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Ultrastructural Observations on Tegumental Surface of *Raillietina echinobothrida* and Its Alterations Caused by Root-Peel Extract of *Millettia pachycarpa*

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KEY WORDS cestode parasite; natural product; control; ultrastructure

ABSTRACT *Millettia pachycarpa* Benth (Leguminosae) has a usage in traditional medicine system practiced among the Lushai tribes of Mizoram, a state in North East India, who customarily consume the aqueous extract of the root peel of the plant to get rid of intestinal worm infections. The crude ethanol, methanol, and acetone fractions of the plant were assayed against *Raillietina echinobothrida*, the intestinal cestode parasite of domestic fowl, to authenticate the putative anthelmintic efficacy and cestocidal potential in particular of the plants. In vitro exposure of the worm to the extract at a concentration of 25 mg/mL phosphate buffered saline (at 37°C ± 1°C) revealed distortion and disruption of mitochondria, nucleus, nucleolus, nuclear membrane, basal lamina, and tegumental vacuolization in the distal cytoplasm leading to scar formation in the surface. The possible use of the plant as a potential anthelmintic against cestode parasite is discussed. *Microsc. Res. Tech. 00:000–000, 2008.* © 2008 Wiley-Liss, Inc.

INTRODUCTION

Traditional system of healing from parasitic diseases has been in use with variable success since ancient times and some traditional potions are still used world wide (Dhar et al., 1968; Didier et al., 1988; Goto et al., 1990; Kar et al., 2004; Robinson et al., 1990; Singh et al., 1982; Soh et al., 1980). Lushai people in Mizoram, a north-east state of India, traditionally consume *Millettia pachycarpa*, a leguminous climbing plant, (Family: Leguminosae) to get rid of suspected intestinal helminth infections. The plant is also used as blood purifier or anticancer agent by Chinese people. Earlier stereoscan observations on the surface topography of cestode exposed to crude alcoholic extract of the plant proved the vermicidal properties of the plant (Roy et al., 2007b). The aim of this work was to study the effects of the root-peel extracts of the plant on the cestode *Raillietina echinobothrida*. Ultrastructural alterations in the tegumental layer of the treated worms were taken as the parameters for the study.

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MATERIALS AND METHODS

Preparation of Extract

Fresh peels of root of *M. pachycarpa* were collected from neighboring villages of Aizawl (Mizoram, India). They were washed gently with distilled water to remove any extraneous materials and dried at 50°C in an oven. After grinding, the material was placed in a reflux flask having rectified spirit (100 g/L) for 8 h at 60°C, and the cooled suspension was filtered through Whatman filter paper No.1 to remove the small particulate plant parts and then distilled. Different fractions of the crude alcoholic extract were prepared by dissolving the crude extract with solvents like acetone and methanol in a separating flask, followed by filtration, and then evaporation of solvent in an oven at 50°C.

Experiment

R. echinobothrida were collected in 0.9% phosphate buffered saline (PBS) from freshly sacrificed fowl at local abattoirs. The live worms were incubated at 37°C ± 1°C with 5, 10, and 25 mg of crude and fractions of crude extract/mL of PBS (three replicates for each concentration) in 0.1% dimethylsulfoxide (DMSO). Control incubation consisted of worms in PBS with 0.1% DMSO only. Praziquantel was used as the reference cestocidal drug in concentrations similar to those used for the crude extract. The time taken for the complete inactivation of the parasites was recorded and the death was confirmed by dipping such worms in slightly warm water. Soon after paralysis, one specimen from different concentrations of different fractions along with one set of control was fixed in 4% gluteraldehyde buffered with 0.1 M sodium cacodylate (pH 7.4) at 4°C for 4 h, followed by secondary fixation in 1% osmium tetraoxide in the same buffer for 1 h at 4°C. Specimens were dehydrated in graded series of acetone, cleared in propylene oxide, and embedded in araldite in beem capsules. Pieces of plastic embedded tissue were reoriented, and ultrathin sections (600–900 Å) were cut on an RMC ultra microtome, MT-X, with a diamond knife. The specimens were collected on an uncoated copper grids, stained with 5% aqueous uranyl-acetate solution for 10 min at 40°C followed by lead citrate (Reynolds, 1963) and examined with a JEM 100 CXII transmission electron microscope (Jeol) at an electron acceleration voltage of 80 kV.

