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Anthelmintic efficacy of *Flemingia vestita* (Leguminoceae): Genistein-induced alterations in the activity of tegumental enzymes in the cestode, *Raillietina echinobothrida*

Papri Pal, Veena Tandon*

Department of Zoology, North-Eastern Hill University, Shillong 793 022, India

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Abstract

The crude root-tuber peel extract of *Flemingia vestita*, an indigenous leguminous plant of Meghalaya, and its active chemical component, i.e. genistein, have shown paralyzing effects on soft-bodied helminth parasites of trematode and cestode groups. With a view to investigating the mode of action of the plant-derived component, its effect on the activity of tegumental enzymes of the parasite was studied. Acid phosphatase (AcPase), alkaline phosphatase (AlkPase), adenosine triphosphatase (ATPase) and 5′-nucleotidase (5′-Nu) are predominantly distributed in the tegument, subtegument, and somatic musculature. After exposure to the crude extract (50 mg/ml of the incubation medium) or genistein (0.5 mg/ml), a pronounced decline in the visible stain intensity was noticeable indicating very little or no activity in these sites. Quantitatively the activity of AcPase, AlkPase, ATPase and 5′-Nu was found to be suppressed by 97, 95, 88, and 57%, respectively, following genistein treatment. The reference drug, praziquantel (0.01 mg/ml) also caused a reduction in the enzymatic activities, somewhat at par with the genistein treatment. The results suggest that the tegumental enzymes of the parasite may be an important target of action for genistein, which appears to act transtegumentally.

Keywords: *Flemingia vestita*; Anthelmintic activity; Phytochemical; Genistein; Tegumental enzymes; Cestode; *Raillietina echinobothrida*

* Corresponding author. Tel.: +91 364 250105; fax: +91 364 250076; e-mail: vtandon@nehus.ren.nic.in

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1. Introduction

Flemingia vestita Benth and Hooker (Family Leguminoceae) is an indigenous medicinal plant of Meghalaya, north-east India, the tuberous roots of which are considered to have anthelmintic properties and hence eaten unpeeled by the natives as a popular cure against worm infections. In-vitro treatment of the adult trematodes namely, *Fasciolopsis buski* and *Artyfechinostomum sufrartyfex*, with the crude extract of the root-tuber peel of this plant induces paralysis and pronounced tegumental damage and disruption in the flukes [1]. While the crude extract of the root-tuber peel seems effective against trematode and cestode parasites, it did not show any effect on the viability of the nematode parasites [2]. The major active component of the root peel which has been identified as an isoflavone, genistein [3], induces paralysis and deformity in the tegument of the cestode, *Raillietina echinobothrida* [2]. The plant-derived components were also shown to cause alterations in the acetylcholinesterase activity of the parasite [4].

Activities of acid phosphatase (AcPase), alkaline phosphatase (AlkPase), adenosine triphosphatase (ATPase), and 5′-nucleotidase (5′-Nu) have been detected by biochemical and histochemical means in a number of helminth parasites wherein these enzymes are found in close association with the tegument, subtegument, somatic musculature, gut and cuticle [5–15]. All the tegumental enzymes are believed to be involved in the digestive and/or absorptive function [16–18] in cestodes. Some drugs alter the metabolism and inhibit the formation of mitochondrial energy and deprive the parasite of ATP [19–21]. Isatin, hexachlorophene, praziquantel, luxabendazole and thiabendazole bring about a change in the activities of AcPase and AlkPase in cestodes [22,23], such as *Echinococcus multilocularis* and *Hymenolepis diminuta*, and trematodes [6,11,24]. However, aqueous crude extract of some medicinal plants namely, *Butea monosperma*, *Embelia ribes* and *Roltleria tinctoria* caused a reduction in both AcPase and AlkPase activity in *Paramphistomum cervi* in vitro [25].

