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In vitro anthelmintic activity of root-tuber extract of *Flemingia vestita*, an indigenous plant in Shillong, India

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Abstract The in vitro activity of root-tuber-peel extract of *Flemingia vestita*, an indigenous plant consumed by the natives in Northeast India, was tested against helminth parasites. Live parasites (nematode: *Ascaris suum* from pigs, *A. lumbricoides* from humans, *Ascaridia galli* and *Heterakis gallinarum* from domestic fowl; cestode: *Raillietina echinobothrida* from domestic fowl; trematode: *Paramphistomum* sp. from cattle) were collected in 0.9% physiological buffered saline (PBS) and maintained at 37 ± 1 °C. In vitro treatment of the parasites with the crude extract (50 mg/ml) in PBS revealed complete immobilization of the trematode and cestode in about 43 and 20 min, respectively. However, the cuticle-covered nematodes did not show any change in physical activity and remained viable even after a long period of exposure to the extract. Exposure of *R. echinobothrida* to genistein (0.5 mg/ml), an active principle isolated from the root-tuber peel, caused spontaneous loss of movement (paralysis) in 4.5 h, which was slower than the time required for praziquantel, the reference flukicide and cestodicide. The treated parasites showed structural alteration in their tegumental architecture. This study suggests the vermifugal activity of this plant extract against trematodes and cestodes.

Introduction

A number of plants have been tested for their anthelmintic efficacy (Dhar et al. 1968, 1973; Dhawan et al. 1980; Aswal et al. 1984; Bhakuni et al. 1988, 1990; Chhabra et al. 1990; Robinson et al. 1990; Akhtar and Riffat 1991; Akhtar and Ahmad 1992; Chatterjee et al. 1992; Than et al. 1993; Chakrborty et al. 1995). In Meghalaya (Northeast India), numerous indigenous plants are used by the natives, who believe them to be curative against worm infections (Rao 1981). *Flemingia vestita* Benth and Hooker, locally known as Sohphlang, is a leguminous root crop commonly found in the north-eastern region of India. Its fleshy tuberous roots along with the peel are consumed raw by the Meghalayan local tribal people to cure intestinal helminth infections. In a preliminary study the crude extract of the whole root tubers of this plant was reported to be effective against *Ascaris suum* in vitro (Yadav et al. 1992). Tegumental alterations and deformity were also observed in digenean flukes treated with the crude peel extract of the roots (Roy and Tandon 1996). The active principles of the root-tuber peels have been isolated and purified by Rao and Reddy (1991); genistein, an isoflavone, is the main component identified. The aim of the present work was to study the effects of the tuber-peel extract and active principles of this plant material on helminth parasites. Changes in physical motility and histomorphological alterations in the treated worms (at the light and electron microscope level) were the parameters for this investigation.

Materials and methods

Extract from *Flemingia vestita*

Fresh tuberous roots of *F. vestita* were collected in October-November from neighboring villages of Shillong (Meghalaya). They were washed thoroughly with water and thinly peeled. The peels were dried at 50°C in an oven and placed in rectified spirit (100 g/l) in a reflux flask. After reflux for 8 h at 60°C, the cooled suspension was filtered and distilled for removal of the solvent. In all, 28 g crude extract was obtained from 100 g dried peels.

Purified compound

The purified active component was obtained from the crude extract

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extract was mix supernatent solution was decanted. This process was repeated 20 times, and the extract was reduced in volume through distillation and passed through a silica-gel column using hexane, benzene, and ethyl acetate as solvents. The column was prepared using a hexane and benzene mixture (6:4) and silica gel of 100–200 mesh (about 350 g). The sample (10 g) was mixed with silica gel of 60–120 mesh and loaded on top of the column. Elution was done with a hexane : benzene mixture (ratio 6:4, 5:5, ... to pure benzene) and thereafter with benzene : ethyl acetate (ratio 9:1, 8:2, 7:3, ... to pure ethyl acetate) using about 300 ml of mixed solvent at each ratio.

At a benzene : ethyl acetate ratio of 7:3, genistein was eluted. Genistein solution was concentrated through distillation in a water bath and collected as pure genistein (approx. 15 mg). The residue (i.e., crude extract minus genistein) was eluted with methanol and contained a mixture of other isoflavones, namely, a formononetin and pseudobaptigenin mixture and diadzein (Rao and Reddy 1991). In all, 6.5 g residue was obtained from 10 g crude extract. Synthetic genistein (Sigma, code G6649) was used in addition to the pure genistein extracted from the root peel. Praziquantel was used as the reference substance.