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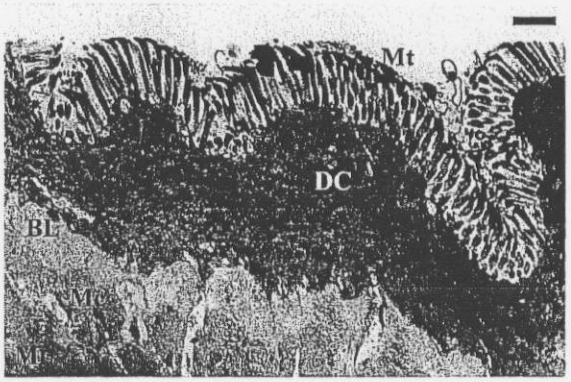

Fig. 1. Transmission electron micrograph of *R. echinobothrida*—Control. Tegument with intact microthrix layer (Mt); distal cytoplasm (DC) electron-dense with tegumental discs, nondisrupted basal lamina (BL), and well organized subtegumental circular and longitudinal muscle blocks (Mc), (ML). Scale bar 0.5 μm.

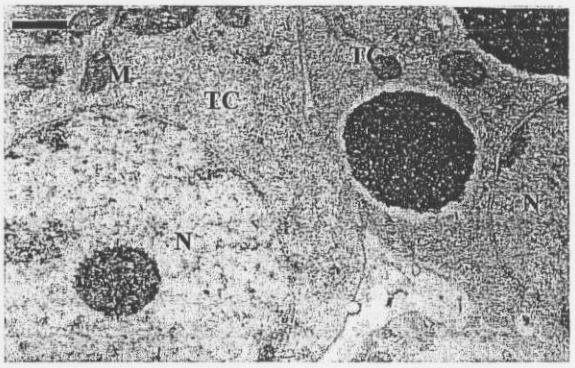

Fig. 3. Transmission electron micrograph of *R. echinobothrida*—Control. Tegumental cytons (TC) retaining normal connections with each other and having abundant GER and mitocondria (M) and other cell inclusions; nucleus with no clumping of chromatin. Scale bar 0.5 μm.

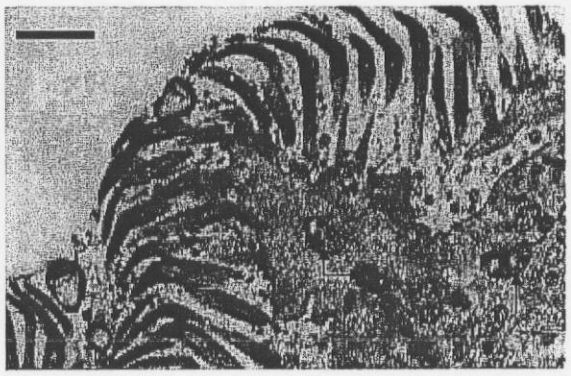

Fig. 2. Transmission electron micrograph of *R. echinobothrida*—Control. Magnified view of the microthrix layer along with the distal cytoplasm showing transport of food transtegumentally in pinosomes (arrows). Scale bar 0.5 μm.

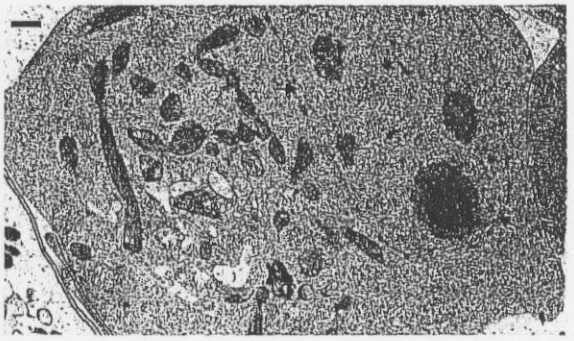

Fig. 4. Transmission electron micrograph of *R. echinobothrida*—Control. Tegumental cyton showing a prominent nucleus having a double layered membrane (arrows). Mitochondria and other cell organelles can also be seen to be in abundance within the cytoplasm. Scale bar 0.5 μm.