In view of the functional significance of tegumental enzymes in digestion and/or absorption in cestodes, we studied the alterations, if any, in the AcPase, AlkPase, ATPase and 5′-Nu activities in *R. echinobothrida* following exposure in vitro of the root-tuber peel extract of *F. vestita* and its active component, genistein. Histochemical localization and biochemical quantification of the above-mentioned enzymatic activities form the basis of the present communication.

2. Materials and methods

2.1. Drugs

The root-tuber peel extract and genistein were obtained from *F. vestita* following the method as described previously [2]. Synthetic genistein (Sigma, code No G6649) was also used besides the pure genistein extracted from the plant material.

2.2. Experimental parasite and treatment

The adult cestodes, *Raillietina echinobothrida* were collected from the intestine of domestic fowl in 0.9% phosphate buffered saline (PBS, pH 7–7.3), from freshly slaughtered hosts at local abattoirs in Shillong. They were incubated at 37 ±1°C for treatment with 50 mg/ml crude extract, 0.5 mg/ml genistein or 0.01 mg/ml praziquantel, all made in PBS (0.9%) with 1% dimethylsulfoxide (DMSO); at these doses of treatment, the paralytic effect in the worm was attained within a shorter time as compared to when lower concentrations of the test materials were used as described earlier [2]. Three replicates for each incubation medium were used. After exposure to the treatment the cestode, immediately after it attained a paralytic state, was processed for histochemical and biochemical studies along with one set of control specimens (maintained in 1% DMSO in PBS).

2.3. Histochemical studies

The following enzymes were investigated histochemically using fixed frozen sections cut at a

thickness of 10–15 μm in a SLEE (London) HR cryostat.

For the detection of AcPase activity, the modified lead nitrate method of Takeuchi and Tanoue [26] was employed using sodium-β-glycerophosphate as the substrate. Sections were incubated in a freshly prepared medium pH 5–5.2, at 37°C for 1 h.

A modified coupling azo-dye method [26] was used for determination of the AlkPase (pH 10) activity at room temperature (17–20°C) for 40–50 min. The color was brown with fast violet B. Sections were subsequently mounted in glycerin jelly.

Demonstration of ATPase activity was achieved through the use of the calcium method of Maenguen-Davies et al. [26]. The preparations were incubated in the medium (pH 9.9) for 30 min at 37°C, dehydrated and mounted in glycerin jelly.

For the study of 5′-Nu activity, the lead method [27] was employed using adenosine monophosphate (AMP) as the substrate. Sections were incubated in a freshly prepared medium (pH 8.3) at 37°C up to 30 min and mounted in glycerin jelly.

2.4. Biochemical studies

Biochemical assays were done for AcPase, AlkPase, ATPase, and 5′-Nu activity.

AcPase and AlkPase activities were assayed following the method as described by Plummer [28]. A 10% (w/v) tissue homogenate was prepared in sodium acetate buffer (pH 4.5) and sodium glycine buffer (pH 10.5) for AcPase and AlkPase, respectively, and centrifuged at 5000 rev./min for 20 min. The supernatant was taken as the enzyme source. *p*-Nitrophenol phosphate (62.5 mM) was used as the substrate. The decrease or increase in optical density in a UV-visible spectrophotometer (Beckman Model-26) at 405 nm was recorded and the enzyme activity calculated with reference to a standard curve of *p*-nitrophenol (1 mM).

ATPase was assayed by a previously established method [29]. A 10% (w/v) tissue homogenate was prepared in glycine buffer (0.2 M, pH 9.1), sonicated for 30 s and then used for enzyme assay. The assay mixture of 2.8 ml contained glycine buffer (0.2 M), $CaCl_2$ (0.1 M), ATP (50 mM) and 0.05 ml tissue extract; incubated for 15–20 min at 37°C. The liberated inorganic phosphate (pi) was estimated [30].

5′-Nu was assayed following the method of Bunitian [31]. A 10% (w/v) tissue homogenate was centrifuged at 3000 rev./min at room temperature and the supernatant was used as the enzyme source. The reaction mixture containing Tris–HCl buffer (40 μM, pH 7.5), $MgSO_4$ (10 μM), AMP (5 μM), and tissue extract (0.1 ml) was incubated at 37°C for 1 h and the liberated pi was estimated [30].

