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Scanning electron microscopic observations on the *in vitro* anthelmintic effects of *Millettia pachycarpa* on *Raillietina echinobothrida*

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ABSTRACT

The ethanolic extract from the root bark of *Millettia pachycarpa*, traditionally used as a remedy for gastrointestinal infections among the Mizo tribes of north-east India, was tested *in vitro* to evaluate its anthelmintic activity on the poultry intestinal tapeworm, *Raillietina echinobothrida*. On treatment of the parasites with varying concentrations of the plant extract, viz, 0.5, 1, 2, 5, 10 and 20 mg ml^{-1}, a dose-dependent lethal efficacy was observed. Scanning electron microscopy revealed extensive distortion and destruction on the surface fine topography of the worm. Focal truncation with formation of pits and vacuoles on the tegument were evident. Deformities on the scolex with its suckers were particularly conspicuous at the anterior extremity. The observations hereby suggest that there is credibility to the use of *M. pachycarpa* root bark as an anthelmintic agent.

KEY WORDS - Anthelmintic; *Millettia pachycarpa*; scanning electron microscopy; *Raillietina echinobothrida*.

INTRODUCTION

Global crisis over rapid emergence of drug resistance in helminth parasites of livestock animals virtually to all classes of pharmaceutical anthelmintics, and the danger of its development in human parasites has turned the research attention to an enthusiastic search for alternative measures (1). Commercial anthelmintics also have additional disadvantages in their expensive cost, limited supply and restriction in organic farming due to their adverse effects on non-target organisms. Among the active fields, experimental evaluation of ethnomedicinal plants plays a large role. This is primarily due to general richness of traditional practices using plants as anthelmintic, and these plants ostensibly offer easily accessible, environmentally acceptable and cost-effective means for an effective control of helminthiasis (2). Thus, it becomes important to have scientific assessments of the many known phytomedicines.

Millettia pachycarpa Bentham (Synonym *Millettia taiwaniana* Hayata) is a perennial climbing tree belonging to the family Fabaceae, and is well-known throughout south-east Asia, where it is found endemic. In Chinese medicine it is commonly used as blood tonic and anticancer agent (3). Various tribal people of north-east India use the crude extract for stupefying fish and for treatment of sexual disorders, and is an indigenous anthelmintic among the Mizo tribes inhabiting the remotest region of north-east India (located between latitude 21° 58' to 24° 34' N and longitude 92° 15' to 93° 25' E).

A number of novel bio-active chemicals were identified including several prenylated isoflavonoids, dihydroflanonol and chalcones from the seed, rotenoids such as rotenone; *cis*-12*a*-hydroxyretenone; rot-2′-enonic acid; *cis*-12a-hydroxyrot-2′-enonic acid from the root of *M. pachycarpa* (4-7). Five prenylated isoflavones including erysenegalensein E, isoerysenegalensein E, 6,8-diprenylorobol, furowanin A and auriculasin from the leaves were all determined to have antiestrogenic activity (8,9). Three novel isoflavonoids, named millewanins G (1) and H (2) and furowanin B (3), were also isolated from the leaves, which were all shown to possess antiestrogenic property (10). Thus, its feasibility as anticancer agent has been well-established; however, no scientific evidence has been reported as to the credibility of this plant as an anthelmintic. Therefore, the present

investigation is an attempt to evaluate the alleged anthelmintic property of the extract of *M. pachycarpa* root bark on the common fowl cestode, *R. echinobothrida*, in terms of lethal efficacy and to describe fine topographical alterations, if any, on the cestode.