RESULTS

The control worms maintained in PBS showed physical activity up to 64–72 h, following which they became immobilized. The worms exposed to 5, 10, and 25 mg of ethanol, methanol, and acetone extract of the plant per mL of PBS became paralyzed taking 3.5–4.6, 2.8–4.0, 1.2–1.6; 5.2–5.8, 3.1–3.5, 1.8–2.4; and 3.0–3.26, 2.4–3.1, 1.3–2.3 h, respectively. The worms treated with 25 mg extract/mL of PBS were selected for an observation because of the early lethal effect of the dose when compared with the other low concentrations. The untreated controls revealed a normal tegumental morphology with densely packed unidirectional and conical microtriches having glycocalyx cover (Fig. 1). Surface invaginations forming pinosomes were distinct in the distal cytoplasm. Some of the food vacuoles were also found to be attached to lysosomes in the syncytial mass (Fig. 2). In the portion between glycocalyx surface membrane and the basal lamina lay the anucleate syncytium having secretory bodies, mitochondria, and mucopolysaccharide mass. Inner plasma membrane was seen clearly attached to the basal lamina. Below the basal lamina lay the circular and longitudinal muscle layers. Cell bodies, also known as tegumental cytons, consisted of smooth and rough endoplasmic reticulum, Golgi complex, as well as secretory bodies. Nuclei with double-layered nuclear membrane, nucleolus, granular nucleoplasm, and chromatin bodies were also observed to be present (Figs. 3 and 4). Mitochondria retained their normal configuration with an intact mitochondrial membranes and prominent cristae (Fig. 5).

When treated with 25 mg crude ethanol extract per mL of PBS, the syncytial layer appeared more electron lucent when compared with the untreated controls (Fig. 6). The tegument had stripped off along with the basal lamina and the muscle layer beneath it was cracked up at many places (Fig. 7). The nuclei appeared misshapen and the karyoplasm was lysed in some regions of the nucleus. There were chromatin

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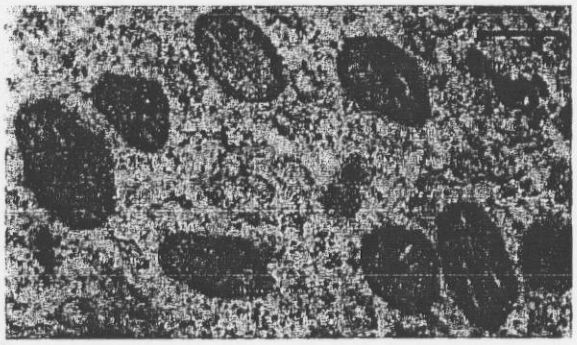

Fig. 5. Transmission electron micrograph of *R. echinobothrida*—Control. Electron-dense mitochondrial matrix with prominent cristae. The mitochondria here are seen to lie in a granular parenchyma suggesting the presence of abundant glycogen. Scale bar 0.5 μm.

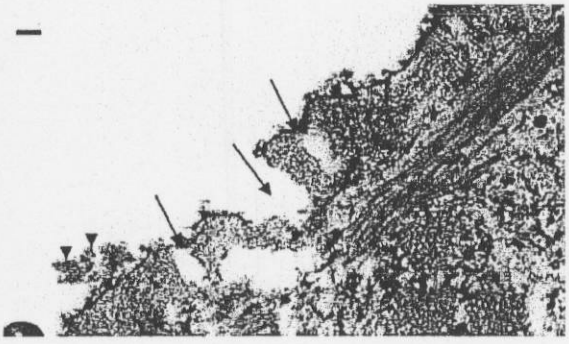

Fig. 7. Transmission electron micrograph of *R. echinobothrida* treated with crude alcoholic extract of *M. pachycarpa* at 25 mg/mL. Magnified view of the tegument showing the tear in the muscle layers (arrows) and the remnants of the basal lamina left behind (arrowheads). Scale bar 0.5 μm.

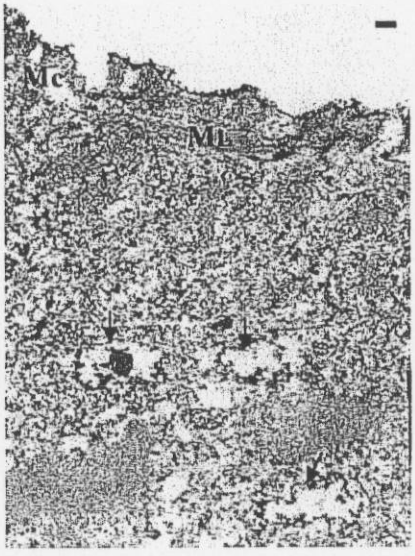

Fig. 6. Transmission electron micrograph of *R. echinobothrida* treated with crude alcoholic extract of *M. pachycarpa* at 25 mg/mL. Stripping up of tegument down to the muscle layer. The circular muscle blocks can be seen to be torn up, whereas the longitudinal muscles are stacked up loosely verging on loss of integrity. Nuclei swollen up and misshapen (arrows) with blotches of heterochromatin. Scale bar 0.5 μm.