Protein content for all the enzyme assays was measured according to the method prescribed in Lowry et al. [32].

3. Results

The distribution and intensity of the enzyme reaction products observed in the various tissues of the parasite are given in Table 1. Representative sections for the enzymes tested are illustrated in Figs. 1–15.

3.1. Control

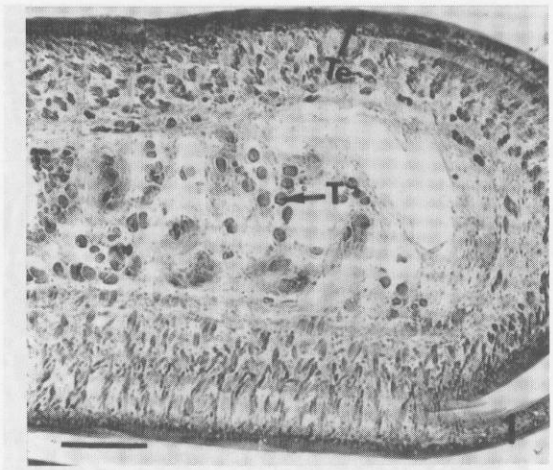
The tegument, subtegument, somatic musculature and gonads displayed strong to moderate reaction to AcPase (Figs. 1 and 2); the enzyme activity was highest in the tegument. Intense AlkPase activity was restricted mainly to the tegument and somatic musculature; the gonads showed almost negative staining, although weak to moderate reaction was observed in the subtegumental region (Fig. 5). The ATPase activity was noted in the tegument, subtegument and somatic musculature where moderate to intense enzyme staining was demonstrated; lower enzyme activity was observed in the subtegument, testes and ovary (Fig. 8). The 5′-Nu activity was limited almost entirely to the tegument and subtegument (Fig. 12).

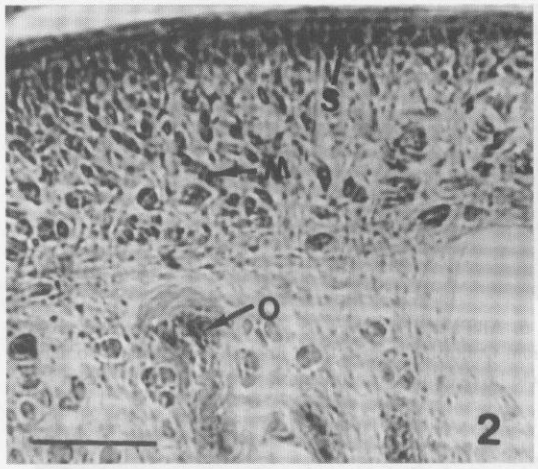
No stain or enzyme activity was found in the tissues when sections were incubated without the substrate.

Table 1
Activities of AcPase, AlkPase, ATPase and 5′-Nu in the various structures of *R. echinobothrida* in vitro: histochemical localization

Treatment (mg/ml)	Distribution and enzyme intensity															
	AcPase				AlkPase				ATPase				5′-Nu			
	Te	S	M	T/O	Te	S	M	T/O	Te	S	M	T/O	Te	S	M	T/O
1. Control (in 0.9% PBS)	+ + + +	+ + +	+ + +	+ + / + +	+ + +	+	+ +	+ / −	+ + + +	+ +	+ + +	+ / +	+ + + +	+ +	+ +	+ / +
2. Crude extract (50.0)	−	−	−	−	−	+ +	+ +	−	+	−	+	+ / +	+		+ +	− / −
3. Genistein (0.5)	−	−	−	+ / −	+	+ +	−	+ / +	−	−	−	−	−	−	−	−
4. Praziquantel (0.01)	−	−	+	−	+	−	−	−	+	−	−	−	−	−	−	−

Abbreviations. + + + +, Very intense activity; + + +, intense activity; + +, moderate activity; +, mild activity; −, no activity; T, tegument; S, subtegument; M, muscle layer; T/O, testis/ovary.