MATERIALS AND METHODS

Preparation of Plant Extract

The fresh roots of *M. pachycarpa* were collected from the nearby forest of Aizawl, Mizoram, India in July 2005. The specimens were identified by Dr. H. S. Thapa, plant taxonomist, Department of Botany, Mizoram University, India, and a voucher specimen (PUC-BOT-M 036) was deposited to the same department. The barks were peeled off, thoroughly washed with deionized water, cut into small pieces, and dried in a hot air oven at 50ºC. The dried root parts were crushed to fine powder and then refluxed with ethanol (100g/l) for 8 h at 60ºC, following the procedure described earlier (11). The solution obtained was filtered through Whatman filter paper (No. 1) and then evaporated to complete dryness at 50ºC. The crude extract was obtained as a deep brown powdered precipitate, which was then refrigerated at 4ºC until further use. The net yield from such extraction was 7.07%.

Chemicals and Drug

All the chemicals used were standard analytical grades, obtained from Merck or Central Drug House, India. Ethanol was supplied by Bengal Chemicals, India, and the reference drug albendazole is a product of GlaxoSmithKline Pharmaceuticals Ltd., India.

Recovery and Treatments of Parasites

Live local fowls (*Gallus domesticus* Linn) were obtained from the local abattoir in Aizawl, Mizoram, India. They were sacrificed and on immediate autopsy, live worms, *R. echinobothrida* (Megnin, 1880) were recovered from the intestines. The cestode species was identified at the Parasitology Laboratory, Department of Zoology, North Eastern Hill University, India. They were collected in 0.9% neutral phosphate-buffered saline (PBS, pH 7) and then incubated at 37±1ºC in a glass-chambered digital BOD incubator. 1 h prior to *in vitro* experimental assay, different concentrations of the extract, viz, 0.5, 1, 2, 5, 10, and 20 mg ml^{-1}, were prepared by dissolving them in 0.9% PBS, supplemented with 1% dimethylsulfoxide (DMSO), and that were all maintained at 37±1ºC. The fresh worms were directly introduced to the different concentrations of the plant extract in separate petri dishes. Similar treatment was performed for albendazole as a reference drug. From the final concentration (20 mg ml^{-1}) of the drug, serial dilutions were made with PBS to get varying concentrations, viz, 0.5, 1, 2, 5, 10, and 20 mg ml^{-1}, into which the worms were introduced. One group is maintained in a medium containing only PBS with 1% DMSO as control. Each experiment consisted of 5 replicates.

Motility, Mortality and Scanning Electron Microscopic Studies

Motility of the worms were observed, time taken for paralysis and death was recorded, as previously described (11, 12). Paralysis is defined as complete loss of movement upon physical provocation of the worms. Dipping the parasites in tepid PBS (~45ºC) induced movement in sentient worms; if no movement occurred upon such stimulation, death was confirmed. From each medium, worms were divided into two groups. Parasite specimens were selected from the control and extract-treated medium for scanning electron microscopy. The worms were immediately fixed in 10% cold-buffered formaldehyde at 4ºC at least for 12 h. The fixed specimens were dehydrated through ascending concentration of acetone and then specifically air-dried in tetramethylsilane following the standardized method of Roy and Tandon (13) for helminth parasites. After coating with gold in a fine-coat ion sputter, JFC-1100 (JEOL) and mounted on metal stubs, the specimens were studied using a LEO 435 VP scanning electron microscope at an electron accelerating voltage of 20 kV.

Data Analysis

All data were presented as means plus or minus the standard error of the mean (SE). Comparison of the statistical values was made using unpaired Student's *t*-test, and the probability value of significance was considered at $p < 0.05$.

