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

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To ascertain the anthelmintic efficacy of *Flemingia vestita* (an indigenous leguminous plant of Meghalaya, having putative anthelmintic usage), its crude root-tuber peel extract and active chemical principle, genistein, were tested *in vitro* with reference to esterase activity in the fowl tapeworm, *Raillietina echinobothrida*. With the localization of non-specific esterases (NSE) and cholinesterase (ChE), the organization of the cholinergic components of the nervous system in toto could be visualized in the cestode. The specific ChE in the parasite is acetylcholinesterase (AChE). Both NSE and ChE were found in close association with the central and peripheral nervous components, besides being present in the tegument and muscular parts of the terminal male genitalia. The whole tissue homogenate of the parasite also showed a high AChE activity. After exposure to the crude peel extract (50 mg/ml of the incubation medium) and to genistein (0·5 mg/ml), a pronounced decline in the visible stain intensity in the cholinergic components of the nervous system and in the tegument was noticeable, indicating extremely reduced activity of NSE and ChE in these sites. The total AChE activity was also reduced to 49·07% and 56·77%, following treatment with the peel extract and genistein, respectively. The reference drug, praziquantel (0·01 mg/ml) also caused reduction in the enzyme activity, somewhat at par with the genistein treatment. Genistein appears to have a transtegumental mode of action. Alteration in the AChE activity points towards acetylcholine, an inhibitory neurotransmitter in cestodes, as the potential target of action.

1. Introduction

Flemingia vestita Benth and Hooker (Family: Fabaceae) is an indigenous medicinal plant of Meghalaya, North-East India, the tuberous roots of which are considered to have anthelmintic properties and hence eaten unpealed by the natives as a popular cure against worm infections. *In vitro* treatment of the adult trematodes, viz., *Fasciolopsis buski* and *Artyfechinostomum sufrartyfex*, with the crude extract of the root tuber peel of *F. vestita* induces paralysis and pronounced tegumental damage and disruption in the flukes (Roy and Tandon 1996). Besides the crude peel extract, the major active component of the root peel that has been identified as an isoflavone, genistein, induces similar effects in the cestode, *Raillietina echinobothrida*; using scanning and transmission electron microscopy genistein-induced tegumental damage in this cestode was demonstrated (Tandon *et al* 1997).

In view of the functional significance of esterases in nervous coordination of helminths, we studied the alterations in the non-specific esterase (NSE) and acetylcholinesterase (AChE) activity in *R. echinobothrida*, following exposure *in vitro* to the crude root-tuber peel extract of *F. vestita* and its major active chemical principle, genistein. Histochemical localization of NSE and AChE (so as to reveal mainly the cholinergic nervous elements) and biochemical quantification of the AChE activity form the basis of the present communication. Alterations in the activity of the tegumental enzymes of the parasite as influenced by the plant derivative will be dealt with later.

Keywords. Anthelmintic; *Flemingia vestita*; genistein; esterase activity; cestode; *Raillietina echinobothrida*

