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Effects of *Millettia pachycarpa* on the trace metals and tegumental enzymes of *Raillietina echinobothrida*

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ABSTRACT

The root bark extract of *Millettia pachycarpa* Bentham is known to certain Mizo tribes of north-east India as a curative to gastrointestinal infestations. An *in vitro* treatment of the poultry gastrointestinal cestode *Raillietina echinobothrida* Megnin with the crude ethanolic extract of the plant part reportedly indicate remarkable cestocidal effects on the survival and morphological structures of the worm. In an attempt to further explore the anthelmintic activity of the plant and with a view to understand the primary mode of action, the parasites were exposed to 20 mg mL^{-1} of the different extracts (viz ethanol, methanol and acetone) of *M. pachycarpa* root bark till they reached paralytic state. Similar dose of albendazole was used as a reference standard drug. The levels of the vital trace elements such as calcium, magnesium, potassium and sodium, and the activities of tegumental enzymes such as acid phosphatase (AcPase) and alkaline phosphatase (AlkPase) of the worms were assessed. The plant extracts clearly indicated significant reduction ($P < 0.05$) in the levels of the trace metals compared to control worms. The enzymatic activities of AcPase and AlkPase were also significantly inhibited. Among the three extracts, the ethanol extract was the most potent in causing these biochemical alterations, and the effects were comparable to those of albendazole. The overall results show that the plant extracts exert anthelmintic activity by acting trans-tegumentally, inhibiting the major enzymes to induce tegumental damages, and depleting the trace metals to bring about flaccid paralysis and mortality of the cestodes.

KEY WORDS - Acid phosphatase (AcPase); alkaline phosphatase (AlkPase); anthelmintic; *Millettia pachycarpa*; *Raillietina echinobothrida*; trace metals.

INTRODUCTION

Millettia pachycarpa Bentham (family Fabaceae) is a leguminous perennial climbing tree endemic to south-east Asia, where it is acclaimed with a wide range of medicinal applications in various traditional practices. The juicy extracts of the root bark and leaf are commonly used in the treatment of infertility, and as a blood tonic and anticancer agent (1). A large number of bioactvie compounds have been identified from it, of which isoflavones such as erysenegalensein E, isoerysenegalensein E, 6,8-diprenylorobol, millewanins G and H, furowanin A and B, and auriculasin were all demonstrated to have antiestrogenic activity (2–4), confirming the anticancer potentials of the plant.

Following the ethnomedicinal usage of the Mizo tribes of north-east India, we have earlier shown that the crude ethanolic extract of the root bark exhibited a potent anthelmintic activity against mature Raillietina echinobothrida Megnin, the intestinal cestode of domestic fowl (5). The plant extract caused dose-dependent paralysis and mortality, associated with inexorable degenerative effects on the tegument all over the body surface of the cestode. The present study is an attempt to disclose the probable primary route of action of the plant extracts by investigating the biochemical changes in the helminth physiology such as the activities of the vital tegumental enzymes and trace metals. Trace metals have been posited to play a significant role in the physiology, growth and development, the sequestration of free radicals and in the cellular antioxidant defense system, metabolism, host-parasite interactions and immuno-tolerance of helminth parasites (6).

Despite successful experimental validation of a large number of medicinal plants traditionally used in different parts of the world as anthelmintic agents against a variety of helminth parasites, the global crisis of helminthic infestation is far from being ameliorated (7–9). This is primarily due to the fact that

though medicinal plants exhibit anthelmintic properties, their chemical nature, safety and, above all, mode of action remain poorly understood. Particularly when applied on large-scale clinical trials, plant extracts can exert undesirably serious consequences. Several lines of recent evidences have posited that even some highly acclaimed medicinal plants and their products are highly toxic to the host and without any appreciable practicable value in clinical and veterinary applications (10–14). Thus, there still remains a veritable hindrance for medicinal plants at large to find their way as subtle alternatives to pharmaceutical drugs.