Parasites and hosts

Live parasites (nematode: *Ascaris suum* from pigs, *A. lumbricoides* from humans, *Ascaridia galli* and *Heterakis gallinarum* from domestic fowl; cestode: *Raillietina echinobothrida* from domestic fowl; trematode: *Paramphistomum* sp. from cattle) were collected in 0.9% phosphate-buffered saline (PBS; 8 g NaCl, 0.34 g KH_2PO_4, and 2.1 g K_2HPO_4 in 1 l of distilled water, pH 7–7.3) from freshly slaughtered hosts at local abattoirs.

Experiments

The above-mentioned adult nematode and platyhelminth parasites were incubated at 37 ± 1°C in media containing no extract (control) or crude extract at 0.5, 1, 2, 5, 10, 20, 40, and 50 mg/ml and residue at 10, 20, 40, and 50 mg/ml in PBS supplemented with 1% dimethylsulfoxide (DMSO). All the parasites except for *Ascaris* spp. were also tested against pure genistein (0.2 and/or 0.5 mg/ml). *R. echinobothrida* was further tested against the formononetin-pseudobaptigenin mixture (0.5 mg/ml) and 0.001-, 0.005-, and 0.1 mg/ml concentrations of praziquantel (Droncit; Bayer, Leverkusen). Three replicates were used for each concentration. The time required for complete inactiveness or paralysis and death of the parasite was recorded. After exposure to the treatment the paralyzed cestode (treated with peel extract at 50 mg/ml and genistein at 0.5 mg/ml) was processed for microscopy along with one set of control specimens (maintained in 1% DMSO in PBS).

Light and electron microscopy

The parasites were fixed in 3% glutaraldehyde buffered with 0.1 *M* sodium cacodylate, (pH 7.2) at 4°C for 4 h. The samples were postfixed in 1% OsO_4 in the same buffer and were then dehydrated in graded acetone, transferred to propylene oxide, and embedded in Araldite. Semithin sections measuring 1 µm in thickness were stained with toluidine blue for light microscopy. For transmission electron microscopy (TEM), ultrathin sections were stained with uranyl acetate and lead citrate and studied in a JEOL-JEM-100 CXII transmission electron microscope.

For scanning electron microscopy (SEM) the material fixed in 10% neutral buffered formalin at 4°C for 4 h was repeatedly washed in double-distilled water, dehydrated through acetone, critical-point-dried using liquid CO_2, metal-coated with gold-palladium, and viewed in a JEOL-JSM-35 CF scanning electron microscope at an electron accelerating voltage of 10–15 kV.

Results

Treatments

The mode of treatment and observations concerning different species of parasites are given in Tables 1 and 2. The results indicate that the crude extract of *Flemingia vestita* showed more activity on trematode and cestode worms than on nematode parasites. All the nematode species except for *Heterakis gallinarum* showed a negligible effect of the treatment as they survived for a considerably long period in the presence of varying concentrations of the crude extract. The survival time of the residue-treated worms was longer than that of those treated with genistein-containing crude extract (Table 1). *Paramphistomum* sp. showed a complete loss of movement after varying intervals, depending on the concentration of the test medium, but did not indicate any revival when removed from the medium, indicating that paralysis did not precede death in this case (Table 2). However, in contrast, the cestode, *Raillietina echnibothrida* initially became paralyzed in the test medium. The time taken for paralysis showed an orderly decline; whereas paralysis occurred after about 25 h of incubation in a 0.5 mg/ml concentration of crude extract, at 50 mg/ml it took only 20 min to become evident. After being removed from the test medium and dipped in slightly warm water and on gentle stimulation, the paralyzed parasite regained considerable motility and death occurred after 6.5 h of incubation at 37°C (Table 2). The residue extract showed less activity against both trematode and cestode parasites as compared with the crude extract containing genistein.

In view of the apparently conspicuous effect on the motility and physical state of *R. echinobothrida*, further experiments were performed only with cestodes. Exposure of *R. echinobothrida* to genistein (0.5 mg/ml) caused paralysis within about 4.5 h, which was much faster than the time required for the formononetin-pseudobaptigenin mixture but slower than that required for praziquantel (Table 2).

