AN IN-VITRO STUDY ON THE ANTHELMINTIC EFFICACY OF THE ROOT TUBER PEEL EXTRACT OF *FLEMINGIA VESTITA* AGAINST THE CESTODE, *RAILLIETINA ECHINOBOTHRIDA*

ABSTRACT

BY

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SUBMITTED

IN

FULFILMENT OF THE REQUIREMENT OF THE DEGREE OF

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OF

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ABSTRACT

The present work incorporates a study on the anthelmintic efficacy of *Flemingia vestita* Benth and Hooker, an indigenous leguminous plant consumed by the natives in North East India. The in-vitro activity of the root- tuber peel extract of this plant and its active chemical principles was tested against the cestode, *Raillietina echinobothrida*. Changes in physical motility, histomorphological alterations, activity of non-specifiic and specific esterases that are associated with the nervous system, activity of some tegumental enzymes and changes in the levels of free amino acid pool and tissue ammonia are the parameters for this study.

(I) Live parasites from domestic fowl were collected in 0.9% physiological buffered saline (PBS) and maintained at 37 ± 1°C. In- vitro treatment of the parasite with the crude extract (50 mg/) in PBS revealed complete immobilization of the cestode in about 20 min. 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 cestodicide.

(II) Stereoscan and transmission electron microscopic observations revealed several tegumental alterations and deformity in the treated worms. Alterations in the contour of microtriches and disorganization of the tegumental region were conspicuously evident; the microtriches showed deformity and clumping in the parasite exposed to the crude peel extract. The tegument, inner subtegumengtal region and muscle layers were the sites predominantly affected by the genistein treatment (0.5 mg/ml); the severe distortion and disorganization occurred in the region of microtriches, and the inner subtegumental region showed pronounced vacuolization in comparison to controls.

(III) With the localization of non-specific esterases and cholinesterases, the organization of cholinergic components of the nervous system in toto could be visualized in the cestode; a pattern in conformity with the general plan of nervous system in cyclophyllidean cestodes was revealed. The cholinesterase in the parasite is acetylcholinesterase. Both non-specific esterases and cholinesterase 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 acetylcholinesterase activity. After exposure to the crude peel extract (50 mg/ml) and genistein (0.5 mg/ml), a pronounced decline in the visible stain intensity in the cholinergic components of the nervous system and tegument was noticeable, indicating extremely reduced activity of nonspecific esterases and cholinesterase in these sites. The total acetylcholinesterase 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 a reduction in the enzyme activity, somewhat at par with the genistein treatment.

Alteration in the acetylcholinesterase activity points towards acetylcholine, an inhibitory neurotransmitter in cestodes, as a potential target of action.

(IV) Acid phophatase, alkaline phosphatase, adenosine triphosphatase and 5'- nucleotidase are predominantly distributed in the tegument, subtegument and somatic musculature. After exposure to the crude extract and

genistein, a pronounced decline in the visible stain intensity was observed indicating very less or no activity in these sites. Quantitatively the activity of AcPase, AlkPase , ATPase and 5'-Nu was found to be supressed by 97%, 95%, 88% and 57%, respectively, following genestin treatment. The reference drug, praziquantel also caused a reduction in the enzyme activity, somewhat similar with the genistein treatment.

(V) Using high performance liquid chromatography phosphoserine, taurine, phosphoamine, threonine, serine, glutamate, proline, glycine, alanine, citrulline, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, β-alanine, α-aminobutyric acid, γ- aminobutyric acid, tryptophan, histidine, ornithine, arginine and ammonia were detected in the tissue homogenate of the parasite both qualitatively and quantitatively. After exposure to the crude extract (50 mg/ml) and genistein (0.5 mg/ml), alterations were noticeable in the free amino acid pool. Following genistein treatment, quantitatively the contents of phosphoserine, taurine, β-alanine,α- aminobutyric acid, tryptophan, histidine and valine were significantly lower

and glutamate, methionine, isoleucine, γ- aminobutyric acid and ammonia were slightly higher than those in the control; phosphoamine, citrulline and ornithine were not detectable. The reference drug, praziquantel (0.01 mg/ml) also caused a quantitative reduction in the free amino acid contents of the parasite, somewhat at par with the genistein treatment.

(VI) The active principle (genistein) of *F. vestita* root peel suggestively acts transtegumentally on the parasite. In view of the known effects of genistein on cellular physiology, anthelmintic role of this chemical of plant origin needs to be further ascertained.

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DEDICATED TO MY SISTER
(LATE) MISS L. R. PAL

THE NORTH-EASTERN HILL UNIVERSITY

SHILLONG- 793 022

18 May, 1998.

I, Papri Pal, hereby declare that the subject matter of thesis is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other university/ Insitute.

This is being submitted to the North-Eastern Hill University for the Degree of Doctor of Philosophy in Zoology.

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PREFACE

In many traditional medicine systems use of herbal medicine for health care has been in practice since long and is well documented. Plant-derived products are generating global interest and emerging as potential alternative to current medical practices that involve chemotherapy. In north-east India, Meghalaya in particular, many plants are used for their medicinal value. The fleshy tuberous roots of *Flemingia vestita*, *or Soh-phlang* as known vernacularly, have popular usage as a putative anthelmintic, curative against intestinal worm infections. In an earlier preliminary study the crude root peel extract of this plant was found effective against soft- bodied trematode and cestode parasites. This finding stimulated further investigation on the anthelmintic efficacy of *F. vestita* to ascertain vermifugal and/ or vermicidal activity of the plant-derived components, the results of which are provided in the present study.

For most anthelmintics which are being currently used in chemotherapy, the parasite body surface (an important host- parasiteinterface),energy metabolism, or neuromuscular

function seem to be the most potential target for action by the drug. The present study provides some clues to the mode and possible targets of action of the active principles of the plant part that exhibits anthelmintic properties in *vestita*.

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CONTENTS

INTRODUCTION

In many tropical, subtropical and traditional medicine systems, a number of plants have their usage in curing worm infections (Perry, 1980; Dharma, 1985; Chhabra et al., 1990). In India a large number of plant species have been reported to have medicinal properties (Chopra et al., 1956; Dhar et al., 1965,1968, 1973; Dhawan et al., 1980; Rao and Krishnaiah, 1982; Bhakuni et al., 1988; Shilaskar and Parashar, 1989). Biological screening of many among such plants has revealed them to be efficaceous as vermicidal or vermifugal (Asswal et al., 1984), either in vivo or in vitro conditions and in a few cases their efficacy has also been compared with that of the synthesized anthelminitics (Dhar et al., 1965; Arslanov and Serov, 1983). There are a number of plants viz., *Zanthoxylum alatum* (= tejbal), *Aegle armelos*, *Callistemon viminalis*, *Cassia aubreviuei*, *Ficus impida*, *Photolocca dodecandra*, *Salsola somalensis*, *Uvaria narum*, to name a few, which have shown anthelmintic efficacy against various helminth parasites (Singh et al., 1982; Singh et al., 1983; Garg and Kasera, 1983; Kilian et al., 1990; Philips, 1990; Turchi and Belli, 1990; Abegaz and Woldu, 1991; Hisham et al.,1992). Qureshi and Sabir (1979) reported the anthelmintic efficacy of *Embellia* seeds

(= babarang) against tapeworms of poultry in vivo. Sharma (1979) gave an account of the preliminary trial on the efficacy of *Punica granatum* root bark against *Raillietina* spp. of poultry. In another study, Ghafghazi et al. (1980-81) reported the aqueous extract of stem and leaves of *Trigonella foenum graceum* (=fenugreek)in vitro against *Hymenolepis nana* and *Syphcia obvelata*. Charoenlarp et al. (1981) found the crude extract of *Artocarpus lakoocha* wood effective against tapeworm infections in human population of Thailand. Kalyani et al. (1989) tested in vitro the anthelmintic activity of essential oil from the fruits of *Zanthoxylum limonella* against tapeworms and hookworms and reported it to be better than that of piperazine phosphate.