Moreover, like the commercial drugs themselves, majority of anthelmintic plants are helminth specific, showing activity against a particular species or group of the parasites (15, 16). Thus the choice of available medicinal plant is restricted with the type of helminthic infection. Besides, plant extracts are prepared using different solvents so that the form of extraction can also reflect the anthelmintic efficacy (17–19). 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 for each plant in order to discover the active principle; thus the aim of the study is to assess the changes, if any, on the levels of vital trace metals and activity of the tegumental phosphatases of R. echinobothrida upon treatment with the extracts of M. pachycarpa root bark.

MATERIALS AND METHODS

Preparation of Plant Extract

Collection, authentication and preparation of the crude extract of Millettia pachycarpa was reported previously (5). The fresh root barks were peeled off, thoroughly washed with deionized water, cut into small pieces, macerated and dried in a hot air oven at 50ºC. The dried parts were pulverized to fine powder and a pre-weighed amount was refluxed with ethanol (100g/L) for 8 h at 60ºC, following the methods of Tandon et al. (15) and Roy (20). Refluxing was repeated thrice. The solution obtained was filtered through Whatman filter paper (No. 1) and then evaporated to complete dryness at 50ºC. The alcoholic extract was obtained as a deep brown powder. The net yield from such extraction was 7.1%.

A portion of the alcoholic extract was macerated with methanol (100g/L) in a fractionating flask, with several changes of the solvent and then vigorously mixed in a rotary shaker for 24 hours. The resultant solution was filtered and refluxed as before. After complete evaporation of the solvent, solid precipitates were obtained as the methanol extract. The total yield was 2.5%. Similarly, another portion of the alcoholic extract was mixed with acetone to get the acetone extract with a net yield of 1.3%. The different extracts were refrigerated at 4ºC until further use. Similar extractions were also attempted using ethyl acetate, diethyl ether, chloroform and benzene, but the crude extract was strictly insoluble in these organic solvents, thus, no extracts were obtained.

1 hour before the actual experimental assay, 20 mg mL-1 of the ethanol, methanol and acetone extracts were separately prepared by dissolving them in 0.9% neutral phosphate buffered saline (PBS, pH 7-7.3), supplemented with 1% dimethylsulfoxide (DMSO). The solutions were then maintained at 37 ± 1ºC in a glass-chambered incubator.

Chemicals and Drugs

All the chemicals used were of standard analytical grades, obtained either 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.

In Vitro Treatments of Parasites

Native bred live local fowls (Gallus domesticus Linnaeus) were purchased from the local abattoir in Aizawl, Mizoram, India. They were sacrificed and on immediate autopsy, live worms, R. echinobothrida Megnin, were recovered from the intestines. Only the live adult worms with more or less body length (7.4 ± 0.8 cm) were selected and collected in 0.9% PBS. Batches of fresh worms were directly introduced to the media containing 20 mg mL-1 of the ethanol, methanol and acetone extracts of M. pachycarpa dissolved in PBS with 1% DMSO. Similar treatment was performed for albendazole at its commercial dosage (20 mg mL-1) 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 Metals

Persistence on the motility of the worms were observed, time taken for the onset of paralysis was recorded, as previously described (15, 20). The onset of paralysis was defined as complete loss of motor activity even after physical stimulation of the worms in culture. The parasites subjected to treatments were collected the moment they indicated paralysis.

Sentient worms in the control medium were directly taken for comparison with the treated groups. They were washed with double distilled water and quick-dried in an incubator at 50°C. 2 g of the powdered dry worms taken from each incubation medium was digested in 10 ml of concentrated HNO_3 overnight at 50°C. The fully digested solution was evaporated on a hot plate at 70°C. 10 ml of deionized water was then added and filtered through Whatman filter paper (110 mm Φ). The volume was finally made to 100 ml with deionized water, which was used for quantitative analysis of trace elements using an atomic absorption spectrophotometer (model Chemito AAS-201, India) at the absorbance wavelengths of 422.6nm for calcium, 285.2 nm for magnesium, 589.0 nm for sodium, and 766.5 nm for potassium.