SEM observations

Control specimens

The whole-body tegument is densely covered with fine microtriches, giving it a velvety appearance (Figs. 3, 5). The entire surface of the scolex, including the tegument of the suckers, is covered with dense microtriches (Figs. 1, 2). Those in the neck region are thin and slender and have pointed ends (Fig. 3). The strobilar surface (from immature to gravid proglottides) is covered with thick, unidirectional conical microtriches that are wider at the base and taper slightly toward the tip (Fig. 5).

Table 1 Efficacy of whole crude peel extract; residue, i.e., crude extract minus genistein; and genistein of *Flemingia vestita* on *Ascaris lumbricoides, A. suum, Ascaridia galli,* and *Heterakis gallinarum* in vitro

Treatment: concentration (mg/ml)	Time (h) taken for complete loss of movement[a]			
	A. lumbricoides	*A. suum*	*A. galli*	*H. gallinarum*
Crude extract:				
0.5	240.4 ± 0.04	335.08 ± 0.04	144.4 ± 0.05	120.1 ± 0.04
1.0	215.4 ± 0.04	300.6 ± 0.06	131.2 ± 0.1	84.1 ± 0.04
2.0	204.6 ± 0.06	288.2 ± 0.2	120.6 ± 0.06	71.1 ± 0.2
5.0	192.4 ± 0.04	276.4 ± 0.2	120.2 ± 0.2	48.1 ± 0.06
10.0	168.5 ± 0.3	264.6 ± 0.06	108.08 ± 0.04	35.08 ± 0.3
20.0	–	192.7 ± 0.06	96.1 ± 0.2	23.1 ± 0.5
40.0	–	96.1 ± 0.01	84.3 ± 0.2	18.08 ± 0.5
50.0	–	84.6 ± 0.05	71.1 ± 0.04	16.4 ± 0.06
Residue:				
10.0	–	–	130.5 ± 0.25	47.2 ± 0.1
20.0	–	–	109.2 ± 0.11	33.5 ± 0.17
40.0	–	–	99.4 ± 0.18	23.0 ± 0.35
50.0	–	–	86.7 ± 0.09	21.7 ± 0.2
Genistein:				
0.5	–	–	108.0 ± 0.1	10.5 ± 0.15

[a] Data represent mean values ± SD for three experiments; Student's *t*-test insignificant. Parasites incubated in control medium showed physical activity as follows: *A. lumbricoides* 288 ± 0.08 h, *A. galli* 168 ± 0.08 h, *A. suum* 384 ± 0.1 h, *H. gallinarum* 120 ± 0.04 h (– Not tested)

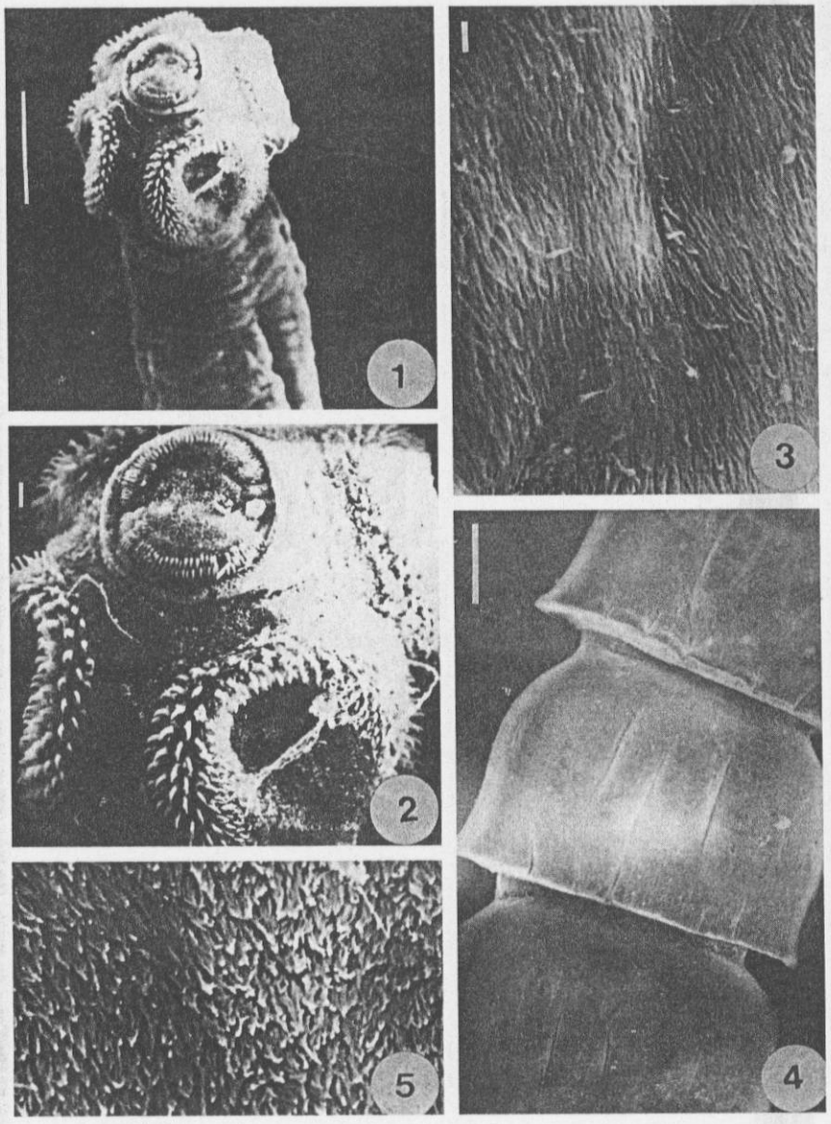
Table 2 Efficacy of whole crude peel extract; residue extract, i.e., crude extract minus genistein; and active components of *F. vestita* and reference drug on *Paramphistomum* sp. and *Raillietina echinobothrida* in vitro