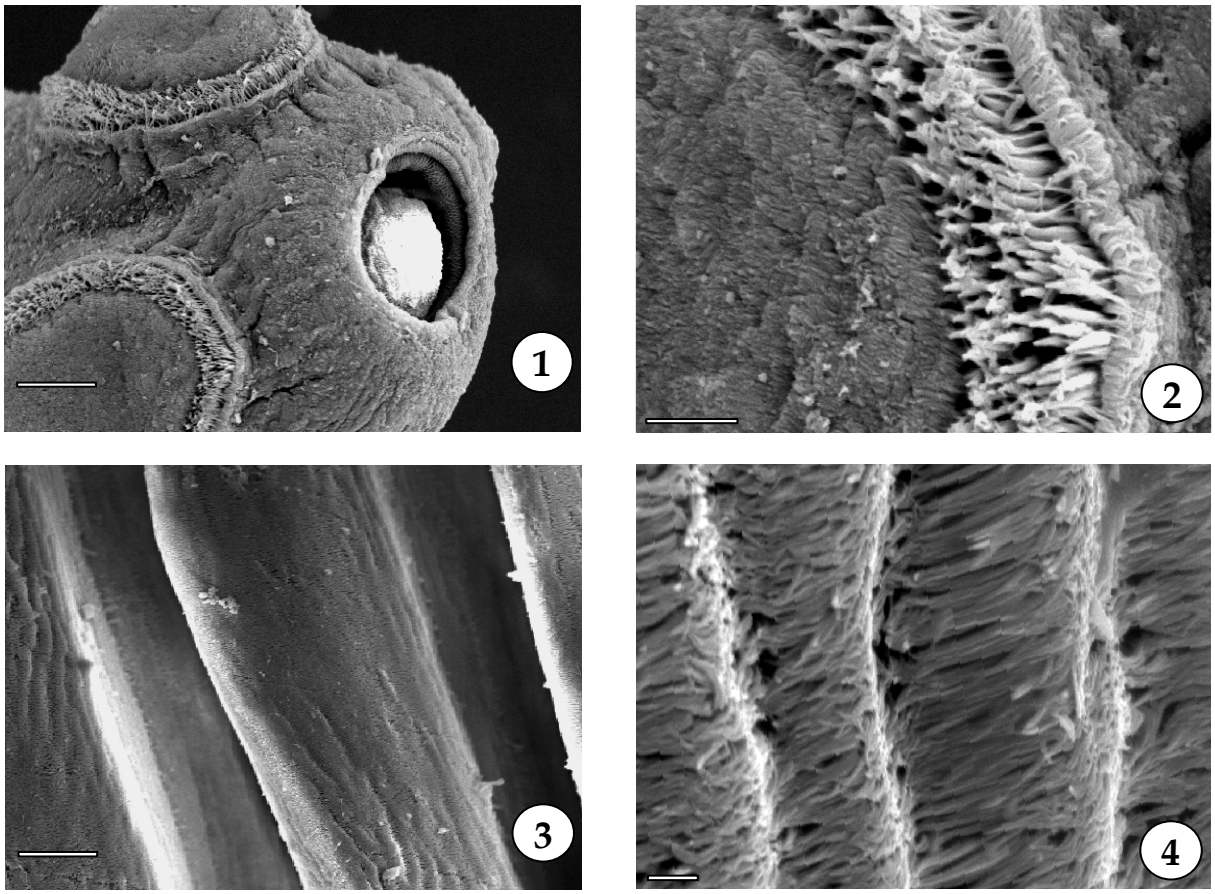
RESULTS AND DISCUSSION

Observations of the efficacy of the plant extract and the drug on the viability of the cestode parasites are represented in Table 1. *R. echinobothrida* maintained as control in only PBS with 1% DMSO survived very well up to 54.78 ± 0.65 hours. The results indicate that the plant extract showed dose-dependent lethal cestocidal efficacy. Those incubated with 0.5, 1, 2, 5, 10, and 20 mg of *M. pachycarpa* extract per ml of PBS showed complete loss of life in 52.99 ± 0.52, 42.77 ± 0.55, 28.42 ± 0.69, 6.32 ± 0.56, 5.23 ± 0.39 and 3.37 ± 0.50 h, respectively. Albendazole took 27.10 ± 0.72, 18.25 ± 0.49, 12.98 ± 0.51, 5.72 ± 0.44, 3.22 ± 0.34 and 1.85 ±