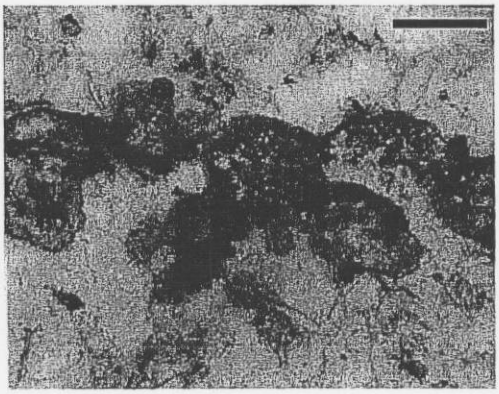

Fig. 8. Transmission electron micrographs of *R. echinobothrida* treated with crude alcoholic extract of *M. pachycarpa* at 25 mg/mL. Severe dilation and vesiculization of the cristae and inner matrix of mitochondria. The rupture of the mitochondrial membrane is to be noted. Scale bar 0.5 μm.

aggregations scattered throughout the karyoplasms (showing cell inactivity) or at the nuclear margin of the nuclei, whereas many did not have any chromatin material at all. Disruption and disintegration of nuclear membrane was observed at many places. At some places, the cytoplasm was completely free from inclusions. Mitochondria of the tegument and subtegumental layer increased in size and this increase in size was associated with severe dilation and vesiculization of their cristae and inner matrix. The mitochondrial membranes had sloughed off and/or ruptured releasing their contents (Fig. 8). The normal surface texture of the general tegument was completely lost because of shedding off of the microtriches and consequent scar and pit formations.

Worms treated with the acetone fraction of the crude extract showed severe vacuolization and disruption even after the shortest incubation time in the lowest drug concentration (Figs. 9–11). The methanol fraction of the plant caused the microtrich layer to strip off from the tegument (Fig. 12) and brought about intense loss of glycogen. The cellular components otherwise remain visible though the nuclei appear swollen and the mitochondria seem to be slightly extracted (Fig. 13).

DISCUSSION

In cestodes, the general body surface and microtriches are known as vital structures in terms of nutrient uptake, immunoprotection, osmoregulation, and sensation. Both of these structures were found to be

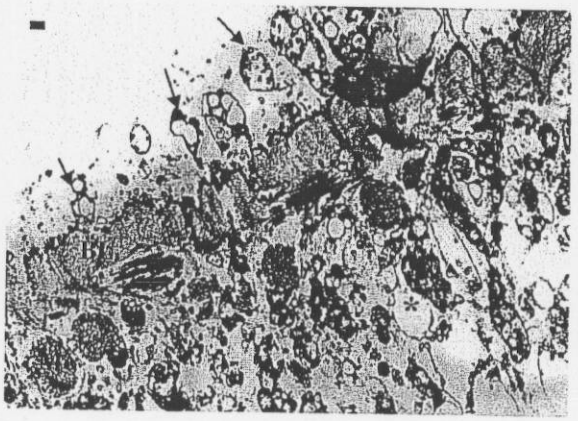

Fig. 9. Transmission electron micrograph of *R. echinobothrida* treated with acetone fraction at 25 mg/mL. Depletion of the tegument leaving the basal lamina exposed. Intense vacuolization (*) of the parenchyma along with lack of orientation of the filaments of muscle fibers. Arrows show the remnants of the distal cytolasm adhering to the basal lamina. Scale bar 0.5 μm.

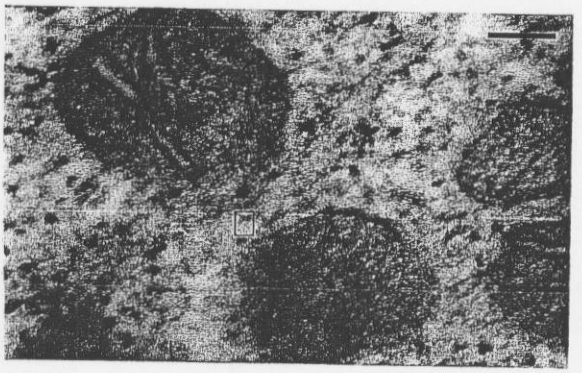

Fig. 11. Transmission electron micrograph of *R. echinobothrida* treated with acetone fraction at 25 mg/mL. Ruptured mitochondria with very few cristae. Scale bar 0.5 μm.