Treatment: concentration (mg/ml)	Time (h) taken for paralysis (P) and death (D) of the worm postincubation[a]		
	Paramphistomum sp. D	*R. echinobothrida* P	D
Crude extract:			
0.5	13.5 ± 0.08	25.1 ± 0.1	78.08 ± 0.5
1.0	3.2 ± 0.08	16.08 ± 0.6	65.5 ± 0.5
2.0	2.1 ± 0.07	9.2 ± 0.5	51.2 ± 0.5
5.0	1.7 ± 0.02	5.9 ± 0.5	46.1 ± 0.04
10.0	1.3 ± 0.02	4.08 ± 0.10	33.1 ± 0.6
20.0	1.1 ± 0.008	2.03 ± 0.08	28.1 ± 0.5
40.0	0.8 ± 0.02	0.9 ± 0.01	18.08 ± 0.5
50.0	0.7 ± 0.02	0.3 ± 0.01	6.5 ± 0.4
Residue:			
10.0	4.08 ± 0.05	8.15 ± 0.15	46.15 ± 0.14
20.0	3.16 ± 0.17	3.5 ± 0.05	39.2 ± 0.08
40.0	1.58 ± 0.08	2.35 ± 0.12	29.4 ± 0.07
50.0	1.31 ± 0.11	2.1 ± 0.06	13.35 ± 0.11
Genistein:			
0.2	5.92 ± 0.11	6.7 ± 0.04	49.2 ± 0.08
0.5	3.04 ± 0.09	4.4 ± 0.07	19.08 ± 0.02
Formononetin – pseudobaptigenin mixture:			
0.5	–	29.2 ± 0.03	47.4 ± 0.05
Praziquantel (Droncit):			
0.001	–	2.9 ± 0.05	9.8 ± 0.17
0.005	–	0.89 ± 0.04	7.9 ± 0.19
0.01	–	0.47 ± 0.07	6.1 ± 0.34

[a] Data represent mean values ± SD for three experiments. Student's *t*-test insignificant. Worms incubated in control medium showed physical activity as follows: *Paramphistomum* sp. 48 ± 0.02 h, *R. echinobothrida* 72 ± 0.05 h (– Not tested)

Treated worms

In vitro exposure of the parasite in media containing crude extract at 50 mg/ml caused distinct damage to the whole-body surface within 20 min. The change/damage was apparent over the whole worm (Figs. 6–9). While the surface of the worm appeared distorted, the general tegument appeared distorted. The microtriches all through the body exhibited a clumpy appearance, with precipitate-like lumps covering them (Fig. 9). The tip of the microtriches appeared somewhat swollen, blunt, and stouter after treatment with genistein at a concentration of 0.5 mg/ml in the PBS medium



Figs. 1–5 Scanning electron micrographs of *Raillietina echinobothrida* – control. **Fig. 1** Anterior region, showing the scolex and neck region. *Bar* 100 µm. **Fig. 2** Enlargement of the scolex, showing two rows of rostellar hooks in the rostellum and suckers with spined rims. *Bar* 10 µm. **Fig. 3** Neck region. *Bar* 10 µm. **Fig. 4** Gravid proglottides. *Bar* 100 µm. **Fig. 5** Microtriches in the mature proglottid. *Bar* 1 µm.