Bagherwal and Nanavati (1990) studied the comparative efficacy of panfugal and taenil against *Raillietina tetragona* infection in laying birds. *Salsola somalensis* is used in Ethiopian medicine for curing tapeworms infections by implying the root parts as tooth sticks and swallowing the juice (Woldu and Abegaz, 1990). Akhtar and Riffat (1991) gave an account of field trial of *Saussurea lappa* roots and *Nigella sativa* seeds against cestodes, namely, *Taenia saginata* and *H. nana* in children; the efficacy was adjudged

on the basis of percentage reduction in the faecal egg counts. Aqueous extracts of *Albizzia anthelmintica* and *A. lebbek* barks showed anthelmintic activity against *Hymenolepis diminuta* in rats (Galal et al.,1991). Soffar and Mokhtar (1991) gave an evaluation of the antiparasitic effects of aqueous garlic (*Allium sativum)* extract against *H.nana* in vitro.The crude extract of root of *Matteuccia orientalis* showed anthelmintic effect on bovine *fascioliasis* (Shiramaizu et al.,1993).

Akhtar and Ahmad (1992) studied the efficacy of *Mallotus philippinensis* fruit (= lotus or Kamala) against gastrointestinal cestodes on Beetal goats and found 90% reduction in faceal egg count by day 15 in treated animals. Chatterjee et al. (1992) tested the microfilaricidal activity of stem bark of *Streblus asper* and its major active constituents in *Litomosoides carinii*- infected *Sigmoden hispidus,* and *Brugia malayi* and *Acanthocephalonema vitae* - infected *Mastomys natalensis*. 8he essential oil obtained from the dried leaves of *Buddleia asiatica* was reported to possess a good in-vitro anthelminticity against *Taenia solium* (Garg and Dengre, 1992). In- vitro studies reported that the essential oil of piper bettle was highly

effective against *T.solium* and hookworms, *Bunostomum trigonocephalum* than the synthetic anthelmintics piperazine phosphate and hexylrisorcinol (Garg and Jain, 1992). Ibrahim (1992) reported that the aqueous extracts of *Balanites aegyptiaea, Sesbania sesbam, Albizzia anthelmintica, Cymbopogon narvatus, Abrus precatorium, Rhynchosia minima, Striga hemontheca* and*Anogeissus leiocarpa* showed anthelmintic activity against *Caenorhabditis elegans* in vitro. Ghosh et al. (1992) reported antifilarial effect of the plant, *Zingiber officinale* on *S. cervi* in rats. The aqueous and alcoholic extracts of *Centratherum anthelminticum* seeds showed antifilarial activity against *Seteria cervi* in vitro (Singhal et al., 1992). Amarsinghe et al. (1993) reported anthelmintic effects of some plants viz., *Caesalpinia bonducalla* (seeds), *Embelia ribes* (fruits), *Ptychetic* sp. (seeds), *Myristica fragrans* (seeds), *Caryophyllum aromaticum* (fruits) and *Cuminum cyminum* (seeds) against *Enterobius vermicularis* and *Ascaris lumbricoides* in children but not against *Ancylostoma duodenale.* Padmaja et al. (1993) studied anthelmintic activities of root barks of *Uvaria lookeri* and *U. narum* against *Haemonchus contortus.*

Recently, Asuzu and Onu (1994) gave an account of anthelmintic activity of the ethanolic extract of *Piliostigma thanningi* bark in *Ascaridia galli*- infected chickens. Javed et al. (1994) reported comparative anthelmintic efficacy of *Caesalpinia crista* seed and *Piperasine adipate* in chicken with artificially induced *Ascaridia galli* infection. Anthelmintic activity of *Melia azedaracha* (= bakain) and papaya latex was reported to be effective against gastrointestinal nematodes in sheep, *Ascaris suum* in pig and *Heligmosomoides polygyrus* in mice (Pervez et al., 1994; Satrija et al., 1994,1995). Chakraborty et al. (1995) worked on herbal drugs viz., *Acacia auriculiformis* used in canine dirofilariaris.

Very recently Asuzu and Njoku (1996) tested the aqueous extract of *Alstonia boonci* bark and *Nauclea latifolia* leaf on *Trichostrongylus* infective larvae. The methanol extract of roots of *Longipedicelata* collected from Nigeria exhibited activity against *Taenia solium* and *Ascaris lumbricoides* in vitro; the actiivity of the extract was 2-4 times lower than that of the reference compound, piperazine citrate (Ayaiycoba and Okogun, 1996). Bogh et al. (1996) studied the anthelmintic effect of an extract of the dried

fruits of *Embelia schimperi* against *H. diminuta* in rats in vivo. The ethanol extract and saponins obtained from the funicles of *Acacia auriculiformis* were reported to be effective on cysticercoid of *H.diminuta*; adult worms were expelled within 5 days from rats (Ghosh et al., 1996). Jost et al. (1996) found anthelmintic effect of *Mallotus philippinensis* (=Kamala) fruit against gastrointestinal nemotodes in goats.

In Meghalaya, a number of indigenous plants are used by the natives believing them to be curative against worm infections (Rao, 1981). *Flemingia vestita* Benth and Hooker (family Leguminoceae), locally known as Soh-phlang, is widely used by the native tribal population of Meghalaya, who consume the pulpy tuberous roots for their anthelmintic activity against intestinal worm infections. In a preliminary study, the whole root-tuber crude extract was reported tobe effective against *Ascaris suum* in vitro (Yadav et al., 1992). The active principles of the root-tuber peels have been isolated by Rao and Reddy (1991); these are isoflavones and have been identified as genistein (0.25%), formononetin (0.033%), pseduobaptigenin (0.015%) and diadzein (0.01%).

Most of the mentioned studies carried out so far pertain to alterations in the physical activity of the test worms following their exposure to the plant crude extract. The mode of action of anthelmintic drugs is known only in respect of few . The tegument (body surface) of the parasite, the interface between the parasite and the environment of the host, seems to be the foremost potential target for action by an anthelmintic. In-vitro treatment of the adult *Artyfechinostomum sufrartyfex* and *Fasciolopsis buski* with the crude extract of the root- tuber peel of *F.vestita* induces paralysis and pronounced tegumental damage and disruption in the tegument (Roy and Tandon, 1996). Observations of alteration in the tegumental organization of the affected worms are suggestive that the plant-derived materials might be acting transtegumentally.