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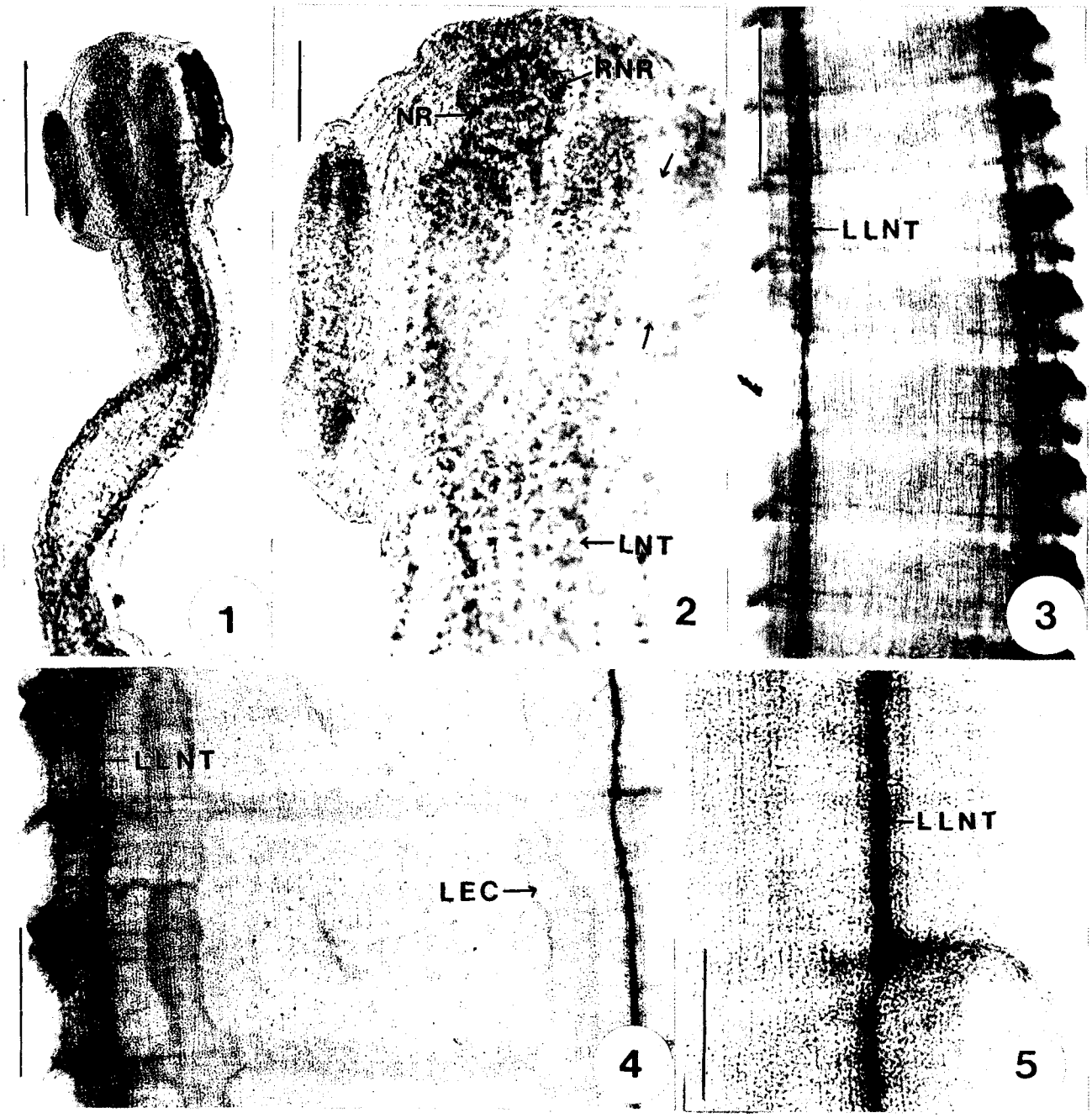
2. Materials and methods