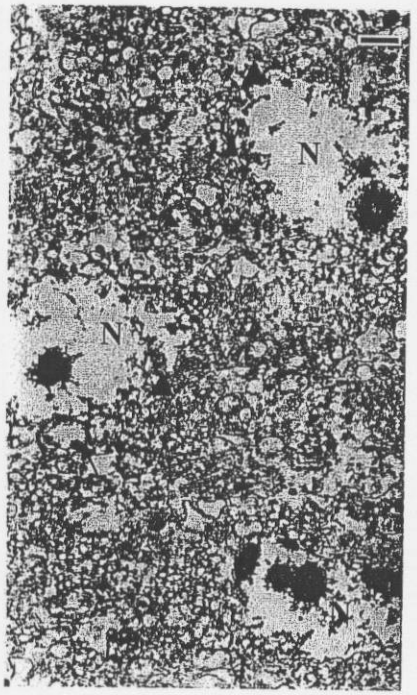

Fig. 10. Transmission electron micrograph of *R. echinobothrida* treated with acetone fraction at 25 mg/mL. Distortion of the nuclei and cytoplasm along with the disappearance of cell organelles. The cytons show no proper boundary and mitochondria appear to be mildly extracted (arrows). Scale bar 0.5 μm.

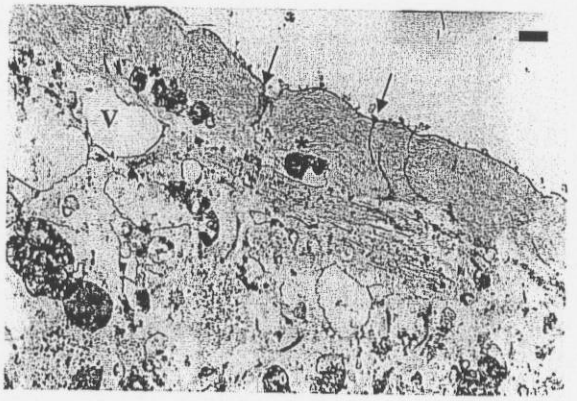

Fig. 12. Transmission electron micrograph of *R. echinobothrida* treated with methanol fraction at 25 mg/mL. Intense loss of glycogen and accumulation of debris in the vacuoles lying beneath the basal lamina (*). Scale bar 0.5 μm.

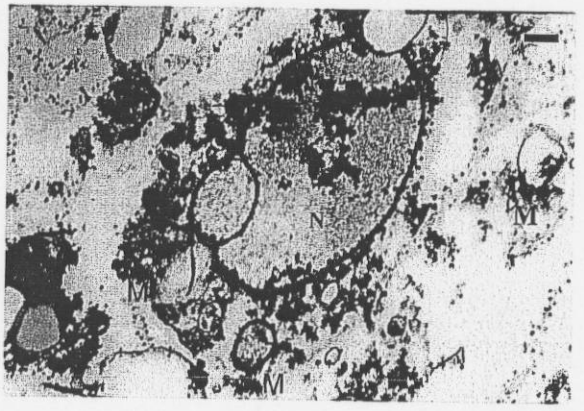

Fig. 13. Transmission electron micrograph of *R. echinobothrida* treated with methanol fraction at 25 mg/mL. Extremely swollen and vesiculated nucleus with extracted mitochondria (M) lying in the cytoplasm. Scale bar 0.5 μm.

affected and altered by the test plant materials. Earlier stereoscan observations on the surface alteration of the parasite exposed to the crude alcoholic extract of the plant revealed contraction, scar, and pit formation through out the body surface including suckers of the

worm (Roy et al., 2007b). Similar to this observations, destruction of the tegument was also caused by synthetic drugs like praziquantel and oxyclozanide, and alcoholic extract of several botanicals in cestodes and trematodes (Roy and Tandon, 1996, 1999; Roy et al., 2007a; Tandon et al., 1997). It is established that contraction and disintegration of flat worms surface tegument treated with praziquantel are closely related to the disturbances in ion flux across the membrane, whereas necrosis of the worm tissue is due to altered membrane transport and osmoregulation together with accelerated autophagy and myelin degeneration (Gorchilova and Spaldonova, 1988).