TEM Observations

Controls

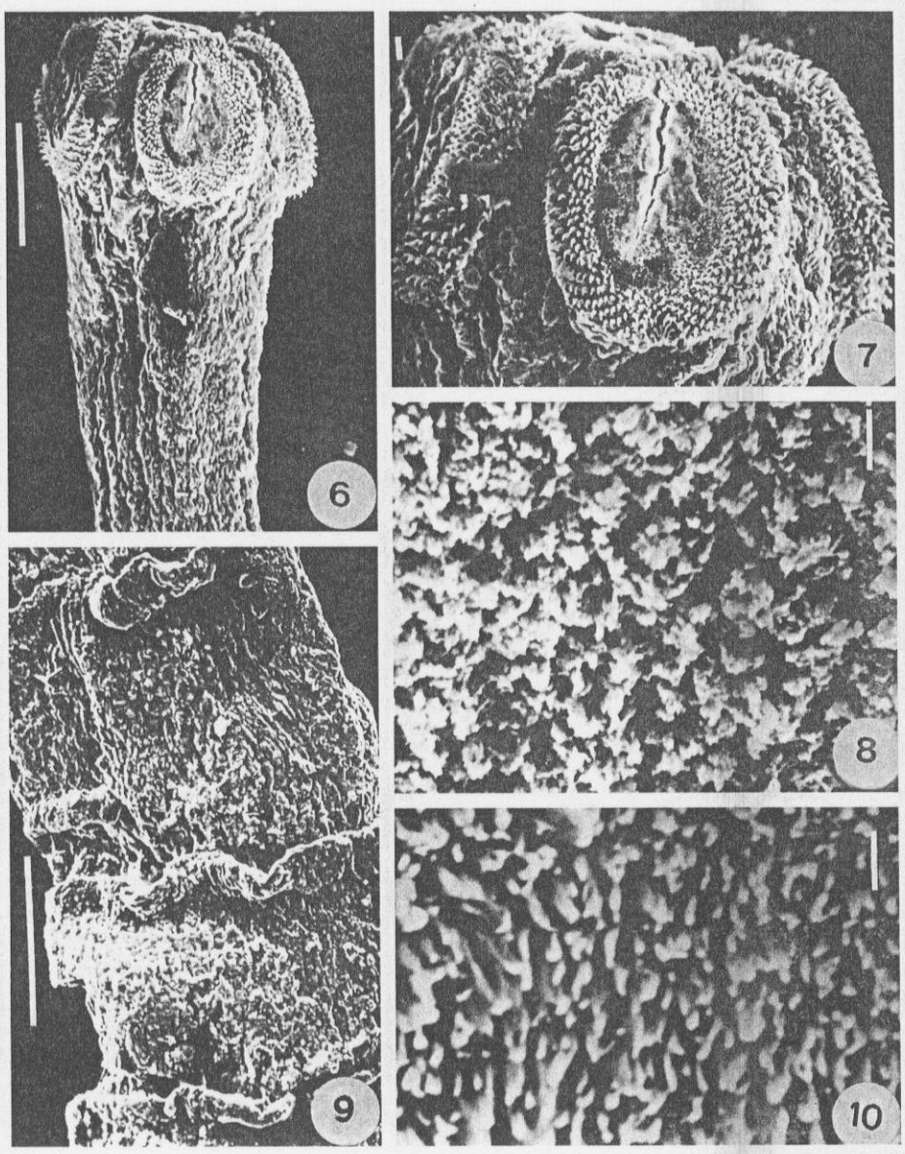
Histologically, the body of the cestode is covered with a thin tegument. The tegumental and subtegumental regions exhibit dense granules and vesicular bodies (Figs. 11, 13). The tegumental musculature is conspicuously developed (Fig. 13).