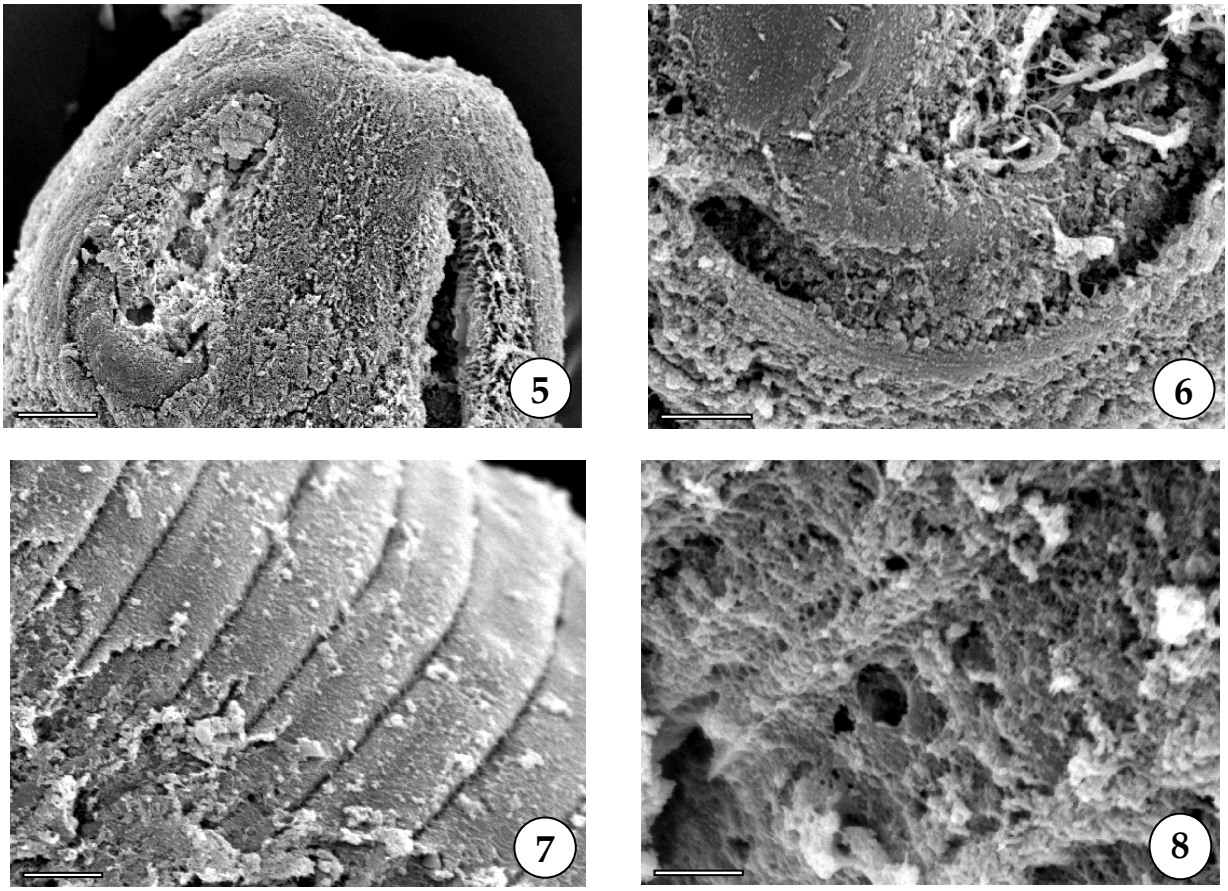
Table 1. *Cestocidal efficacy of the ethanol extract of M. pachycarpa root bark and albendazole.*