Figs. 1, 2. AcPase activity in *R. echinobothrida*; cryostat sections, showing very intense activity in the tegument (Te), subtegument (S), somatic muscle layer (M), testes (T) and ovary (O) in the control worm (scale bars = 0.2 mm, 0.1 mm, respectively).

3.2. Treatment

After exposure of *R. echinobothrida* to the root-peel crude extract or genistein, there was a pronounced decline in the visible stain intensity of all the enzymes under study. No or very little enzyme activity was observable in the tegument (Figs. 3, 4, 6, 7 and 9–10) after treatment with genistein but a varying degree of activity for 5′-Nu was noticeable in the subtegument and somatic musculature (Figs. 13 and 14).

Quantitatively also, the AcPase, AlkPase, ATPase and 5′-Nu activity was reduced by 96, 86, 71 and 28%, respectively, following treatment with genistein (Table 2). Changes were also found in

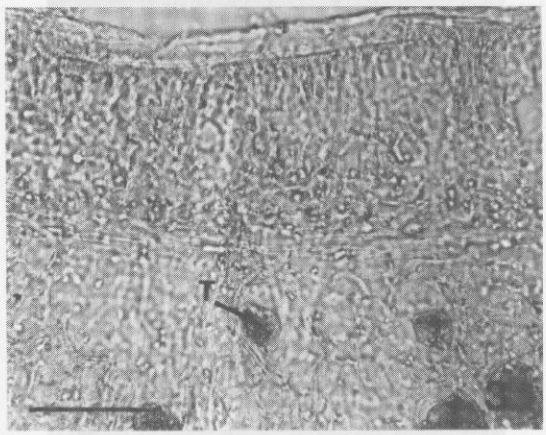


Fig. 3. AcPase activity in *R. echinobothrida*, cryostat cross section. Section showing negative staining for AcPase in the tegument and subtegument, whereas pronounced activity persists in the testes after treatment with genistein (scale bar = 0.1 mm).

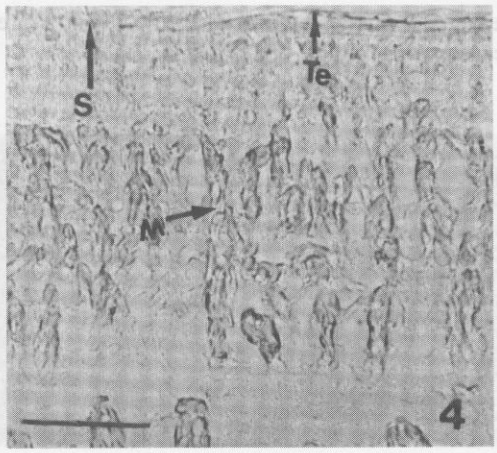


Fig. 4. AcPase activity in *R. echinobothrida*, cryostat cross section. Section showing no AcPase activity in the tegument after treatment with praziquantel (scale bar = 0.1 mm).

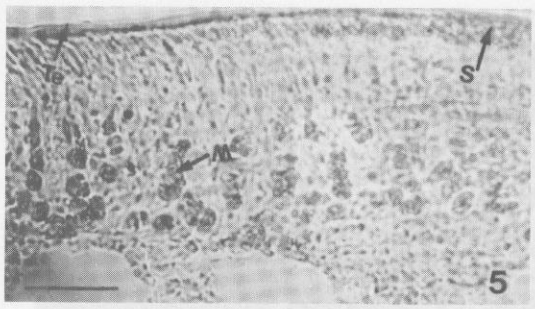


Fig. 5. AlkPase activity in *R. echinobothrida*, cryostat cross section. Control, strong activity in the tegument and somatic muscle layer (scale bar = 0.1 mm).