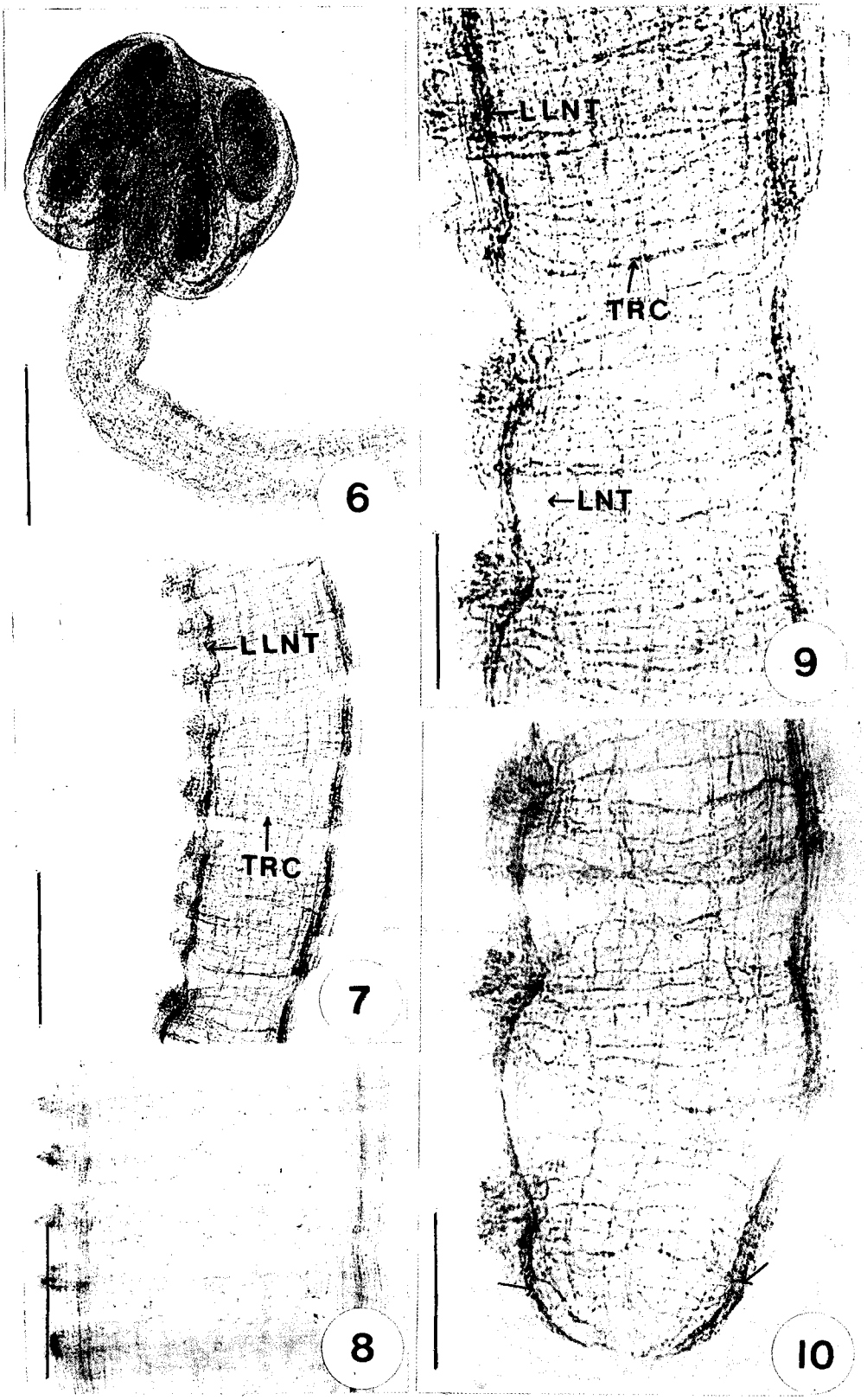
2.1 *Drugs*

The root-tuber peel extract and active component (genistein) were obtained from *F. vestita* following the method of Rao and Reddy (1991) and the detailed procedure previously described by Tandon *et al* (1997). Synthetic genistein (Sigma, code No. G6649) was also used besides the pure genistein extracted from the root-tuber peel of *F. vestita*.

Praziquantel was used as the reference substance.



Figures 1–5. Nervous system in *Raillietina echinobothrida* (photomicrographs, Bromoindoxyl acetate method). (**1**) Scolex and neck regions, showing an intense NSE reactivity revealing the nervous system. Scale bar = 0·2 mm. (**2**) Magnified view of the scolex, showing the nerve ring (NR), the rostellar nerve ring (RNR), the longitudinal nerve trunks (LNT) and nerves innervating the suckers (arrows). Scale bar = 0·1 mm. (**3**) Innervation in the region of immature proglottides; two prominent lateral nerve trunks (LLNT) are conspicuous. Scale bar = 0·5 mm. (**4–5**) Region of mature proglottides; several closely clustered nerves constitute the LLNT, each lying external to the longitudinal excretory canal (LEC). Intense NSE reactivity in the region of the genital pore is evident. Scale bar = 0·5 mm and 0·2 mm, respectively.



Figures 6–10. Nervous system in *R. echinobothrida* (photomicrographs, Acetylthiocholine iodide method). (**6**) Scolex region, showing ChE activity. Scale bar = 0·2 mm. (**7, 8**) Immature proglottides showing the LLNT and the other longitudinal nerves, several transverse commissures (TRC) joining these nerves form a fine nerve net. Scale bar = 0·5 mm. (**9**) A magnified view of the mature proglottides, showing the fine nerve arrangement comprising the LLNT, LNS and transverse commissures. Scale bar = 0·5 mm. (**10**) Terminal proglottides, showing all the longitudinal nerves joining together at the extreme posterior end. Scale bar = 0·5 mm.