Treated worms

At the histological and ultrastructural levels, changes were observable in the tegumental organization. Conspicuous vacuolization of the tegument became obvious after 20 min of incubation in media containing crude extract at 50 mg/ml (Figs. 12–14). Vacuolization became more pronounced after 60 min of incubation, indicating a time-dependent effect. The subtegumental region showed severe distortion with disorganization of the tegumental musculature (Fig. 14).

Discussion

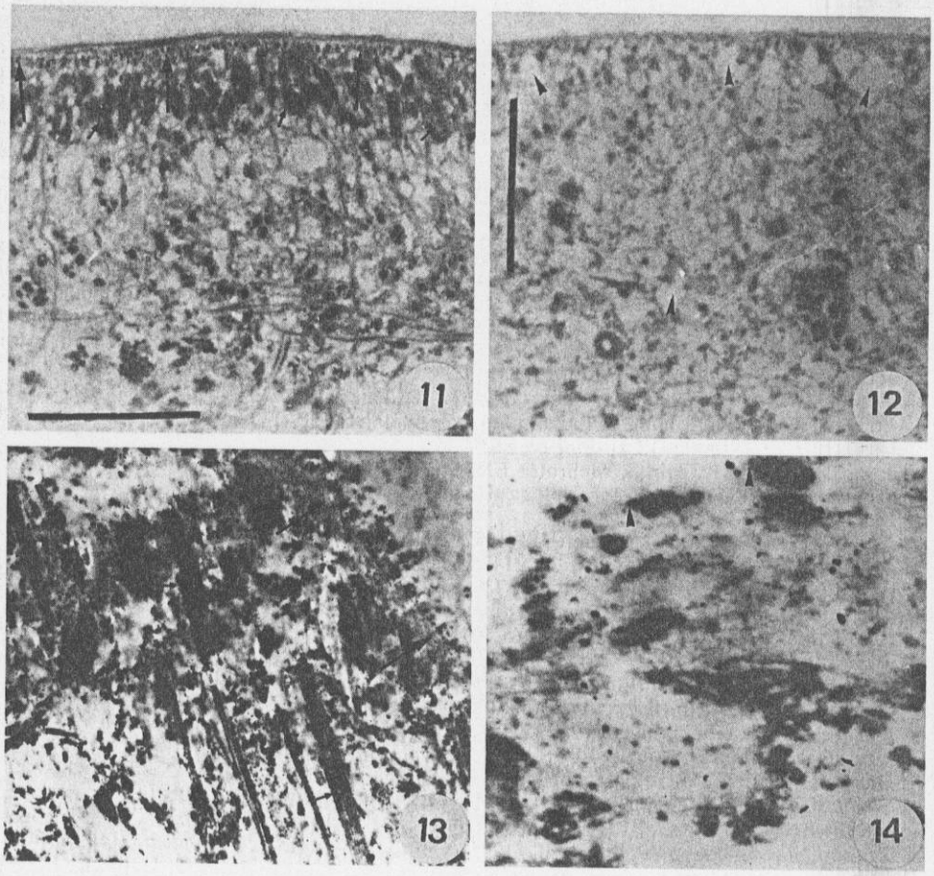
The efficacy of plant materials has been judged on the basis of the loss of spontaneous movement and/or complete destruction of the worm in in vitro studies (Goto et al. 1990; Robinson et al. 1990; Togo et al.1992). The present investigation indicates that the nematode species tested are less sensitive to the root-tuber-peel extract of *Flemingia vestita,* since they could survive in the test medium for considerably long periods. Whereas the longer nematode species survived for about 3 days



Figs. 6–9 Scanning electron micrographs of *R. echinobothrida* treated with crude root-tuber-peel extract of *Flemingia vesita* at 50 mg/ml. **Fig. 6** Deformity in the anterior region. The scolex and neck region are evident. *Bar* 100 μm. **Fig. 7** Close view of the scolex, showing cracks in the tegument of the sucker. *Bar* 10 μm. **Fig. 8** Tegumental surface of the gravid proglottid after exposure. *Bar* 100 μm. **Fig. 9** Surface of the mature proglottid. Microtriches are conspicuously clumped. *Bar* 1 μm. **Fig. 10** Tegumental surface of the mature region after treatment with genistein at 0.5 mg/ml. A change in the contour of microtriches (in comparison with controls) is noticeable. *Bar* 1 μm

nematode *Heterakis gallinarum* could survive for more than 16 h at the extract concentration of 50 mg/ml. In a previous study the whole root-tuber ethyl acetate extract (the peel and the fleshy part together) of *F. vestita* (0.36%) was found to be effective against *Ascaris suum* since a decrease in motility followed by paralysis within 5–8 h and disorganization of the cuticle and body musculature in the treated worms were observed (Yadav et al. 1992). It may be that some active principles that might be present in the root-tuber pulp are lost during the extraction procedure. The peel extract appears to be effective against *Paramphistomum* sp., since within a considerably short interval, complete inactivation and irreversible immobilization was observed in the treated worms, indicating a lethal effect on the parasite.