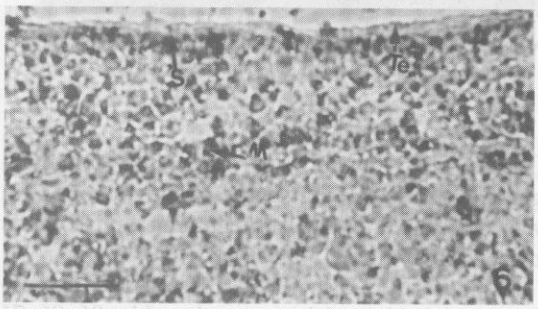


Fig. 6. AlkPase activity in *R. echinobothrida*, cryostat cross section. AlkPase activity is shown in the tegument, subtegument and somatic muscle layer after treatment with the crude root peel extract (scale bar = 0.1 mm).

the activity of these enzymes in the praziquantel-treated worms (Figs. 4, 11 and 15, Table 2).

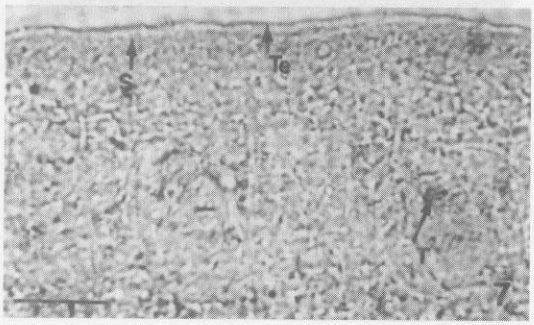


Fig. 7. AlkPase activity in *R. echinobothrida*, cryostat cross section. Mild AlkPase activity in the tegument, subtegument and testes after treatment with genistein (scale bar = 0.1 mm).

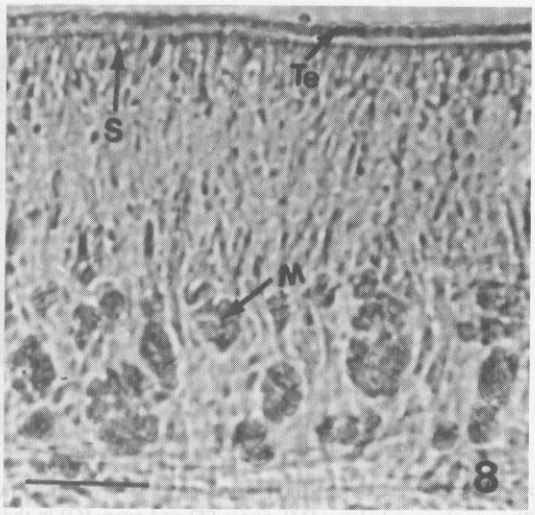


Fig. 8. ATPase activity in *R. echinobothrida*, cryostat cross section. Intense enzyme activity shown in the somatic musculature and tegument (control) (scale bar = 0.1, mm).

4. Discussion

The four enzymes, AcPase, AlkPase, ATPase and 5′-Nu were clearly detectable in the parasite

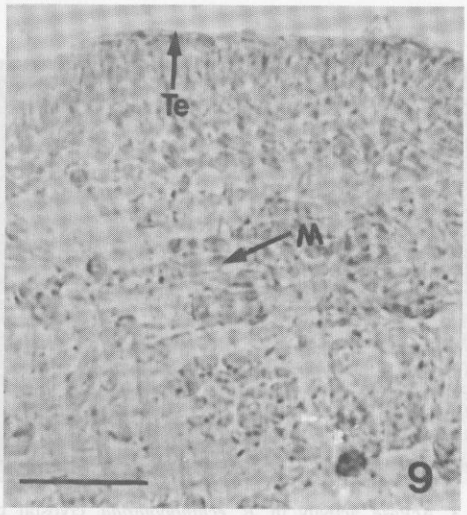


Fig. 9. ATPase activity in *R. echinobothrida*, cryostat cross section. Mild ATPase activity, as shown by light staining, after treatment with the crude root peel extract (scale bar = 0.1 mm).