At structural and cellular levels, alterations were also significantly observable in the tegument of helminth parasites (Gonnert and Andrews,1977; Grazywacz, 1980; Imai et al.,1981; Mehlhorn et al., 1983; Schmahl and Mehlhorn, 1985; *Schmahl and Taraschewskhi*,1987; *Bogoyavlenskii et al.*, 1988; Semenkov and Akilzhanov, 1988; Zheng and Zhang, 1988; Delabre et al., 1989; Gijon et al., 1989; Xiao et al.,

1989).Destructive, degenerative and necrotic alterations to the absorption surfaces of *Fasciola hepatica* were prominent after treatment with luxabendazole (Gorchilova et al., 1990). Jiang and Xia (1992) found ultrastructural alterations as the effect of praziquantel and albendazole on *Paragonimus heterticemus* in rats. Perez et al. (1994) worked on the effects of albendazole and albendazole sulphoxide combination therapy on *E. granulosus* in vitro and found ultrastructural changes induced by the former and latter. The drug was shown to provoke significant damage to the tegument of *F.hepatica* treated with luxabendazole (Stoitsova and Gorchilova, 1994) in vivo. Xu-Lin et al. (1994) reported tegumental damages in adult *Schistosoma japonicum* after in -vivo treatment with levo-praziquantel. Anderson and Fairweather (1995) found ultrastructural changes to the tegument of juvenile flukes, *F.hepatica* following incubation in vitro with the deacetylated (amine) metabolite of diamphenethide.

On exposure of male *Onchocerca volvulus* to Mel W and milbemycin in vitro, the muscle of the body wall showed severe damage and the other tissue revealed degenerative changes (Storte et al., 1990). Kozar and Shlikas (1993)

reported structural changes in the tissues and systems of the 4th stage larvae of the nemotode,of *Onchotheca caudin flata* caused by the anthelmintic tetramisole. Rothwell and Sangster (1996) investigated the effect of closantel treatment on the ultrastructure of *Haemonchus contortus* in vitro.

Certain drugs are known to act by binding to the structural proteins of the parasite, particularly those of the muscle and tubulin protein cytoskeleton (Eckert, 1986). Many of the broad spectrum anthelmintics markedly affect the worms by blocking their central nervous system, altering the metabolism and inhibiting the formation of mitochondrial energy, i.e.,oxidative phosphorylation.

The presence of acid phosphastase (AcPase), alkaline phosphatase(AlkPase), adenosine triphophatase (ATPase) and 5'- nucleotidase (5'-Nu) has been detected by histochemical and biochemical means in a number of helminth parasites wherein it is found in close association with the tegument, subtegument, somatic musculature, gut and cuticle (Gupta , 1970; Sharma and Gupta, 1970; Gupta and Sharma, 1973; Maki and Yanagisawa 1980a, 1980b; Barrett, 1983; Pappas, 1988; Gorchilova et al., 1990; Mishra and Srivastava, 1990; Pappas

and Univ,1991; Omar and Raoof, 1994; Keehoon and Changhwan , 1996). All the tegumental enzymes are believed to be involved in the digestion and /or absorptive function in the cestodes (Jones et al., 1979; Roy, 1982; Poljakova et al., 1983). Some drugs such as phenothiazine, diethylcarbamazine and centperazine alter the metabolism and inhibit the formation of mitochondrial energy, i.e., ATPase and deprive the parasite of adenosine triphosphate (ATP) in *Seteria cervi* (Agarwal et al., 1990). Parveen et al. (1992) reported that rafoxamide (RFX) affects the key dehydrogenases involved in the carbohydrate metabolism of *Trichuris globulosa* by blocking the glycolytic pathway and deprives the parasite of energy. It has also been suggested that RFX and albendazole cause high leucine amino transferase activity in *Fischoedereious cobboldi* compared to that of aspartate transferase, suggesting the diversion of the glycolytic cycle of the pyruvate level towards the CO_2 fixation (Vanaja and Rao, 1992). McCracken and Taylor (1983) investigated biochemical effects of thiabendazole and cambendazole on *H. diminuta* in vivo; the failure of glucose uptake could lead to the depletion of parasitic AlkPase activity. Pappas (1988) studied AcPase activity in

the isolated brush border membrane of the tapeworm, *H.diminuta* and found that the AcPase activity was inhibited specifically by levamisole, 2-mercaptoethanol and ethylene diamino tetra- acetate (EDTA). Gorchilova et al. (1990) reported the functional characteristics of the tegument and intestinal wall in mature *F. hepatica* after treatment with luxabendazole and found alterations in the activity of AcPase, ATPase, inosino triphosphatase and succinate dehydrogenase. Similarly, thiabendazole and mebendazole changed the activity of AcPase in *Nippostronglyus brasiliensis* in infected mice (Mishra and Srivastava, 1990). However, the aqueous extract of some medicinal plants viz., *Butea monosperma, Embelia ribes* and *Roltlesia tinctoria* caused a reduction in both AcPase and AlkPase activities of *Paramphistomum cervi* in vitro (Chopra et al., 1991). Likewise, praziquantel induced AlkPase activity on the surface of the adult *Schistosoma mansoni* (Fallon et al., 1994 b).

Many drugs are also suggested to affect the neuromuscular system by inhibiting the neuromodulators (viz., bioamines, acetylcholine and neuropeptides), and causing thereby paralysis or death of the treated worm

(Cox, 1994; Raether, 1988). The presence of cholinesterase (ChE) has been detected by biochemical and histochemical means in a number of cestode species, wherein it is found in close association with the tegument, subtegument and parts of the reproductive system (Lee et al., 1963; Lee and Hodsden, 1963; Lee and Tatchell, 1964; Kotikova and Kuperman, 1977, 1978; Gustafsson, 1984; Ramakrishna et al., 1989; Fairweather et al., 1990; Rahemo, 1993). Acetylcholine (ACh), is thought to serve as an inhibitory neurotransmitter (Wilson and Schiller, 1969; Lumsden and Specian, 1980) and have potent inhibitory effect on the muscle activity in *Hymenolepis diminuta* and *H.microstoma* (Thomson and Mettrick, 1984). Acetylcholinesterase (AChE) is the enzyme responsible for removing ACh at synapses in helminths (Bryant and Behm,1989). Some anthelmintics such as praziquantel, are reported to cause enhanced activity of nonspecific esterases (NSE - which are associated with the tegumental membrane) as evident from the quantification in *Schistosoma mansoni* of drug-induced exposure of the enzyme (Fallon et al., 1994 a). However, active components of some medicinal plants, viz., *Holarrhena antidysenterica* and *H. pubescens* are reported to have a high AChE activity (Tin et

al., 1994).

As major constituents of biological materials and with a wide range of properties and functions, amino acids have recently emerged as potential targets for anthelmintic development. Differences between the pathways of amino acid metabolism in helminths and their mammalian hosts are being exploited in newer drug designs. Newer information is now accumulating with regard to amino acid metabolism in helminths (Barrett, 1991), more so for cestodes.There are reports that in *H. diminuta* the major free amino acid is alanine (Daugherty, 1952; Foster and Daugherty, 1959; Campbell, 1963; Chappel and Read, 1973; Wack et al., 1983; Webb, 1986). There are published reports on the free amino acid pool of several cestode species (Dabrowski, 1980; Soutter et al., 1980; Pathak et al., 1980; Gaur and Agarwal, 1981; Nanda et al.,1981; Bhalya et al.,1983, 1984,1985; Niyogi and Agarwal, 1983) and evidences suggest that free amino acids may be involved in osmotic regulation (Lussier et al., 1978; Wack et al., 1983). Of the hitherto studied helminths, most have been found to excrete significant amounts of nitrogen in the form of amino acids, peptides or proteins. In cestodes, too, free amino acids constitute a

large proportion of the excreted amino nitrogen. The exeretion of amino acids can provide a means of detoxifying ammonia. Most of the intermediates in amino acid synthesis or catabolism are precursors for other pathways involving decarboxylation and hydroxylation reactions. Ammonia is known to have a neurotoxic affect and also to cause various neurological disorders (Cooper and Plum, 1987; Campbell, 1991). Paralysis of the worm may be one such manifestation of the same.