Test group	Dose (mg ml^{-1})	Time (h) taken for		Student's *t*-test
		Paralysis	Death	
Control	0		54.78 ± 0.65	
M. pachycarpa extract	0.5	27.38 ± 0.62	52.99 ± 0.52	N.S.
	1	17.77 ± 0.27	42.77 ± 0.55	$p < 0.05$
	2	11.46 ± 0.38	28.42 ± 0.69	$p < 0.05$
	5	4.05 ± 0.32	6.32 ± 0.56	$p < 0.05$
	10	3.37 ± 0.35	5.23 ± 0.39	$p < 0.05$
	20	1.72 ± 0.29	3.37 ± 0.50	$p < 0.05$
Albendazole	0.5	17.07 ± 0.55	27.10 ± 0.72	$p < 0.05$
	1	12.94 ± 0.63	18.25 ± 0.49	$p < 0.05$
	2	9.62 ± 0.60	12.98 ± 0.51	$p < 0.05$
	5	3.40 ± 0.38	5.72 ± 0.44	$p < 0.05$
	10	1.32 ± 0.22	3.22 ± 0.34	$p < 0.05$
	20	1.12 ± 0.33	1.85 ± 0.43	$p < 0.05$

Values are expressed as mean ± SE (n=5).
P value significant at < 0.05 for comparison of treated against control groups.
N.S. = not significant (i.e. P ≥ 0.05).



Figs. 1–4 *Scanning electron micrographs of untreated control R. echinobothrida.* ***Fig. 1*** *The anterior scolex bearing apical rostellum with surrounding suckers (acetabula). x 600. Bar 20 µm.* ***Fig. 2*** *A sucker bordered by spines (hooklets). x 2,000. Bar 10 µm.* ***Fig. 3*** *The tegument of mature proglottids. x 800. Bar 20 µm.* ***Fig. 4*** *Microtriches on the tegument. x 6,000. Bar 2 µm*



Figs. 5–8 *Scanning electron micrographs of R. echinobothrida treated with 20 mg/ml of ethanol extract of M. pachycarpa root bark.* ***Fig. 5*** *Severe surface erosion of the scolex. x 600. Bar 20 µm.* ***Fig. 6*** *Damage on the sucker. Spines are sloughed off. x 2,000. Bar 10 µm.* ***Fig. 7*** *Portions of the tegument peeled off. x 600. Bar 20 µm.* ***Fig. 8*** *Complete destruction of microtriches. Conspicuous pits and vacuoles are evident. x 4,000. Bar 5 µm*

0.43 h, respectively, to effectively kill the cestodes. Though the time taken for each concentration of the plant extract to effectively kill the parasite is comparatively longer than that for the reference drug, the results are highly significant. It becomes apparent that the cestocidal activity increases with increased concentration of the plant extract and albendazole.

For scanning electron microscopic observations, worms treated with 20 mg ml^{-1} of *M. pachycarpa* extract were selected since the most obvious alterations were shown at this concentration with compared to the control worms. Scanning electron micrographs of the morphological organization revealed a normal body contour on the control worm (Figs. 1-4). The cestode body is whitish in colour and greatly elongated terminating into a knob-like anterior end termed scolex (Fig. 1). Four suckers are located radially around the proximal end of the scolex. Each circular sucker is marked with an array of short but thick pointed spines along its rim (Fig. 2). Centrally around the four suckers is located a rostellum possessing circularly arranged double set of hammer-shaped hooks. Spines and hooks are the organs of attachment to the host intestinal wall. The body proper called strobila is an elongated ribbon-like structure composed of a series of segments or proglottids (Fig. 3). The entire tegument is densely covered with posteriorly-directed microvillar filaments called microtriches, giving the worm surface a velvety appearance (Fig. 4).

The cestode treated with the plant extract showed irrevocable destruction all over the general topography of the body (Figs. 5-8). The tegumental surface of the scolex was extensively damaged with appearance of cracks and truncations (Fig. 5). The spines were dislocated from the suckers and the surrounding teguments were sloughed off (Fig. 6). The tegument was

extensively damaged throughout the strobila with formation of cracks and pits, with intermittent focal peeling off of the surface (Fig. 7). The microtriches were eroded and clumped into irregularly distributed clustered protuberances (Fig. 8).

On assessment of the survivability and scanning electron micrographs, it becomes apparent that the crude extract of *M. pachycarpa* indeed possessed anthelmintic property against the test parasite, *R. echinobothrida*. The primary evaluation for anticestodal efficacy generally involves observations on the loss of spontaneous movement and/or complete immobilization of the worms upon exposure to the plant extracts in *in vitro* studies, and following, several ethomedicinal plants have been established for their anthelmintic potency. (11, 12, 14–18).