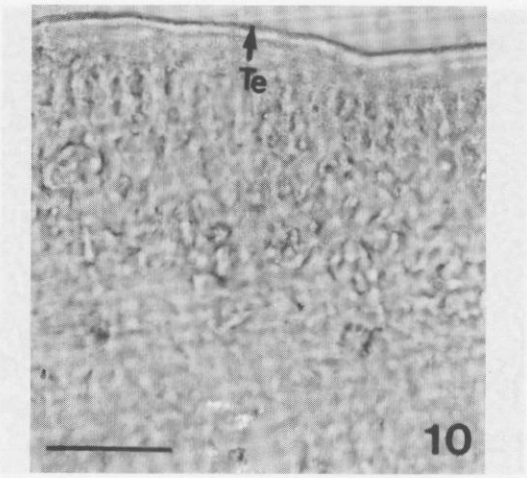


Fig. 10. ATPase activity in *R. echinobothrida*, cryostat cross section. The ATPase activity is absolutely negative in the tegument after treatment with genistein (scale bar = 0.1 mm).

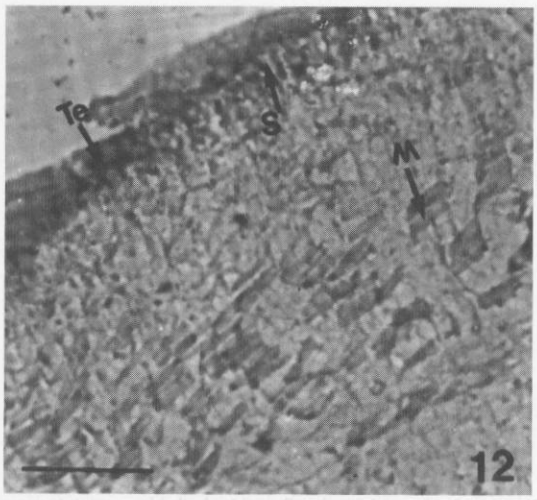


Fig. 12. 5′-Nu activity in *R. echinobothrida*, cryostat cross section. Control, showing pronounced enzymatic activity in the tegument, subtegument and somatic muscle layer (scale bar = 0.1 mm).

at several sites, such as the tegument, subtegument, somatic musculature and reproductive organs; there was no enzyme activity detectable in the parenchyma cells. The widespread and impressive amounts of certain tegumental enzymes demonstrated in several cestodes suggest that they might play a highly significant role in digestion and or absorption in the distinctive tissues [33]. In cestodes like *H. diminuta* the phosphatases are

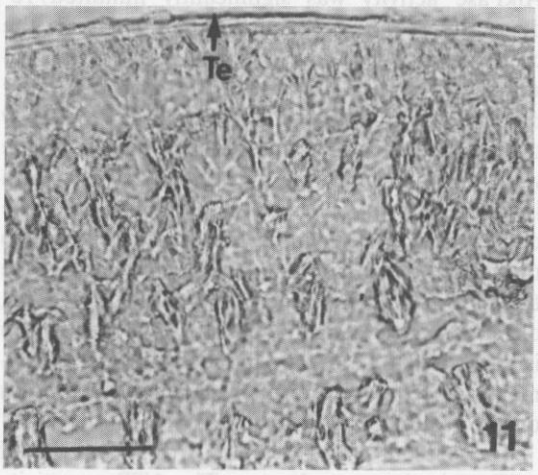


Fig. 11. ATPase activity in *R. echinobothrida*, cryostat cross section. The ATPase activity is absolutely negative in the tegument after treatment with praziquantel (scale bar = 0.1 mm).