Estimation of Tegumental Enzyme Activities

The acid phosphatase (AcPase; EC 3.1.3.2) and alkaline phosphatase (AlkPase; EC 3.1.3.1) activities were estimated using *p*-nitrophenol product from an enzyme source following the method of Plummer (21) with slight modifications in the concentration of the buffer and substrate. For AcPase, a 10% (w/v) of the cestode was homogenized in sodium acetate buffer using a Potter-Elvehjem homogenizer and centrifuged at 5,000 rpm at 4°C for 20 minutes in a cooling centrifuge (REMI C4, India). The supernatant obtained was used as the enzyme source for estimation of AcPase activity. For AlkPase, a 10% (w/v) of the cestode was homogenized in glycine buffer and 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 enzymes, *p*-nitrophenyl phosphate was used as the substrate. Incubation was carried out at 37 ± 1°C and the reaction was stopped after 15 min through addition 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*. (22) using bovine serum albumin as the standard protein and Folin-Ciocalteau reagent as the substrate.

Data Analysis

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

RESULTS

The quantitative observations of vital trace metals in *R. echinobothrida*, those in control experiment and those treated with albendazole and the ethanol, methanol and acetone extracts of *M. pachycarpa* are shown in Table 1. The data clearly indicate that the cestodes exposed to 20 mg mL^{-1} each of albendazole and all the three extracts of *M. pachycarpa* resulted in significant reduction in the concentration 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 ± 5.7, 953.0 ± 4.8, 435.7 ± 2.2 and 132.8 ± 1.1 µg/g dry tissue weight, respectively.

The most effective reduction was caused by albendazole reducing the levels of the trace metals to 128.6 ± 4.7, 629.5 ± 1.2, 268.3 ± 2.8 and 87.0 ± 2.1 µg/g dry tissue weight, respectively. Of the three extracts of the plant, ethanol appeared to exert the most abrupt effect resulting in a decrease to 174.6 ± 3.7, 653.1 ± 3.6, 326.4 ± 2.9 and 92.3 ± 3.4 µg/g dry tissue weight, respectively; and the least effect was with the acetone extract. Therefore, albendazole and all the three extracts of *M. pachycarpa* caused highly significant reduction in the levels of vital trace metals in *R. echinobothrida*.

The enzymatic activities of AcPase and AlkPase in *R. echinobothrida* maintained as untreated control, and those treated with albendazole and the three extracts of *M. pachycarpa* are presented in Table 2. Cestodes in control group indicated high activity of AcPase and AlkPase, 10.3 ± 1.8/1.7 ± 0.2 and 37.6 ± 1.2/3.4 ± 0.4 total activity/specific activity, respectively. The result also indicates that between the two enzymes, AlkPase is the dominant tegumental enzyme in *R. echinobothrida*.

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 *M. pachycarpa*, highest inhibition on the tegumental enzymes was observed for the ethanol extract, which indicated 37.9% and 47.3% inhibition of AcPase and AlkPase activities, respectively. Worms treated with the methanol extract showed 35% and 43.9% reduction of the enzymes, respectively. While the acetone extract was noted to affect inhibition of AcPase by 31.1% and AlkPase by 42.8%. Therefore, the three extracts of *M.*

pachycarpa significantly inhibited the activities of the tegumental enzymes in *R. echinobothrida*.

DISCUSSION

The presence of trace metals such as cadmium, calcium, cobalt, copper, iron, lead, nickel, magnesium, manganese, nickel, potassium, selenium and zinc has been adequately reported in different helminth parasites, ranging from trematodes (23, 24), nematodes (24–26) to cestodes (26, 27), including *R. echinobothrida* (28, 29). These trace elements were documented to 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 (6). For instance, it has been shown that glucose transport is coupled to sodium cations in cestodes like *H. diminuta* (30). This condition also reflects 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 (6).