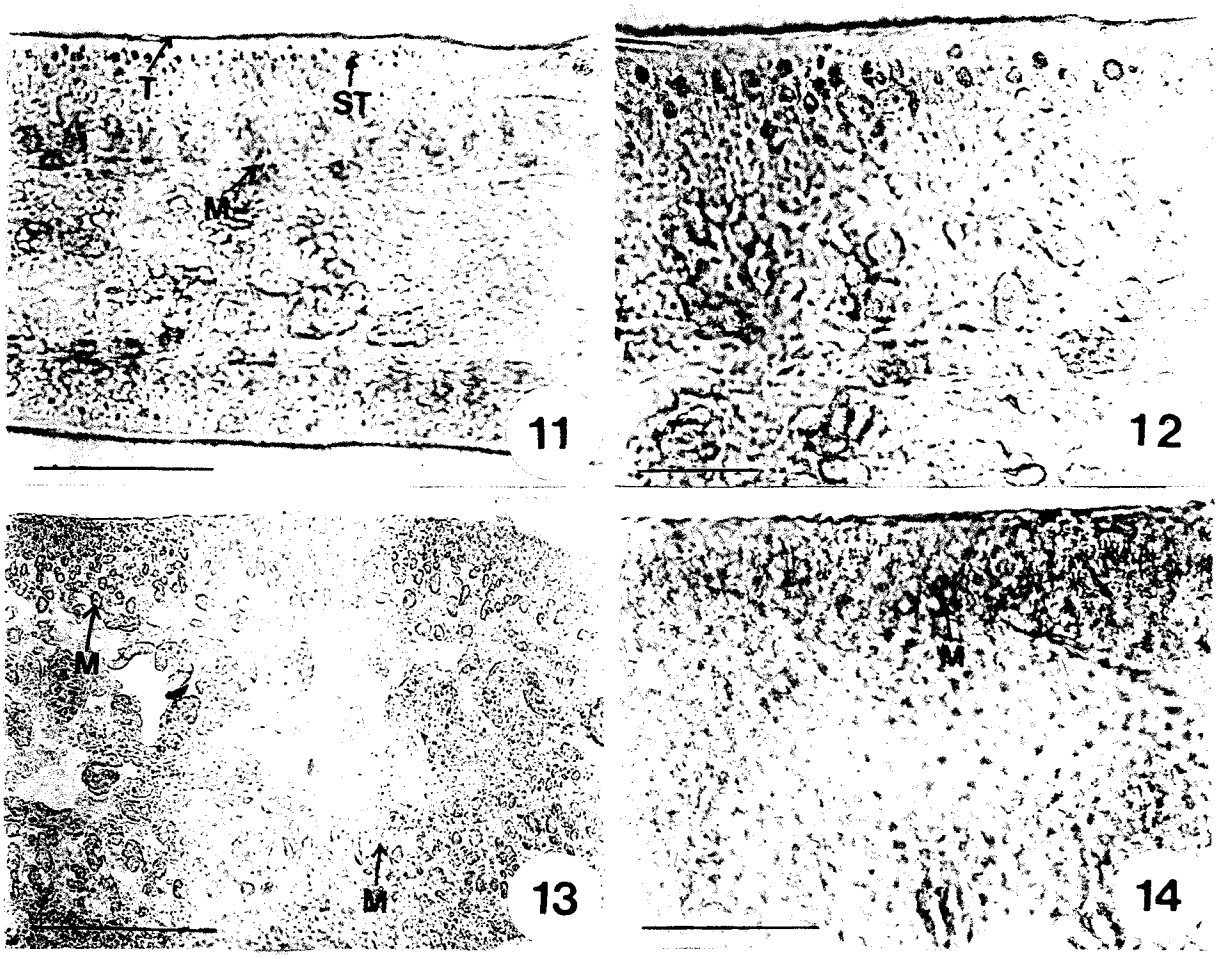
2.2 *Experimental parasites and treatment*

The adult cestode, *R. echinobothrida* (Megnin 1888) 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 and 0·01 mg/ml praziquantel, all made in PBS with 1% dimethylsulphoxide (DMSO) (three replicates for each incubation medium). After exposure to the treatment the paralysed cestodes were processed for histochemical and biochemical studies along with one set of control specimens (maintained in 1% DMSO in PBS).