The present study also provides the lethal efficacy of the extract of *M. pachycarpa*, which is associated with severe degenerative effects on the morphological integrity of the helminth parasite. The dose-dependent effect of the plant extract indicated potent mortality on the cestode comparable to that of the standard drug albendazole. Similar anthelmintic activity for several anthelmintic plants used by Naga tribes of the northeast India including *Psidium guajava*, *Houttuynia cordata* and *Lasia spinosa* were demonstrated on different cestodes; while many of the traditionally acclaimed plants did not show significant efficacy (19, 20).

The different concentrations of the plant extract applied in the experiment exerted significant mortality effect ensuing paralysis when compared to the untreated control worms. Although significant mortality was not observed for the lowest concentration (0.5 mg ml^{-1}), still a considerably significant paralysis occurred. It has been well-documented that anthelmintic efficacy is not based exclusively on mortality effects, but immobilization of the worms is often sufficient. In fact, the standard drugs like macrocyclic lactones act via paralytic effect on the parasites within the host intestinal tract. The immobile worms are simply expelled by the peristaltic movement of the host along the faeces (21). In can be inferred from this that the plant material tested herein exhibit significant anthelmintic activity at all level tested.

The parasite tegument has been ascertained as the principal target site of different classes of synthetic drugs and natural anthelmintic products (17, 21–23). The tegument in cetodes serves as a unique interface to the environment and vital functions such as absorption, secretion and sensory reception at the interface occur through it (21, 23). Drugs like albendazole and its related compounds are known to enter the parasite tegument through simple diffusion and then disrupting the tegumental and muscle layers (24, 25). Localized blebbing on the tegument and accumulation of secretory bodies in the tegumental cells have been conspicuously demonstrated (26). Morphological and structural changes caused by anthelmintic agents on different helminths had been well-documented (21, 27–30).

R. echinobothrida treated with the plant extract (20 mg ml^{-1}) clearly showed permanent damages in the structure of the microtriches and affected general disorganization of tegumental appearance; while the microtriches were severely deformed and clumped, the tegument surface exhibited extensive distortion in the form of erosion and vacuolization. Tandon et al. (17) had firmly demonstrated similar destruction using crude tuber root extract of *Flemingia vestia* on *R. echinobothrida*. Isoflavonoids such as genistein had been firmly established as the principal anthelmintics from *F. vestia* acting as potent cestocidal and trematocidal (11, 31). Further, the primary target site is evidently the tegument and the tegumental enzymes appeared to be the vital, if not primary, target of genistein (31). In general, the teguments of cestodes and trematodes, and the cuticle of nematodes are known to be the basic entry route and primary site of activity of anthelmintic drugs (23, 24).

It becomes apparent that the structural deformities observed in the present experiment conform to those that were demonstrated for genistein. *M. pachycarpa* is a rich source of bio-active isoflavonoids, which are demonstrated to exhibit potent anitestrogenic and anticancer activities (9, 10), therefore, it may be speculated that isoflavonoids are the active ingredients responsible for the cestocidal activity of *M. pachycarpa*. However, the specific compound(s) and the precise mode of action behind such anthelmintic activity are not determined from the present study, and remain to be investigated.

CONCLUSION

Results from the present study provide definitive evidence that the ethanolic extract of the root bark of *M. pachycarpa* exhibited significant anthelmintic activity on the cestode, *R. echinobothrida*. The plant extract caused dose-dependent cestocidal effect comparable to the reference drug, albendazole. Scanning electron microscopy revealed extensive

morphological damages on the cestode after treatment with the plant extract. Thus, the data support the plausibility of this plant as an anthelmintic agent. Further investigation is, however, required to ascertain the active compound(s), mode of action and effective dose before proper adoption in human and veterinary use.

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