The cestode proved to be the most obviously affected parasite, in which paralysis could ensue soon after in- curred after a long delay. Exposure of the cestode parasites to various concentrations of the crude extract and a 0.5-mg/ml concentration of genistein did not cause any mortality for some time, and acquisition of the paralytic state was also not irreversible. It might be suggested that even though the plant products may not have a cestodicidal effect, they may be vermifugal in nature and the inactiveness caused would last long enough for the parasites to be swept out of the host's body (Cox 1982). In comparison with the other active component, i.e., the formononetin-pseudobaptigenin mixture, the genistein component appeared quite efficacious at a concentration of 0.5 mg/ml, causing paralysis of worms after about 4.5 h; however, though it required about 19 h to kill them, the other component at the same concentration began to induce a paralytic state only after 29 h. The paralyzing effect of praziquantel



Figs. 11, 12 *R. echinobothrida* – semithin sections, light microscopy. **Fig. 11** Section of a mature proglottid (control), showing normal tegumental and subtegumental organization. Densely staining muscular components (*large arrows*) and subtegumental cells (*small arrows*) are abundant near the surface. *Bar* 0.05 mm. **Fig. 12** Section of a mature proglottid after incubation with crude peel extract at 50 mg/ml. Note the occurrence of numerous vacuoles (*arrowheads*) in the tegument and the loss of densely staining components near the surface. *Bar* 0.05 mm.

Figs. 13, 14 Transmission electron micrographs of *R. echinobothrida*.

Fig. 13 Tegument of a control worm. The cytoplasmic zone (*large arrows*) and abundant tegumental musculature (*small arrows*) are conspicuous. × 4000.

Fig. 14 Section through the tegument of a parasite exposed to crude extract at 50 mg/ml for 1 h. Complete disorganization of the surface layer is evident (*arrowheads*). × 4000

0.001 mg drug/ml brought about inactivation of the parasite within 3 h of incubation. It may be assumed that genistein present in the root-tuber peel exerts a reversible action on the neuromuscular system of the cestode (Croll and Mathews 1977), though the effect is irreversible with respect to *Paramphistomum* sp.

In the treated *Raillietinat echinobothrida*, alterations in the contour of microtriches and disorganization of the tegumental region were conspicuous; while the microtriches exhibited deformity and clumping, the tegumental region showed pronounced vacuolization in comparison to the control. Tegumental alterations and severe vacuolization on exposure to flukicidal drugs have been observed in several species of trematodes (Gupta and Sharma 1973; Mehlhorn et al.1983; Schmahl and Mehlhorn 1985; Schmahl and Taraschewski 1987; Schmahl 1993). Structural disruption of the tegumental integrity is one of the early morphological effects also caused by praziquantel (Van den Bossche 1985; Schepers et al. 1988). According to Bricker et al. (1982, 1983), contraction and vacuolization are closely related phenomena and both require Ca^{2+}. Perhaps the chemical component in the tuber peel of *F. vestita* might bring about permeability changes in the tegument of the worm. Disruption of the cuticular interface and/or intestinal epithelium and degenerative changes even in the subcuticular region have been reported in several nematode species exposed to anthelmintics in vitro (Kaur and Sood 1983; Xiao et al.1989; An 1990; Strote et al. 1990). It would be tempting to speculate that the death of the cestode *R. echinobothrida* on exposure to the peel extract or genistein might be related to the changes induced in the tegumental integrity of the parasite.

From the results obtained in the present study it may be presumed that in vivo, under the effect of the plant-tuber peels the paralyzed worms are easily expelled from the host gut by peristaltic movements. Apparently, the active principle, genistein in particular, is effective against trematodes and cestodes but not against roundworms, which have a cuticular body surface. Though the active principle of the test plant may be operating transtegumentally, the precise mode of its action needs to be investigated further.

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