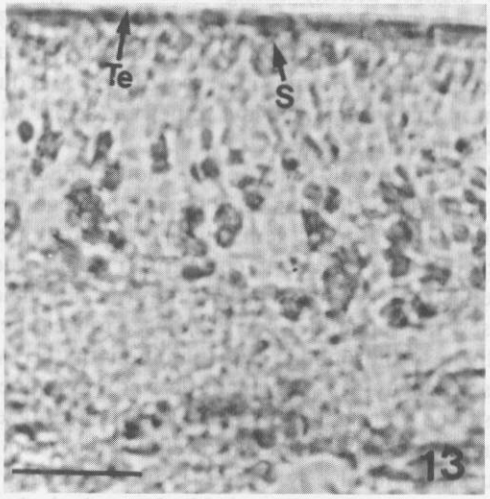


Fig. 13. 5′-Nu activity in *R. echinobothrida*, cryostat cross section. Mild 5′-Nu activity observed in the tegument after treatment with the crude root peel extract (scale bar = 0.1 mm).

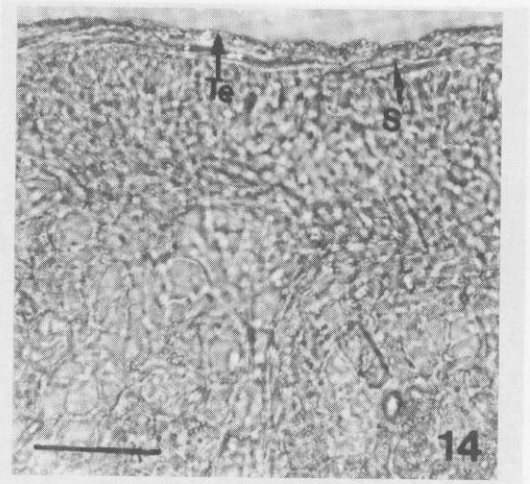


Fig. 14. 5′-Nu activity in *R. echinobothrida*, cryostat cross section. Absence of 5′-Nu reaction in the tegument after treatment with genistein (scale bar = 0.1 mm).

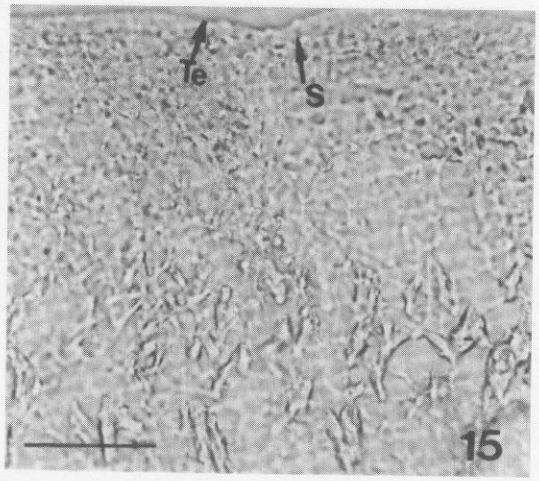


Fig. 15. 5′-Nu activity in *R. echinobothrida*, cryostat cross section. Absence of 5′-Nu reaction in the tegument after treatment with praziquantel (scale bar = 0.1 mm).

thought to be in proximity to the sugar and nucleoside uptake sites [34]. In the present study histochemical and biochemical changes occurred after treatment with the crude root-tuber peel of *F. vestita* or its purified component, genistein. The results of the present investigations indicate that the highest activity change was with regard to AcPase (97%) and the lowest for 5′-Nu (37%) after genistein treatment. The reference drug, praziquantel (0.01 mg/ml) also caused a reduction in the enzyme activity, somewhat at par with the genistein treatment.

Histochemically also, AcPase showed much less or negligible staining reaction in the tegument following treatment; genistein showed greater effectiveness to AcPase in comparison to the crude peel extract. A similar observation was also made in several trematode species exposed to treatment with hexachlorophene [6]. Inhibition of the AcPase activity by the plant-derived component observed during the present study may suggest that the absorption and intracellular digestion of drugs may involve lysosomes [35]. A decrease in the enzyme activity is probably due to its leakage into the medium as a result of the disruption of the absorptive surface [36].

