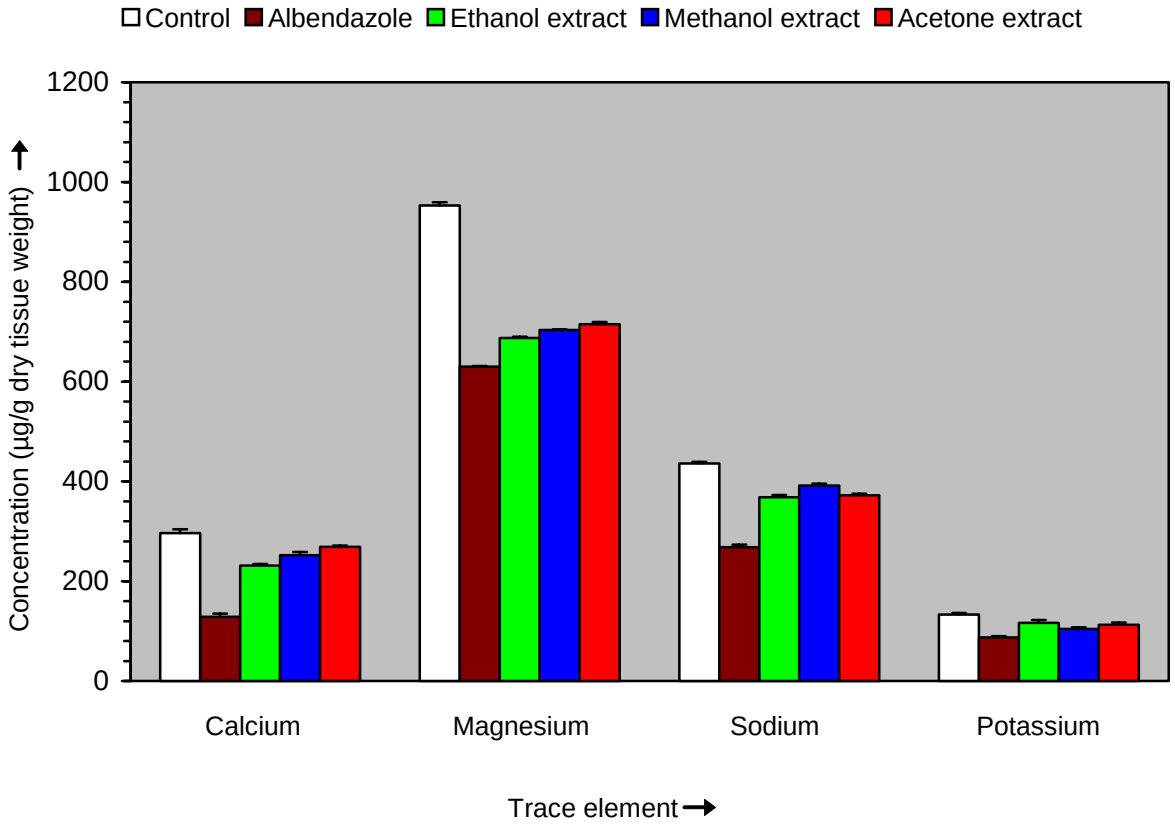

Figure 1. Differences in the levels of trace metals in *R. echinobothrida* after treatment with albendazole as a reference anthelmintic drug and three extracts of *A. oxyphylla*. Columns represent values as mean, and error bars as the corresponding SE.

Table 2. Effects of albendazole and extracts of *A. oxyphylla* stem bark on the activity of the tegumental enzymes of *R. echinobothrida*.

Incubation medium		Total[1]/specific activity[2] of		Percentage (%) decrease of	
		AcPase	AlkPase	AcPase	AlkPase
Control (PBS+DMSO)		10.3 ± 2.4/ 1.7 ± 0.3	37.6 ± 1.8/ 3.4 ± 0.6		
Albendazole (20 mg ml^{-1})		5.7 ± 1.5/ 1.3 ± 0.7[a]	19.3 ± 1.1/ 1.4 ± 0.8[a]	44.7	48.7
A. oxyphylla extract (20 mg ml^{-1})	Ethanol	6.4 ± 0.8/ 0.9 ± 0.3[a]	21.7 ± 2.5/ 1.8 ± 0.7[a]	37.9	42.3
	Methanol	6.7 ± 0.2/ 1.4 ± 0.1[a]	22.1 ± 0.8/ 1.7 ± 0.5[a]	35.0	41.2
	Acetone	7.8 ± 0.6/ 1.3 ± 0.5[a]	22.4 ± 1.3/ 2.2 ± 0.7[a]	24.3	40.4

Values are expressed as mean ± SE (n = 5)
[1] Total enzyme activity is defined as the amount of enzyme that consumes 1.0 µm substrate/g wet wt tissue/h
[2] Specific activity expressed as unit/mg protein/h
[a] P value significant at < 0.05 in treatments compared to control group.