Densely packed rod-shaped and lamellate secretary bodies as observed in the control/normal worms are known to be responsible for renewal of glycocalyx layer of tegument and supplying of raw materials for microtriches synthesis, respectively (Lumsden et al., 1974; Oaks and Lumsden, 1971). A drastic reduction in the number of both rod-shaped and lamellate secretary bodies in the extract treated worm may be correlated with the degeneration and disintegration of two vital structures, namely, the glycolcalyx layer (protective) and microtriches (absorptive), respectively. Further, disintegration of the glycocalyx layer can also be correlated with deformation and disintegration of perinuclear Golgi apparatus which is responsible for the synthesis of the former (Lumsden, 1975).

An intact surface tegument is a prerequisite for smooth ion-transfer with the help of tubular projections of internal plasma membrane of basal lamina toward distal cytoplasm (Morris and Finnegan, 1969; Threadgold and Read, 1970). However, an increase in the tubular projection of inner plasma membrane, as observed in the treated worms, may be an adaptation for more ion transfer from distal cytoplasm toward perinuclear cytoplasm.

This observations clearly indicate that the tegument of *R. echinobothrida* is sensitive to alcoholic crude extract of *M. pachycarpa*. After in vitro incubation with different fractions of the crude extract, the parasite showed similar effects consisting of disruption of the tegument by vacuolization, swollen and disrupted basal lamina, mitochondria, and other cell organelles. However, there are clear differences in the severity of the effect. Worms treated with acetone fraction of the crude extract showed severe vacuolization and disruption even after the shortest incubation time in the lowest drug concentration.

A variety of agents have been found to affect the structure and morphology of cestode and trematodes (Mehlhorn et al., 1983; Pal and Tandon, 1998). Genistein, an active component of *Flemingia vestita*, has been found to induce vacuolization and tegumental disruption in *Raillietina* sp. through suppression of the activity of tegumental enzymes like acid phosphatase, alkaline phosphates, adenosine triphosphatase, and 5′-nucleotidase (Pal and Tandon, 1998). A remarkable level of increase of neurotoxic substances like ammonia and neuromodulatory amino acids namely γ-amino-butyric acids and citrulline were observed in *Fasciolopsis buski*, an intestinal trematode, when treated with genistein (Kar et al., 2004). Further, the chemical is also found to be responsible for alteration of several enzymes of glucose and glycogen metabolism in *R. echinobothrida* (Das et al., 2004a,b; Tandon and Das, 2007; Tandon et al., 2003). Because genistein is also reported to be present in *M. pachycarpa* (Okamoto et al., 2006), probably the chemical suppresses tegumental enzyme activities in *R. echinobothrida* in a similar way leading to deformation and disruption of its tegument. Genistein is also known as specific inhibitor of tyrosine-specific protein kinase (Akiyama et al., 1987). Through in vitro experiment it has been proven that crude extracts of *M. pachycarpa* inhibit DNA polymerase activity (Ono and Nakane, 1989). Okamoto et al. (2006) isolated eight chemicals from *M. pachycarpa* namely, genistein, daidzein, wighteone, alpinumisoflavone, warangalone, auriculasin, 6,8-diprenylorobol, furowanin A, isoerysenegalensein E, and erysenegalensein E. All of these except genistein showed antiestrogenic activity, whereas genistein showed estrogenic activity on ligand-dependent yeast-two hybrid assay.

Mehlhorn et al. (1983) showed that the site of origin of vacuoles was the basal lamina in digenetic trematodes, whereas, in monogeneans it was the surface of the tegument where the vacuoles originates (Schmahl and Mehlhorn, 1985). Disruption of tegumental integrity leading to vacuolization or pit formation is the result of disturbances in ion (Ca^{2+}) flux across the parasite membrane and this was established in different flat worms (Bricker et al., 1982; Mehlhorn et al., 1983; Schmahl and Mehlhorn, 1985). Vacuolization of subtegumental and tegumental layer and erosion of microtriches, as observed in this study, disturbs the tegumental absorption of food and also the ion balance thus making the worm susceptible to the host's defense system.

The crude alcohol, methanol, and acetone extracts of *M. pachycarpa* thus seem to have vermifugal/vermicidal effect. However, detailed biochemical studies on the parasite involving all the known chemicals recovered from the plant is a prerequisite to know the exact chemical/chemicals responsible for anthelmintic activities and their precise mode of action.

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