In view of the plausible vermifugal effect of *F.vestita* root tuber peel against the cestode, *Raillietina echinobothrida* (Megnin, 1880),an in depth investigation on the anthelmintic activity and mode of action of the active principles of this plant material seemed desirable. The present study, therefore, aims to provide clues to the likely targets in the parasite on which the putative anthelmintic component of the test plant might be working.It is intended (i) to ascertain the effect of the plant-derived components on the motility, survival and structural organization in the parasite, (ii) to see alterations in the distribution and also the activity of tegumental enzymes, viz., AlKPase, AcPase, ATPase and 5'-Nu in the treated

parasite with relation to the control, (iii) to find out the changes, it any, in the levels of different free amino acids both qualitatively and quantitatively, and also the levels of collagen in the treated worm with relation to the control, (iv) to study the alterations in the tissue ammonia levels in the parasite, and (v) to observe the effect, if any, on the neuromuscular system of the treated worm.

MATERIALS AND METHODS

EXPERIMENTAL PARASITE AND HOST

The adult cestodes, *Raillietina echinobothrida* (Megnin, 1880), were collected on autopsy from the intestine of domestic fowl in 0.9% phosphate buffered saline (PBS: Nacl 8g; $KHPO_4$ 0.34g and K_2HPO_4 1.21g in one litre of distilled water, pH 7 - 7.3) from freshly slaughtered hosts at local abattoirs in Shillong.

PLANT MATERIALS

Flemingia vestita Benth and Hooker (Family Leguminoceae) is a trailing herb (Fig.1), having a hairy stem of around 80-100 cm height and is cultivated by native population of Meghalaya. The edible part is soft,fleshy tuberous root (white) just below the crown and the peels of this root tuber are considered to be effective against soft-bodied parasites i.e., flatworms and tapeworms (Roy and Tandon,1996).

EXTRACT FROM *F.vestita*

Fresh tuberous roots of *F.vestita* were collected in October-November,1994 from neighbouring villages of Shillong (Meghalaya). They were washed thoroughly with tap water and then with distilled water, and were dried in the sun light and peeled off thinly. The peels were dried at 50°C in an oven and the known weight of the same was placed in rectified spirit (100 g /litre) in a reflux flask. The resulting suspension was refluxed for 8 h at 60°C and the cooled suspension was decanted. This reflux process was repeated at least 3 times. The solution was then filtered first through a fine cloth and then through Whatman filter paper (So-I). The clear rectified spirit solution was put to distillation for removal of the solvent and a semisolid crude extract of the plant material was obtained. The latter was weighed and used for further processing and kept at 4°C. 28 g crude extract was obtained from 100 g of dried peels.

contained a mixture of other isoflavones, namely, formononetin and pseudobaptigenin mixture, and diadzein (Rao and Reddy, 1991). 6.5 g residue was obtained from 10 g crude extracet. Synthetic genistein (Sigma, Code No. G 6649) was also used besides the pure genistein extracted from the root peel of *F.vestita* .

Praziquantel was used as the reference substance.

EXPERIMENTS

The parasites, *R. echinobothrida*, were incubated at 37°± 1°C in media containing 0 (control), 0.5, 1, 2, 5, 10, 20, 40 and 50 mg/ml crude extract and 10, 20, 40, and 50 mg/ml residue in PBS with 1% Dimethylsulphoxide (DMSO). The parasites were also tested against pure genistein (0.2 and 0.5 mg/ml). *R. echinobothrida* was further tested against formononetin- pseudobaptigenin mixture (0.5 mg/ml) and 0.001, 0.005 and 0.01 mg/ml of praziquantel (Droncit,Bayer). One set of petri dishes containing 5-10 ml of 1% DMSO in PBS was maintained as control for each concentration. A particular concentration was tested against batches of 3 worms each having approximately the same size

PURIFIED COMPOUND

The purified active component was obtained from the crude extract following the method of Rao and Reddy (1991). The alcoholic crude extract was mixed well with hexane and the supernatant solution was decanted. This process was repeated 20 times. The extract was reduced in volume through distillation and passed through silica gel column using hexane, benzene and ethyl acetate as solvents. The column was prepared using hexane and benzene mixture (6:4) and silica gel 100-200 mesh (about 350 g). The sample (10 g) was mixed with silica gel 60-120 mesh loaded on top of the column. Elution was done with hexane : benzene mixture at the ratio of 6:4, 5:5 and so forth to pure benzene and thereafter with benzene : ethyl acetate (9:1, 8:2, 7.3, 6:4, 5:5... till pure ethyl acetate) using about 300 ml of mixed solvent at each ratio.

At a concentration of 7:3 benzene: ethylacetate, genistein came out.Genistein solution was concentrated through distillation in a water bath and collected as pure genistein (approximately 15 mg). The residue (i.e.,crude extract minus genistein) was eluted with methanol and

and weight. Three replicates for each concentration were used. Time required for complete inactiveness or paralysis and death of the parasite was recorded. After exposure to the treatment the paralyzed cestode (treated with 50 mg/ml crude extract,0.5 mg/ml genistein and 0.01 mg/ml praziquantel) was processed for microscopical, histochemical and biochemical studies along with one set of control specimens.

The anthelmintic efficacy was determined in terms of motility, survivability and histomorphological changes, if any, in the treated worms. Permanent immobilization of treated and control worms was assayed visually when no motility occurred on physically disturbing them. Time required for complete inactiveness or paralysis and death of the parasite was recorded. The death was ascertained by dipping the parasite in slightly warm water at 40-50°C, which induced movements in the worm still alive.

SCANNING ELECTRON MICROSCOPY (SEM)

For SEM, the material was fixed in 10% neutral buffered formalin at 4°C for 24 h. Following fixation the material

was washed in PBS and dehydrated with ascending grades of acetone to pure dried acetone. The specimens were then critical-point dried using liquid carbon dioxide as the transitional fluid. In lieu of critical- point drying, the material was treated with tetramethyl silane (TMS-$(CCH_3)_4Si$, boiling point 26.3°C, surface tension 10.3 dynes/ cm at 20°C) for 15 min and air dried at 25°C. The dried material was put on metal stub in required orientation and sputtered with gold in a fine-coat ion sputter, JFC-1100 (JEOL). The metal -coated specimens were observed using a JSM 35CF (JEOL) scanning electron microscope at electron accelerating voltage ranging between 10-20 Kv.