It is evident from the present investigation that AlkPase is much more active in *R. echinobothrida* as compared to AcPase and is particularly strong in the tegument. In adult cestodes AlkPase is usually most active, while AcPase tends to predominate in trematodes [34]. AlkPase has been localized on the surface of both male and female *Schistosoma mansoni* worms as a result of praziquantel-induced tegumental damage [37–39]. The AlkPase activity was significantly inhibited/reduced by 86 and 95% after treatment with the crude peel extract and genistein component, respectively. In *Echinococcus multilocularis* metacestodes, inhibition of the AlkPase activity by 23% and complete inhibition of glucose uptake was reported following treatment in vivo with isatin, a known phosphatase inhibitor, and the depletion in the enzyme activity was attributed to the failure of glucose uptake [23]. However, more than twofold increase in the AlkPase activity, observed in the praziquantel-treated *S. mansoni* is attributed to the drug-induced tegumental damage exposing the normally concealed enzymes on the tegumental surface of the worm [24].

The present study revealed a high activity of ATPase in the control worm. The ATPase activity is widely distributed in helminths and probably represents a variety of enzymes [34]. Three classes of specific ATPase have been described: proton

Table 2
Biochemical effects of the root-tuber peel extract and genistein component of *F. vestita* on *R. echinobothrida* in vitro

Treatment (mg/ml)	Enzyme activity (total[a]/specific activity[b])				% Change after treatment			
	AcPase	AlkPase	ATPase	5′-Nu	AcPase	AlkPase	ATPase	5′-Nu
1.Control (in 0.9% PBS)	8.07 ± 0.11/ 0.078 ± 0.001	3512 ± 1.09/ 4.75 ± 0.17	4496.45 ± 1.1/ 4.9 ± 0.12	204 ± 1.13/ 1.5 ± 0.015				
2.Crude extract (50.0)	0.29 ± 0.01/ 0.033 ± 0.001	473.44 ± 1.11/ 3.33 ± 0.21	1291.66 ± 0.05/ 3.81 ± 0.09	146.28 ± 0.21/ 1.25 ± 0.003	96	86	71	28
3.Genistein (0.5)	0.241 ± 0.013/ 0.022 ± 0.0	145.26 ± 1.08/ 2.75 ± 0.19	525.21 ± 1.09/ 2.7 ± 0.11	87.8 ± 1.2/ 0.87 ± 0.17	97	95	88	57
4.Praziquantel (0.01)	0.63 ± 0.03/ 0.015 ± 0.0	101 ± 1.13/ 1.81 ± 0.8	661.08 ± 1.2/ 1.81 ± 0.8	128.27 ± 1.03/ 1.2 ± 0.001	92	97	85	37

Values are given as mean (±S.E.) from three replicates assays.
[a]Enzyme activity expressed as a specific unit which consumes 1.0 μmol substrate/g wet wt. tissue/h.
[b]Specific activity expressed as unit/mg protein/h.

translocating ATPases catalyzing the synthesis of ATP, other ion-transporting ATPases that normally hydrolyze ATP and use the energy released for active ion transport across plasmalemmal membranes, and cytoplasmic ATPases associated with contractile systems [40]. The localization of ATPase in somatic musculature of *R. echinobothrida* strongly suggests that one of the roles of this enzyme is the hydrolysis of ATP in this tissue. This enzyme is known to be related to energy metabolism, active transport and lipid synthesis [41]. The inhibition of ATPase activity in the present study amounted to 71 and 88% with the treatment of crude peel extract and genistein, respectively. Similar observations have also been made for *E. multilocularis* after exposure to mebendazole, thiabendazole, levamisole and acrisoline [19].

5′-Nu activity was observed to decrease by 57% following treatment with genistein in *R. echinobothrida*. The function of 5′-Nu is not well understood, although its role in active transport across plasma membrane has been suggested [42]. In helminths, 5′-Nu may be involved with other enzymes in the uptake of nucleosides or their hydrolysis to pyrimidine and purine bases [34,43].

The alterations observed in the activity of the tegumental enzymes after treatment with the root-tuber peel extract of *F. vestita* and genistein suggest that the tegumental enzymes of the parasite may be a plausible target of action for genistein, which appears to act transtegumentally.

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