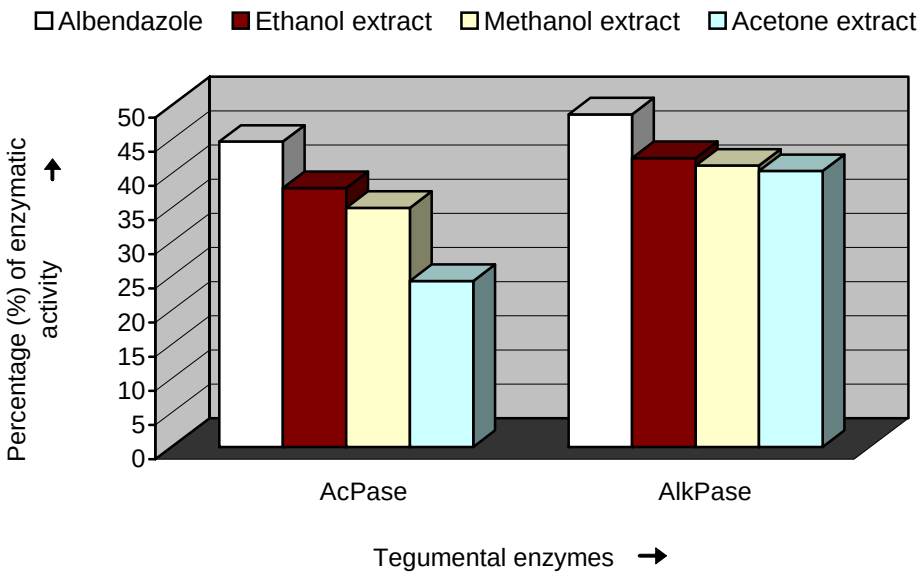

Figure 2. Reduction in the activities of acid phosphatase and alkaline phosphatase in *R. echinobothrida* upon treatment with albendazole and three extracts of *A. oxyphylla*. Clustered columns represent values in percentage (%).

The AcPase activity was decreased by 44.7% upon treatment with 20 mg ml^{-1} of albendazole. The same treatment also resulted in a 48.7% inhibition of AlkPase activity. Among the three extracts of *A. oxyphylla*, highest inhibition on the tegumental enzymes was observed for the ethanol extract. The ethanol extract-treated worms indicated 37.9% and 42.3% inhibition of AcPase and AlkPase activities, respectively. Worms treated with the methanol extract showed 35% and 41.2% reduction of the enzymes, respectively. While the acetone extract was noted to affect inhibition of AcPase by 24.3% and AlkPase by 40.4%. Therefore, the three extracts of *A. oxyphylla* caused highly significant inhibition of tegumental enzymes, and the efficacy is in the order ethanol extract>methanol extract>acetone extract.

Discussion

The ocuurance of trace metals such as cadmium, calcium, cobalt, copper, iron, lead, nickel, magnesium, manganese, nickel, potassium, selenium and zinc has been adequately documented for different helminth parasites, ranging from the amphistomid trematodes [30], nematodes [31,32], to cestodes [33,34], including *R. echinobothrida* [26]. These trace elements play a significant role in the physiology, growth and development, the sequestration of free radicals and in the cellular antioxidant defense system, metabolism and immuno tolerance of parasites. Certain parasites, particularly cestodes of fish, can accumulate heavy metals at concentrations that are orders of magnitude higher than those in the host tissues of the environment [35]. It has been revealed that the glucose transport coupled to sodium cations in cestodes like *H. diminuta* [36]. This condition confirms the manifold role of calcium inside the cell, as its presence regulates the sodium level, maintenance of inter-cellular ionic bridges, neuro-motor functions and several other activities within the cell.

In addition, trace metals are specific indicators of host-parasite interrelationship. Deficiencies of iron, molybdenum, copper, and zinc in host tissues have been associated with higher worm burdens, as have excessive intakes of molybdenum, iron, and copper [37]. The possibility is emerging that there may be an optimum trace element level in the diet above which and below which the parasite is advantaged. Moreover, there is some data to suggest that specific trace elements may be directly toxic to the parasite [34]. Thus, it is understood that not only is there competition for elements between the helminths inside the gut but there is also competition for these elements between the host and the parasites [32].