Moreover, different trace metals are attributed to play critical roles in host-parasite interactions. 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 (31). 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 (32). 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 (33).

From the present investigation, it can be recognized 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*. (27). Additionally, sodium and potassium were also detected at the concentrations of 435.7 and 132.8 µg/g dry tissue weight, respectively. The cestodes treated with albendazole as a reference drug, and the ethanol, methanol and acetone extracts of *M. pachycarpa* root bark evidently caused considerable decrease in the physiological concentration of these trace metals. Therefore, drastic decline in the levels of trace metals probably lead to gradual loss of physical and metabolic activities within the cells, eventually resulting in paralysis, and death. The result comprehensively conforms to that of Lalchhandama *et al*. (28) on the effects of *Acacia oxyphylla* but with a comparatively higher efficacy.

Anthelmintic drugs are known to enter target parasites by either oral ingestion or by diffusion through the external surface (34). The cuticle in nematodes or tegument in cestodes and trematodes is metabolically active, and morphologically specialized interface to perform selective absorption of nutrients, secretion of glycoproteins for immunoprotection, osmoregulation and (insofar as it supports sense organs) sensory perception (35, 36). Trans-cuticular or trans-tegumental passive diffusion is, therefore, the principal mechanism of anthelmintic entry into the helminths (36). Consequently, it has been sufficiently accounted that one of the hallmark effects of any anthelmintic is destruction of the worm's surface (37–39).

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 microtubule proteins, tubulins, causing disruption of the microtubule dynamic equilibrium, and with that, cell lysis (35, 40). By binding specifically to free ß-tubulin, BZs inhibit the polymerization of α- and ß-tubulin molecules and the microtubule-dependent uptake of glucose, ensuing starvation the worms are paralyzed, killed and expelled (41).

The occurrence of vital enzymes, viz acid phosphatase (AcPase) and alkaline phosphatase (AlkPase) has been resolutely demonstrated in a number of helminth parasites, both histochemically and biochemically (42–45), including *R. echinobothrida* (46). 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 (47–49). The present study also revealed a comparatively higher degree of activity of AlkPase over AcPase in control *R. echinobothrida*. It has been positively demonstrated that AcPase and AlkPase are the two vital enzymes of the tegument and subtegumental regions in cestodes, with AlkPase as the dominant enzyme (42, 44, 46). Previous investigations had demonstrated that the enzymes are highly abundant in those parasite compartments crucially involved in interacting with the host (50). Due to its abundance at the host-parasite interface and its high activity, it is conceivable that they represent

Table 1. Effects of albendazole and extracts of M. pachycarpa root 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 ± 5.7	953.0 ± 4.8	435.7 ± 2.2	132.8 ± 1.1
Albendazole (20 mg mL^{-1})		128.6 ±4.7[a]	629.5 ± 1.2[a]	268.3 ± 2.8[a]	87.0 ± 2.1[a]
M. pachycarpa extract (20 mg mL^{-1})	Ethanol	174.6 ± 3.7[a]	653.1 ± 3.6[a]	326.4 ± 2.9[a]	92.3 ± 3.4[a]
	Methanol	211.3 ± 3.6[a]	682.3 ± 2.5[a]	315.6 ± 2.1[a]	103.6 ± 5.7[a]
	Acetone	246.2 ± 2.1[a]	675.7 ± 5.2[a]	349.2 ± 1.2[a]	115.0 ± 3.8[a]

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

Table 2. Effects of albendazole and extracts of M. pachycarpa root bark on the activity of the tegumental enzymes of R. echinobothrida.