2.3 *Histochemical studies*

The live worms were washed in 0·9% PBS and fixed in cold 10% neutral buffered formalin at 4°C and were processed for histochemical localization of NSE and cholinesterase (ChE). Staining for NSE was performed according to the method of Holt and Withers (1952), using standard incubation medium containing 5-bromo indoxyl acetate and following the procedure published earlier (Lyngdoh and Tandon 1992). The method of Gomori (1952) was followed for ChE using acetylthiocholine iodide as the substrate as described by Rahemo and Gorgees (1987). The NSE and ChE were localized in the whole mounts of methylbenzoate-cleared specimens by their deep indigo blue and brown staining, respectively and revealed the organization of the nervous system in toto.

For the distribution and localization of AChE activity, the frozen cross sections of the material, fixed in cold formalin at 4°C overnight, were cut at 14 µm thickness and processed, following the method of Gomori (1952). Using standard incubation medium as described by Pearse



Figures 11–14. (**11, 12**) Control. Transverse sections. (**11**) Showing intense AChE activity in the tegument (T), subtegument (ST) and muscle elements (M). Scale bar = 0·2 mm. (**12**) A magnified view of a portion of the same. Scale bar = 0·1 mm. (**13, 14**) Treated worms. Transverse sections. Treatment with crude root-tuber peel extract. (**13**) Mild AChE activity revealed in the subtegument and muscle; the tegument shows no activity. Scale bar = 0·2 mm. (**14**) A portion of (**13**) in a closer view. Scale bar = 0·05 mm.

(1968) the sections were incubated for 30 min at pH 6–6·2 at 37°C ± 1°C, washed with water and freshly mounted in glycerine jelly. The AChE activity present in the tegumental and other tissue components was observed by their blackish brown colour.