TRANSMISSION ELECTRON MICROSCOPY (TEM)

For TEM, from both control and treated worms, small-sized pieces (1-2 mm^2) of mature proglottides were fixed in 3% glutaraldehyde solution buffered with 0.1M sodium cacodylate at pH 7.2 at 4°C for 2-4 h. Following primary fixation, the specimens were postfixed with freshly prepared 1% OSO_4 in cacodylate buffer for 2 h at 4°C. The materials were washed thoroughly with the same buffer, dehydrated with

ascending grades of acetone for 10 min with 2 changes in each grade, kept in dried acetone for 1 h,cleared with propylene oxide and embedded in araldite medium. First, semithin sections of 1μm thickness were cut using a glass knife in a LKB- 2088 Bromma- microtome and picked up on a clean glass slide with a drop of water. They were dried at 80°C for a few minutes and stained with toluidine blue. The stained sections were washed thoroughly with tap water and then with distilled water and air dried nicely for observation under the light microscope. Ultrathin sections were picked up on copper grids (mesh size 200/300) and stained with uranyl acetate and lead citrate for 10-15 min. Observations with semithin sections were made under Leitz - Ortholux research microscope. Ultrathin sections were observed under a JEOL JEM- 100 CX magnification range of 2,000- 27,000 X and micrographs were taken on Kodak 4439 electron microscope film 100/6.5x 9cm.

HISTOCHEMICAL STUDIES

(a) Nonspecific esterases (NSE)- The live specimens were washed in 0.9% PBS and fixed overnight in 10% neutral

buffered formalin at pH 7.0 at 4°C and were processed for histochemical localization of NSE. Staining for NSE was performed according to the method of Holt and Withers (1952) with minor modification . The materials were washed several times with distilled water at 10°C followed by incubation in an incubation medium at room temperature for a period of 3-24h. The incubation medium consisted of 3 mg of o-acetyl-5-bromoindoxyl dissolved in 0.1 ml ethanol, added to it were 2.0 ml of 0.1 M Tris, 1.0 ml of 0.05 M potassium ferricyanide, 1.0 ml of 0.05 M potassium ferrocyanide and 1.0 ml of 0.1 M calcium chloride; distilled water was added to make a 10 ml final volume at pH range between 5 - 6.

The nervous system stains deep indigo blue and its components are clearly visible in intact worm.

(b) Cholinesterase (ChE)- Staining for ChE was done following Gomori's method (1952) as described by Rahemo and Gorgees (1987). The specimen was washed with several changes of distilled water and then treated with an incubation medium. The latter contained 20 mg of acetylthiocholine iodide dissolved in 1 ml of distilled water and added to 10 ml of stock solution made up of 0.3 g cupric sulphate, 0.38

g glycine, 1g magnesium chloride, 1.75 g maleic acid, 30 ml 4% aqueous sodium hydroxide and 170 ml 48% aqueous (saturated) sodium sulphate. The incubation time was 16 h at pH 6-7.

Following incubation (both for NSE and ChE) the specimens were washed with distilled water, dehydrated in alcohol, cleared in methyl benzoate and mounted in Canada balsam. The NSE and ChE were localized in the whole mounts of methyl benzoate- cleared specimens by their deep indigo blue and brown staining, respectively and revealed the organization of the nervous system in toto.

(c)Acetylcholinesterase (AChE)- For the distribution and localization of AChE activity, the frozen cross sections of the material fixed in cold formalin at 4°C for overnight were cut at a thickness of 10-15 μm. The sections were processed following the method of Gomori (1952), using standard incubution medium containing cupric sulphate (0.3 g), glycine (0.38 g), magnesium chloride (1.0g), maleic acid (1.75 g), 48% aqueous sodium sulphate and 4% aqueous NaOH (30 ml) as described by Pearse (1968). The sections were incubated for 30 min at pH 6-6.2 at 37°C $\pm$ 1°C, washed with

water several times and freshly mounted in glycerine jelly. The AChE activity present in the tegument and other tissue components was observed by blackish brown colour.

(d) Acid phosphatase (AcPase)- For the demonstration and distribution of AcPase activity the modified Lead Nitrate method (after Takeuchi and Tanoue) as described by Pearse (1968) was employed using β-glycerophosphate as the substrate. The live control and treated parasites were fixed in cold formol calcium fluid (prepared with 4% formaldehyde containing 1% $CaCl_2$ at pH 7.0 with additional $CaCl_2$ at 4°C over night. The fixed materials were extensively washed in water and processed for frozen sectioning. Several sections were cut at a thickness of 10-15 μm. For the localization of AcPase, the sections were incubated in a freshly prepared medium containing 2 volumes 2% sodium-β-glycerophosphate, 1 volume 0.1 M acetate buffer (pH 5.0-6.0), 1 volume 2% lead acetate and 0.3 volume 1-5% $MgCl_2$, at 37°C for 2 h. After staining the sections were rinsed in distilled water and developed in ammonical silver nitrate solution (prepared by adding 28% ammonia water drop by drop to 5% aqueous $AgNO_3$ until the precipitate just

dissolved) for 30 min. Then the specimens were rinsed in 5% sodium thiosulphate for 5 min, dehydrated, cleared and mounted in Canada balsom. A brownish precipitate indicates sites of AcPase.

(e) Alkaline phosphatase (AlkPase) - A modified coupling azo-dye method described by Pearse (1968) was used for the determination of AlkPase activity. The specimens were fixed in 10% neutral buffered formalin at 4°C for 10-16 h. The specimens were washed thoroughly with running water and processed for frozen sectioning. Sections were cut at 10-15 μm thickness and mounted on clear slides without adhesive and allowed to dry in air for 1-3 h. Frozen sections were incubated in fast violet B (2- benzoxylamine-4 methoxytoluidine) freshly prepared in 0.1 M stock tris buffer at pH 10.0 at room temperature (17-20°C) for 15-60 min, washed in running water for 1-3 min, counter stained in Mayer's haemalum (1-2 min), again washed in water for 30-60 min and mounted in glycerine jelly. The sites of AlkPase activity are coloured brown with salts, nuclei dark blue.

(f) Adenosine triphosphatase (ATPase)-For the demonstration of ATPase activity, calcium method (after Maengwn - Devies et al., as described by Pearse, 1968) was used. The fresh frozen sections of 12-14 μm thickness were incubated in a freshly prepared incubatiion medium (pH 9.9) containing 0.1M glycine and 0.4 M KCl in saturated sodium acetate, 0.36 M $CaCl_2$, 1.0 M KOH, 0.04 M Na-ATP, distilled water and saturated sodium phosphate for 0.5-3 h. The sections were then washed in 3 changes of 1% $CaCl_2$ in 75% ethanol, transferred to 2% cobalt chloride for 3 min and developed in 1% yellow ammonium sulphide, finally washed in distilled water and mounted in glycerine jelly. The activity of ATPase shows as a blackish brown deposit.

(g) 5'- Nucleotiodase (5'-Nu)- For the study of 5'- Nu activity the lead method (after Wachstein and Meisel, 1957) was employed using adenosine monophosphate (AMP) as the substrate. The live specimens were fixed in cold formol - calcium (pH-7.0) at 4°C for overnight, washed in water and processed for frozen sectioning. Frozen sections were cut at 10-15 μm thickness and incubated directly into the reaction medium (pH 8.3) at 37°C $\pm$ 1°C for 30 min. The reaction

mixture contained 1.25% AMP, 0.2M Tris-buffer (pH 7.2), 0.2% $Pb(NO_3)_2$ and 0.1M $MgSO_4$. The reaction was stopped with the addition of 2 ml of 40% formaldehyde for 30 min. The sections were then treated with dilute yellow ammonium sulphide for 2 min and again rinsed with double distilled water and mounted in glycerine jelly. Yellow deposits of lead sulphide indicate sites of 5'-Nu enzyme activity.