Incubation medium		Enzyme activity (total[1]/specific activity[2])		Percentage (%) decrease of	
		AcPase	AlkPase	AcPase	AlkPase
Control (PBS+DMSO)		10.3 ± 1.8/ 1.7 ± 0.2	37.6 ± 1.2/ 3.4 ± 0.4		
Albendazole (20 mg mL^{-1})		5.7 ± 1.1/ 1.3 ± 0.4[a]	19.3 ± 0.8/ 1.4 ± 0.5[a]	44.7	48.7
M. pachycarpa extract (20 mg mL^{-1})	Ethanol	6.4 ± 0.7/ 0.9 ± 0.2[a]	19.8 ± 1.9/ 1.8 ± 0.5[a]	37.9	47.3
	Methanol	6.7 ± 0.8/ 1.0 ± 0.6[a]	21.1 ± 0.6/ 1.7 ± 0.5[a]	35.0	43.9
	Acetone	7.1 ±0.4/ 1.2 ±0.3[a]	21.5 ± 0.4/ 1.7 ± 0.4[a]	31.1	42.8

Values are expressed as mean ± SD (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 treated group compared to control group.

molecules of considerable importance for parasitic helminths, as it may be involved in the acquisition of nutrients (43) as well as in the modulation of phosphorylation-dependent events at the host-parasite boundary: for instance, those interactions initiated by host-effector cells (50).

Extracts from certain medicinal plants, including *Butea monosperma*, *Embelia ribes*, and *Roltlesia tinctoria* reportedly influenced drastic decrease in the activities of both AcPase and AlkPase in the trematode, *Paramphistomum cervi* (51). The root tuber peel extract and genistein from *Flemingia vestita* similarly caused significant reduction of the enzymes in *R. echinobothrida* (46), and in the fluke, *Fasciolopsis buski* (52), comparable to those of the standard pharmaceuticals, prizquan--tel and oxyclozanide, respectively.

Pharmaceutical drugs such as albendazole, flubendazole, isatin, hexacholorophene, levamisole, luxabendazole, mebendazole, praziquantel and thiabendazole reportedly induce detectable alterations in the activities of the tegumental enzymes in different helminths (45, 53–58). In the present study, R. echinobothrida exposed to albendazole and the ethanol, methanol and acetone extracts of M. pachycarpa were found to be significantly inhibited in their AcPase and AlkPase activities. Similar inhibitions were observed for the cestocidal effects of A. oxyphylla (28). Comparable results were reported in a human tapeworm Echinococcus multilocularis metacestode in which acute inhibition of AlkPase activity by 23% following treatment with isatin was observed (54). The cestocidal effects of albendazole on E. multilocularis, characterized by progressive degeneration and destruction of the tegumental tissue was directly associated with AlkPase activity, indicating that the phosphatase activity is an ideal parameter for performing first-round *in vitro* tests on the efficacy of a large number of antiparasitic compounds (45). Similar result was also obtained for the both the AlkPase

and AcPase activity in *H. dimunita*, where a definite correlation between phosphatase activity and glucose uptake was observed (59). Thus, the observed reduction in the two tegumental phosphatases also might be associated with destruction of the tegumental surface (5) and possibly, inhibition or reduced uptake of glucose of *R. echinobothrida* leading to gradual loss of motor activity due to deprivation of energy source, culminating in to paralysis, and ultimately, death.

CONCLUSION

The present investigation revealed that the anthelmintic activity of albendazole and *M. pachycarpa* root bark on *R. echinobothrida* involved alterations in the enzymatic activities of AlkPase and AcPase, and levels of trace metals such as calcium, magnesium, potassium and sodium. The study substantially demonstrated that the extracts of *M. pachycarpa* root bark caused significant inhibition of the tegumental enzymes and reduction of vital trace metals; and the efficacy is in the order albendazole>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 choice to start with. However, the complete cellular and molecular events underpinning the anthelmintic activity of the plant is yet incompletely defined from this study and need further investigation.

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