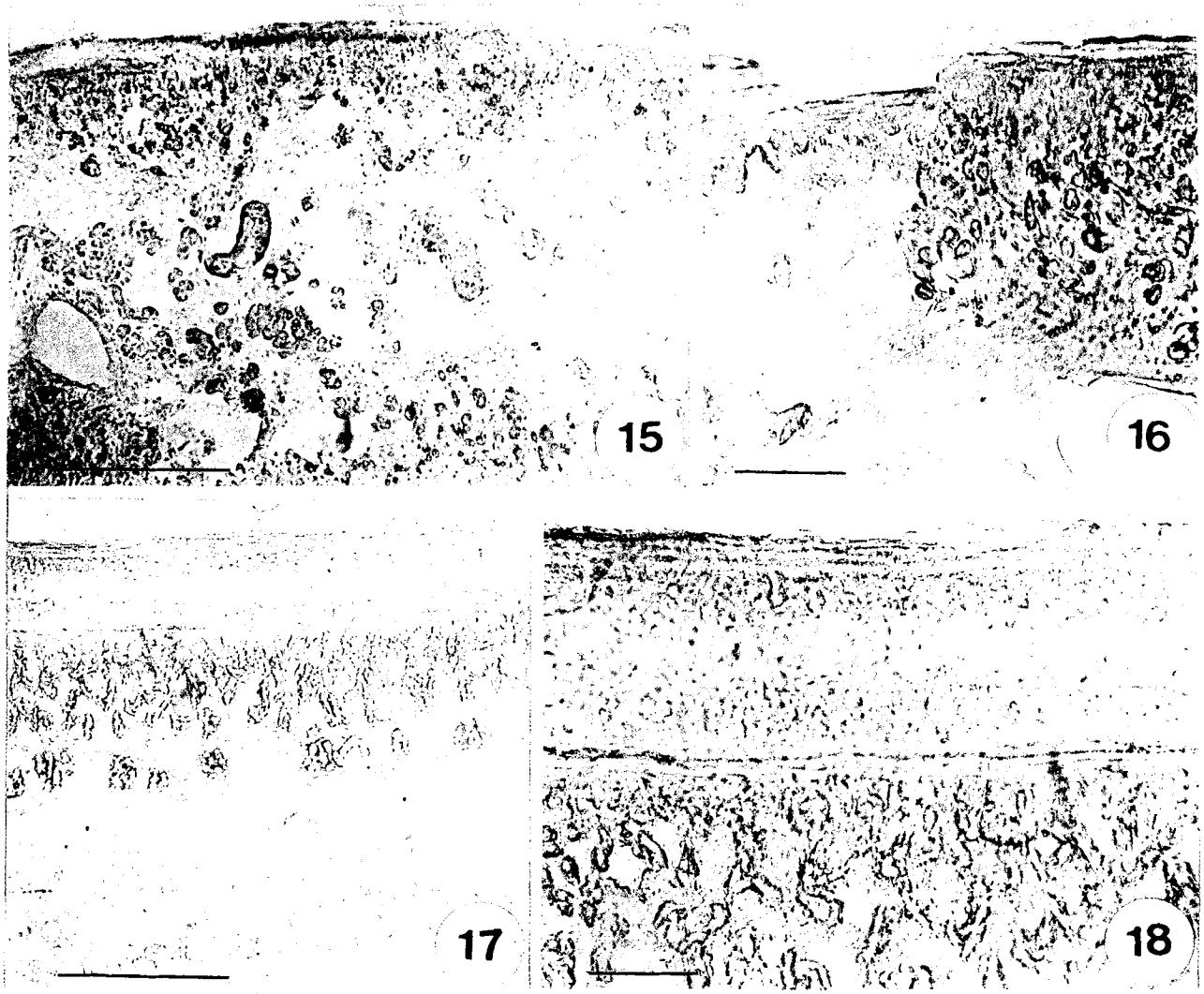
2.4 *Biochemical assay*

AChE activity was assayed following the method of Elman *et al* (1961), as given by Ott *et al* (1975). A 10% (w/v) tissue homogenate was prepared in 0·2 M sucrose solution at 0 ± 2°C, centrifuged at 20,000 *g* at 4°C for 30 min and the supernatant was used for the enzyme source. The substrate solution was prepared fresh on the day it was to be used. The assay mixture of 3·0 ml contained sodium phosphate buffer (150 mM, pH 7·4), acetylthiocholine iodide (10 mM), 5,5′-dithiobis nitrobenzoic acid (DTNB, 1·25 mM), triton, X-100 (0·3%) and enzyme extract (0·1 ml). It was incubated at 37°C and the optical density (OD) value was taken in a uv-visible spectrophotometer (Beckman model-26). The decrease or increase in OD at 412 nm was recorded at 30 s intervals for 4–5 min.

3. Results

3.1 *Control*

With the demonstration of deep indigo blue or brown staining revealing the presence of NSE and ChE, respectively, the entire arrangement of the nervous system



Figures 15–18. Treated worms. Transverse sections. **(15, 16)** Treatment with genistein (0·5 mg/ml). Loss of AChE activity is revealed in the mentioned tissues compared to controls. Scale bar = 0·2 mm and 0·1 mm, respectively. **(17, 18)** Treatment with praziquantel (0·01 mg/ml). Loss of AChE activity, as with genistein treatment in the tissues is evident. Scale bar = 0·2 mm and 0·1 mm, respectively.

in *R. echinobothrida* could be visualized (figures 1–5, 9, 10). NSE and AChE activity in the reproductive system appeared to be limited to the muscularized organs of the male system, viz., the cirrus sac and the genital atrium (figures 4, 6–10). The intense staining reaction with acetylcholine iodide used as a substrate indicated that the cholinergic component in the nervous components is AChE.

Histologically, AChE was found to be present in the tegument, subtegument and muscle layers, with intense activity restricted mainly to the tegument (figures 11, 12); in the whole tissue homogenate high AChE activity was measurable (table 1).

3.2 *Treatment*

After exposure of *R. echinobothrida* to the crude root peel extract of *F. vestita* and genistein (table 1), there was a pronounced decline in the visible stain intensity of the nervous components in the paralysed worm, indicating very little or no activity of NSE and ChE in the parasite; no activity was observable in the tegument (figures 13–16). Quantitatively also, the AChE activity was reduced to 49·07% and 56·77% following treatment with the crude extract and genistein, respectively (table 1). Changes were also noticeable in the praziquantel-treated worm (figures 17, 18; table 1); the reduction in AChE activity was somewhat at par with that of the genistein-treated worms when compared to the control.