(h) Collagen- For localizing collagen, the live specimens were fixed in Zenker's fluid for 24 h at room temperature. and then duly processed for histochemical test.The technique employed is following Emmel and Cowdry (1970). Paraffin sections of 7-8 μm thickness were cut and hydrated through a descending series of alcohol to water. The fixed sections on the glass slide were stained with aqueous acid fuchsin (0.5% Mallory's I) for 1-5 min, rinsed with distilled water and in 1% aqueous phosphomolybdate for 1 min .Then the sections were kept in glacial acetic acid and saturated mercuric chloride (1:1) for 1-3 min. For removal of $HgCl_2$, the sections were kept in Lugol's iodine (1% in 70% alcohol) for 5-10 min, followed by water rinse and bleached in 5% sodium thiosulfate. The sections were again washed in

distilled water and stained in Mallory's II (made with 2% Orange G and 0.5 % aniline blue, 1:1) for 15-20 min. The stained sections were rinsed with water, dehydrated, cleared in xylene and mounted in DPX. The collagen present in the tegument and other tissue components takes blue colour.

BIOCHEMICAL ASSAYS:

(a) Acetylcholinesterase (AChE)-The enzyme activity was assayed following the method of Ellman et al. (1961), as given by Ott et al. (1975), where thiocholine reacts with dithiobis nitrobenzoic acid which was estimated colorimetrically.

(i) *Preparation of 10% tissue homogenate*

A 10% (w/v)tissue homogenote was prepared in 0.2M sucrose solution with a Potter- Elvehjem motor-driven glass homogenizer with a teflon pestle at 0 ± 2°c, centrifuged at 20,000 g at 4°c for 30 min and the supernatant was used as the enzyme source.

(ii) The assay mixture in a final volume of 3 ml contained the following :

Sodium phosphate buffer, pH 7.4 - 150 mM

Acetylthiocholine iodide	-	10 mM
5-5 -Dithiobis nitrobenzoic acid	-	1.25 mM
Triton X- 100	-	0.3%
Enzyme extract	-	0.1 ml

The reaction mixture was incubated in a rectangular quartz cuvette (having 1 cm light path) at 37°± 1°C for 3-5 min in a UV visible spectrophotometer (Beckman Model-26) fitted with peltier type temperature control unit. After adjusting the optical density (O.D) to zero, the required amount of enzyme solution was added to the experimental cuvette. The decrease or increase in O.D. at 412 nm was recorded at 30 sec intervals. The period of linear decrease or increase in O.D. was used for calculation of enzyme activity. One unit of AChE was defined as that amount which catalysed the formation of 1μ mole of 5-thio-2 nitro-benzoic acid/ h at 37°C. The enzyme activity was expressed as the total activity (units/ g wet wt of tissue) and the specific activity (units/ mg protein).

(b) Acid phosphatase (AcPase)- The AcPase activity was assayed by estimating the P- Nitrophenol product following the method as given by Plummer (1988) with necessary

modification in the concentration of the buffer and substrate.

(i) *10% (W/V) tissue homogenate*

A 10% (w/v) tissue homogenate was prepared from the parasite in buffer containing 125 mM sodium acetate and glacial acetic acid at pH 4.5 using a Remi-motor-driven homogenizer fitted with a teflon pestle. The homogenate was centrifuged at 5000 rpm at 0 ± 2°C for 20 min and the supernantant was used for enzyme assay.

(ii) The assay mixture in a final volume of 1 ml contained the following:

Sodium acetate buffer, pH 4.5	-	125 μmole
P-Nitrophenyl phosphate	-	62.5 μmole
Tissue extract as enzyme source	-	0.2 ml

The assay mixture was preincubated for 5 min at 37° ± 1°C before addition of the tissue extract to initiate the reaction. 0.2 ml of the enzyme extract was then added and incubated for 10-20 min at 37°C in a serological water bath fitted with a temperature control unit. After a particular time of incubation the reaction was stopped by adding 5 ml of 0.02 N NaOH solution and the absorbance measured at 405 nm. For blank, similar procedure was followd using 0.2 ml

water instead of enzyme solution. The amount of P-Nitrophenol produced was calculated from a linear standard graph prepared using different concentrations (0.1- 1.0 mM) of P- Nitrophenol. One unit of AcPase activity was defined as that amount which catalysed the formation of 1 mM of P-Nitrophenol/h at 37°C. The enzyme activity was expressed as the total activity (units/ g wet wt. of tissue) and specific activity(units/mg protein).

(c) **Alkaline phosphatasae (AlkPase)**- The AlkPase activity was estimated following the same method as for the AcPase activity.

The reaction mixture of 1 ml contained the following:

P- Nitrophenyl phosphate - 31.25 μmole

Sodium glycine buffer, pH 10.5 - 0.1 μmole

10% tissue extract - 0.1 ml

The incubation was carried out at 37° ± 1°C in glass centrifuge tube for 5 - 20 min and the reaction was stopped by adding of 0.02N NaOH solution to the reaction mixture prior to the addition of enzyme. The decrease or increase in O.D. at 420 nm was recorded and the amount of P- Nitrophenol produced was calculated from a linear standard graph

prepared using different concentrations (0.1- 1.0 mM) of P-Nitrophenol.One unit of AlkPase activity was defined as that amount which catalysed the formation of 1 mM of P- Nitrophenol/h at 37°C ± 1°C . The enzyme activity was expressed as the total activity (units /g wet wt of tissue) and specific activity (unit/ mg protein).

(d) Adenosine triphophatase (ATPase)- The ATPase activity was assayed by estimating the free phosphate released following the method of Kaplan (1957).

(i) *10% tissue homogenate (w/v)*

The parasite tissue was frozen immediately after paralysis at- 20°C until used for estimation. The estimations were completed within three days of sampling during which enzyme activities did not alter.

Each frozen tissue was thawed on ice and 10% homogenate (w/v) was prepared following Kaplan (1957) in glycine buffer at pH 9.1 .The homogenate was sonicated for 30 sec using 10% tissue homogenate and Triton-X (0.5% v/v final concentration at 1:1 ratio) before ATPase assay. This treatment was standardized and found to be optimum.

(ii) The assay mixture in a final volume of 1.8 ml

contained the following:

Glycine buffer , pH 9.1	- 200 μmole
$CaCl_2$	- 100 μmole
Na-ATP	- 100 μmole
Tissue extract as enzyme source	- 0.05 ml

The assay mixture was preincubated for 5 min at $37^{\circ} \pm 1^{\circ}C$ before addition of the tissue homogenate to initiate the reaction. The reaction mixture was terminated after 1 h by addition of 1 ml of 15% trichloroacetic acid (TCA) to the reaction mixture. The tissue blank was prepared for each assay with the addition of TCA prior to the addition of tissue extract. The precipitated protein was separated out by centrifugation at 3000-4000 rpm for 10 min at room temperature. The phosphate formed was estimated in the supernatant following the method of Fiske and Subba Rao (1925). To 0.25 ml of the supernatant was added 0.75 ml water, 0.5 ml 5 N H_2SO_4,0.5 ml ammonium molybdate (2.5% w/v) and 0.1ml freshly prepared Fiske and Subba Rao reducer (2.5% w/v solution in water of the mixture of 6 part sodium sulphate :6 part sodium metabisulfite: 1 part 1- amino-3 napthol-4-sulphonic acid). The reaction mixture was incubated for 10 min and the O.D. taken at 700 nm at room

temperature against zero time tissue blank where TCA was added prior to the addition of enzyme. The amount of phosphate present was calculated from a standard graph prepared using different concentrations of a monophosphate (0.1-1 μmole of KH_2PO_4), which was linear. One unit of ATPase was defined as the amount which catalyzed the release of 1 μmole of phosphate /h at 37°C ± 1°C from ATP. The enzyme activity was expresed as the total activity (units / g wet wt of tissue) and specific activity (units/ mg protein).