From the present investigation, it is comprehensible that calcium and magnesium are present in high proportion in *R. echinobothrida*, 296.2 and 953 μg/g dry tissue weight, respectively, supporting the data of Das et al. [26]. Additionally, sodium and potassium are also detected at somewhat lower concentrations, 435.7 and 132.8 μg/g dry tissue weight, respectively. The cestodes treated with albendazole as a reference pharmaceutical drug, and the ethanol, methanol and acetone extracts of *A. oxyphylla* evidently affected considerable disturbances in the physiological homeostasis of these four trace metals. Therefore, the anthelmintic activities are highly correlated to the levels of trace metals such that significant decline in the amount probably lead to gradual loss of physical and metabolic activities within the cells, eventually resulting in paralysis, and death.

Anthelmintic drugs are known to enter target parasites by either oral ingestion or by diffusion through the external surface (‘cuticle’ in nematodes or ‘tegument’ in cestodes and trematodes), or some combination of both routes [37,38]. The cuticle or tegument is metabolically active and morphologically specialised interface to perform selective absorption of nutrients, secretion of glycoproteins for immunoprotection, osmoregulation and (insofar as it supports sense organs) sensory perception. Trans-cuticular or trans-tegumental passive diffusion is, therefore, the principal mechanism of anthelmintic entry into the helminths [38]. Consequently, it has been sufficiently accounted that one of the hallmark effects of any anthelmintic is destruction of the worm’s surface [27,39-41].

Albendazole and other benzimidazoles are construed to enter the cestode body by passive diffusion through the tegument in which they bind selectively and with high affinity to the β-subunit of the microtubule proteins, tubulins, causing disruption of the microtubule dynamic equilibrium, and with that, cell lysis [42,43]. By binding specifically to free β-tubulin, BZs inhibit the polymerization of α- and β-tubulin molecules and the microtubule-dependent uptake of glucose, ensuing starvation then the worms are paralysed and killed [44,45].

It has been resolutely demonstrated that AcPase and AlkPase are the two vital enzymes of the tegument and subtegumental regions in cestodes, with AlkPase are the dominant enzyme [17,22,23]. The high abundance of these phosphatases implicates that they play an intimately role in the digestion and/or absorption in the tegumental tissues. The present study also revealed a comparatively higher degree of activity of AlkPase over AcPase in control *R. echinobothrida*.

Commercial synthetic drugs such as isatin, hexacholorophene, praziquantel, luxabendazole and thiabendazole reportedly induce detectable alterations in the activities of the tegumental enzymes in different soft-bodied cestodes and trematodes [23,26,46-49], as well as in the hard-bodied nematodes [50,51]. The cestodes exposed to albendazole and the ethanol, methanol and acetone extracts of *A. oxyphylla* were found to be significantly inhibited in their AcPase and AlkPase activities. Comparable results were reported in a human tapeworm *Echinococcus multilocularis* metacestode in which acute inhibition of AlkPase activity by 23% and a complete inhibition of glucose uptake following treatment with isatin, a known inhibitor of phosphatase [47]. Similar result was also obtained for the both the AlkPase and AcPase activity in *H. dimunita* [15,22]. It can be inferred from here that phosphatase activity is related to glucose uptake in cestodes. Thus, the observed reduction in the two tegumental phosphatases also might be associated with inhibition or reduced uptake of glucose of *R. echinobothrida* leading to gradual loss of motor activity due to deprivation of energy source and, thus, culminated to paralysis.

However, the experimental results do not impart sufficient information at the molecular level, particularly in connection with the probable inhibition of tubulin polymerization. Though albendazole is already established to act via tubulin assembly, it still remains uncertain whether the plants extracts follow the same route of action. However, it is hereby definitely evident that they do act trans-tegumentally to cause considerable constraints on the activities of tegumental enzymes.

It is also perceptible that all the three extracts of *A. oxyphylla* stem barks employed in the present study indicated significant activity in inhibiting the tegumental enzymes and decreasing vital trace metals; and the efficacy is in the sequence ethanol extract>methanol extract>acetone extract. This is further suggestive that in order to pinpoint the specific principal ingredient of the plant as an anthelmintic, the ethanol extract would be a good source to start with.

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