4. Discussion

The organization of the nervous system in toto of *R. echinobothrida* could be visualized and discerned with the histochemical localization of the NSE and ChE. The nervous system in this cestode follows the nerve arrangement in conformity with the general plan exhibited among the cyclophyllidean cestodes (Wilson and Schiller 1969; Shield 1969; Rahemo 1993). The specific ChE in the parasite (as revealed by the use of acetylthiocholine iodide as substrate) is AChE. The latter is found closely associated both with the central and peripheral nervous components in cestodes (Maule *et al* 1993). A pronounced decline in the activity of NSE and AChE was noticeable following treatment of *R. echinobothrida* with 50 mg/ml root-tuber peel extract and 0·5 mg/ml genistein; both in toto and histological preparations the indigo blue or brown staining of the cholinergic components was either very faint or totally missing; the biochemical assay of AChE in the treated worms also indicated a considerable loss of enzyme activity.

A reduction in ChE activity following *in vitro* exposure to anthelmintics or potential anthelmintics of plant origin has been reported (Kaur and Sood 1982; Tin *et al* 1994). In contrast, a progressively enhancing NSE activity along with alkaline phosphatase activity in praziquantel-treated *Schistosoma mansoni* as reported by Fallon *et al* (1994) is attributed to the drug-induced tegumental damage that brings about the exposure of these enzymes (normally concealed in the tegument) on the worm surface.

Acetylcholine receptors in platyhelminths are known to have pharmacological properties different from their mammalian counterparts (Mellin *et al* 1983). Anthelmintics including organophosphates and praziquantel possibly have a neuromuscular mode of action (McKay *et al* 1989; Cox 1994) by interfering with transmission at nerve–nerve synapse or at neuromuscular junction by inhibiting the enzyme (AChE) responsible for inactivating the neurotransmitter at the synapse (Bryant and Behm 1989; Raether 1988). Treatment both with the root-peel extract of *F. vestita* and genistein does cause paralysis

Table 1. AChE activity in the various tissues of *R. echinobothrida in vitro*.

Treatment (mg/ml)	Longevity of the parasite (h)	Enzyme activity and intensity (histochemical localization)				Total activity*	Specific activity**	Change after treatment (%)
		Tegument	Subtegument	Muscle	Testis/ovary			
Control (in PBS)	72 ± 0·05	++++	++	+++	+/+	2·45 ± 0·011	0·013	
Crude extract (50·0)	P = 0·3 ± 0·01 D = 6·5 ± 0·4	–	±	+	–	1·25 ± 0·12	0 ± 01	49·07
Genistein (0·5)	P = 4·4 ± 0·07 D = 19·08 ± 0·02	–	–	–	–	1·06 ± 0·02	0·007	56·77
Praziquantel (0·01)	P = 0·47 ± 0·07 D = 6·1 ± 0·34	–	–	–	–	1·08 ± 0·19	0·004	55·84

Values are the mean (+ SE) from three replicate assays. *Enzyme activity expressed as a specific unit which consumes 1·0 µmol substrate/g wet wt tissue/h. **Specific activity expressed as µmol/mg protein/h.
++++, Very intense activity; +++, intense activity; ++, moderate activity; +, mild activity; –, no activity; P, paralysis; D, death.

of the worm (Tandon *et al* 1997) which may be a result of a depolarizing type of neuromuscular blockage and a sustained muscle contraction (Coles *et al* 1975; Aubry *et al* 1980). A flaccid paralysis may also ensue when the drug causes hyperpolarization of the muscle membrane, thus preventing depolarization (Bryant and Behm 1989). Acetylcholine has an inhibitory effect on motility in *Hymenolepis diminuta* (Sukhdeo *et al* 1984; Thompson *et al* 1986). In the treated *R. echinobothrida* also, there may be loss of grip on the site of attachment and hence a vermifugal action may ensue (Bennett and Depenbusch 1984). Since the helminth AChE differs in sensitivity to inhibition from the mammalian counterpart, AChE appears to be a potential target for chemotherapy (Barrett 1981). The alteration in AChE activity observed after treatment with root-tuber peel extract or genistein suggests that *F. vestita*-derived component may be of anthelmintic interest.

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