(e) 5 '- Nucleotidase (5 '-Nu) - 5'- Nu activity was assayed by estimating the free phosphate released following the method of Bunitian (1970).

(i) *Preparation of 10% (w/v) tissue homogenate*

A 10% tissue homogenate was prepared following the method of Bunitian (1970) in tris-HCl buffer at pH 7.45.

(ii) The assay mixture in a final volume of 1 ml contained the following :

Tris- HCl buffer, pH 7.45	- 40 μmole
$MgSO_4$	- 12 μmole
AMP	- 5 μmole

10% tissue homogenate as enzyme source - 0.1 ml

The reaction mixture was preincubated for 5 min at 37° ±1°C before addition of the tissue extract to initiate the reaction. The reaction was terminated after 1 h by addition of 1.5 ml of 10% (w/v) perchloric acid (PCA) to the reaction mixture. The tissue blank was prepared for each assay with the addition of PCA prior to the addition of the tissue extract. The precipitated protein was separated out by centrifugation at 3000 rpm for 10 min at room temperature. The phosphate released was estimated in the supernatant following the method of Fiske and Subba Rao (1925). To 0.5 ml of the supernatent were added 0.5 ml water,0.5 ml 5 N H_2SO_4, 0.5 ml ammonium molybdate (2.5% w/v solution in water of the mixture of 6 part sodium sulphate: 6 part sodium metabisulfite : 1 part 1-amino-2 napthol, 4-sulphonic acid). The reaction mixture was incubated for 10 min and the O.D. taken at 700 nm at room temperature against zero- tissue blank where PCA was added prior to the addition of the enzyme. The amount of phosphate present was calculated from a standard graph prepared using different concentrations of a monophosphate (0.1- 1.0 μmole of KH_2PO_4), which was

linear. One unit of 5'- Nu activity was defined as that amount which catalysed the release of 1 μ mole of phosphate/h at 37°C from adenosine monophosphate. The enzyme activity was expressed as the total activity (units/g wet wt tissue) and specific activity (units/ mg protein).

(f) Ammonia

(i) Concentration of ammonia in the parasite tissue and effluent was measured enzymatically based on the procedure of Kun and Kearney (1974). Immediately after collection, to the sample (1ml of incubation medidum), 10 μl of 2 M PCA was added in each test tube to precipitate out the protein present in the sample. The precipitate was separated by centrifugation and the supernatant was neutralized by adding, 10 μl of 2 M NaOH in each tube before the measurement of ammonia.

(ii) *Preparation of 20% tissue homogenate for ammonia estimation*

The parasite materials were frozen immediately at -20°C until used for estimations. The frozen tissue was thawed on ice and 20% tissue homogenate (w/v) was prepared following Bergmeyer et al. (1974) in tris- HCl buffer (100 mM) at pH

8.0. The homogenate was centrifuged at 600xg for 10 min at room temperature. To the supernatant 5% PCA was added (1 :0.5 ratio) and centrifuged again. The pellet was discarded and the supernatant was neutralized with 2 M NaOH (1:0.5 ratio) and used for ammonia estimation.

(iii) The assay mixture of 1 ml contained the following:

Tris -HCl buffer, pH 8.0	- 66 μmoles
α- Ketoglutarate	- 2.5 μmoles
EDTA	- 0.2 μmole
ADP	- 1.0 μmole
NADH	- 0.4 μmole
GDH	- 20 Units
Incubation medium containing the effluent	- 0.2 ml

The reaction mixture was incubated at 37°C for 30 min. A control was run simultaneously which contained everything in the reaction mixture as mentioned above except the effluent which was replaced by 0.2 ml of distilled water. O.D.was measured at 340 nm both in control and in effluent reaction mixture containing the effluent. The difference in O.D. value obtained between these two was used to calculate the concentration of ammonia present in the effluent taking

6.22×10^3 as the molar extinction coefficient for NADH.

(g) Protein-The protein was estimated following the method of Lowery et al. (1951) using crystalline serum albumin as standard.

(h) High performance liquid chromatography (HPLC)

(i) *Preparation of 10% tissue homogenate for free amino acid analysis (FAA)*

A 10% (w/v) tissue homogenate was prepared in distilled water at 2± 1°C. Protein was precipitated out by adding 5% perchloric acid (PCA, prepared in 0.4N lithium citrate) in the ration of 1:1, followed by centrifugation at 10,000 X g for 20 min at 2 ± 1°C to pellet out the precipitated protein. The clear supernatant was then filtered through Whatman microfilter (0.45 μm pore size) and the pH of the filtrate was adjusted to 2.2 by adding known volume of 0.2N lithium hydroxide. All the processed samples were preserved in deep freeze at -20°C until used for amino acid analysis. All the analyses were completed within 2-3 days after processing the sample.

(ii) *Processing of incubation medium for FAA*

For analysis of amino acids excreted by the parasite in the incubation mediunm, the medium was collected after 1 h of incubation and assumed to have contained the excreted material. The medium was briefly centrifuged at 10,000 x g and 3 ml of supernatant was concentrated to a volume of 0.5 ml, by overnight lyophilization in a lyophilizer . The pH of the lypholized sample was also adjusted to 2.2 with a known volume of 0.2 N lithium hydroxide and passed through Whatman microfilter (0.45μm pore size) before being injected to HPLC for amino acid analysis.

(iii) *Method of FAA analysis*

FAAs and also ammonia, both in the tissue as well as in the incubation medium, were analysed with a Shimadzu HPLC (model LC 4A) with a post-column fluorimetric detection system by using O-phthaldehyde reagent as a fluorescent dye following method of Fujiwara et al.(1987) with certain modifications. A cation- exchange column (Shim-Pack ISC- 07 Li) was used for separation of FAAs. The mobile phases were (A) lithium citrate (0.16 N, pH 2.5) containing 7% of methyl cellusolve, (B) lithium citrate (0.32 N, pH 10.0) containing 12.4% of boric acid, and (C) lithium hydroxide

(0.2 N) . A gradient of buffer A to B for 190 min was used for analysis of FAAs, amino compounds and ammonia. Standard amino acid mixture (Sigma) was also run under identical conditions for both qualitative and quantitative analysis of FAAs.

OBSERVATIONS AND RESULTS

TREATMENTS -PHYSICAL MOTILITY AND LONGEVITY

The worms incubated at 37° ± 1°C in the control medium, i.e.,PBS, showed physical activity for about 72 h. The mode of treatment and observations concerning different concentrations of the test materials are given in Table 1. The results indicate that the crude peel extract of *F. vestita* and its active component, genistein, showed activity on *R.echinobothrida*. Of all the concentrations tested (i.e., 0.5, 1,2,5,10,20,40 and 50 mg /ml of PBS) the concentration of the crude extract as 50 mg/ ml of PBS was effective against *R.echinobothrida* and for genistein it was 0.5 mg/ ml.The cestode initially became paralyzed in the test medium followed by death after a lapse of time. The survival time of the residue (i.e., crude extract minus genistein)-treated worms was longer than that of those treated with genistein -containing crude extract. 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

Table 1. Efficacy of whole crude peel extract; residue extract, i.e., crude extract minus genistin; and active components of F. vestita and refernce drug on *Raillietina echinobothrida* in vitro.

Treatment: concentration (mg/ml)	Time (h) taken for paralysis (P) and death (D) of the worm postincubation	
	P	D
Crude extract:		
0.5	25.1 + 0.1	78.08 ± 0.5
1.0	16.08 + 0.6	65.5 ± 0.5
2.0	9.2 ± 0.5	51.2 ± 0.5
5.0	5.9 ± 0.5	46.1 ± 0.04
10.0	4.08 ± 0.10	33.1 ± 0.6
20.0	2 03 ± 0.08	28.01 ± 0.5
40.0	0.9 ± 0.01	18.08 ± 0.5
50.0	0.3 ± 0.01	6.5 ± 0.4
Residue:		
10.0	8.15 ± 0.15	46.15 ± 0.14
20.0	3.5 ± 0.05	39.2 ± 0.08
40.0	2.35 ± 0.12	29.4 ± 0.07
50 0	2 1 ± 0 06	13 35 ± 0 11
Genistein.		
0.2	6.7 ± 0.04	49.2 ± 0.08
0.5	4.4 ± 0.07	19.08 ± 0.02
Formononetin - pseudobapigenin 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

Data represent mean values ± SD for three experiments. Student's t-test significant. Worms incubated in control medium showed physical activity for 72 ± 0.05h.

P = paralysis : D = death

stimulation the paralyzed parasite regained considerable motility and death occurred after 6.5 h of incubation at 37 ± 1°C. The residue extract showed less activity against *R. echinobothrida* as compared with the crude extract containing genistein.

Worms incubated in the purified chemical component, genistein, at a concentration of 0.5 mg/ ml, showed considerably faster acquisition of a paralytic state. Exposure of the worm 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 at the same concentration. However, the parasites incubated with praziquantel even at lower concentrations (viz., 0.001, 0.005, and 0.01 mg/ ml) showed faster activity as compared to that of the plant product i.e., genistein.

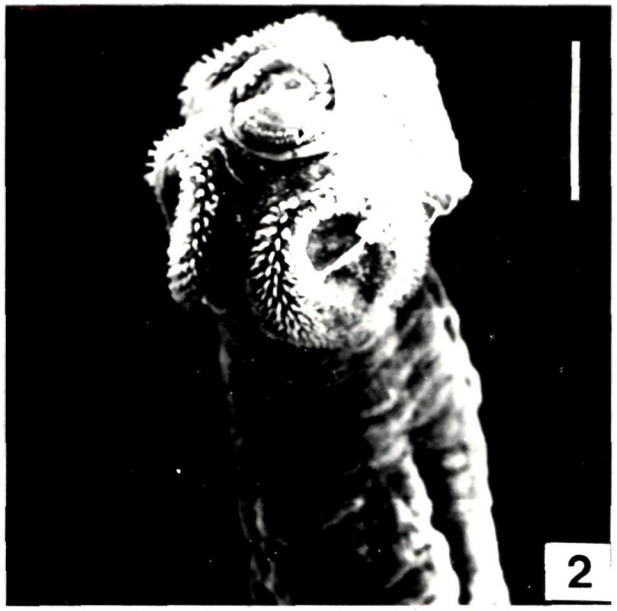
In view of the apparently conspicuous effect of the crude peel extract and genistein component of *F. vestita* on the motility and physical state of *R. echinobothrida*, further experimentation and observatons were continued using the concentration of 50 mg /ml crude extract and 0.5mg/ml genistein test solution, since an early inactiveness or paralysis was noticed at these concentrations.

Fig. 1. Whole Plant of *Flemingia vestita*

Figs.2-6: Scanning electron micrographs of *Raillietina echinobothrida* - Control.

2. Anterior region, showing the scolex and neck region. (Scale bar= 100 μm)
3. Enlargement of the scolex, showing two rows of a rostellar hooks in the rostellum and suckers with spined rims. (Scale bar = 10 μm)
4. Neck region. (Scale bar = 10 μm)
5. Gravid proglottides. (Scale bar= 1μm)
6. Microtriches in the mature proglottid. (Scale bar = 1 μm)


1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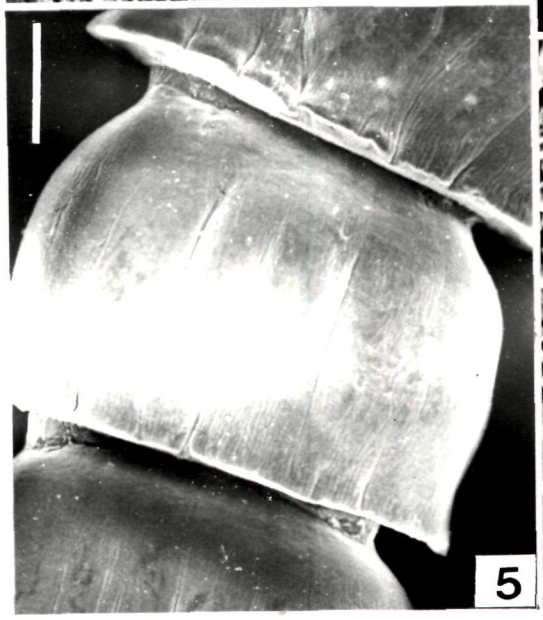
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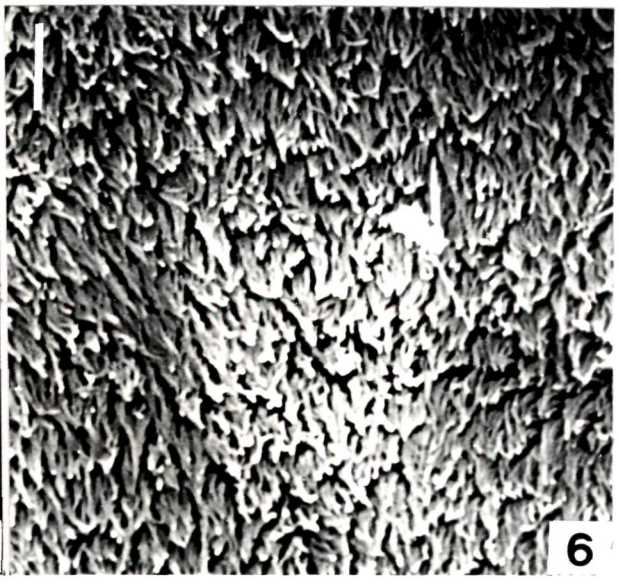

6

MORPHOLOGICAL CHANGES

SEM Obserevations

Control specimens - Observations on the surface topography of the parasite revealed that the whole body of the worm right from the scolex tip to the posterior end is densely covered with fine microtriches giving it a velvety appearence (Figs. 2-6). Under higher resolution the microtriches show structural variations in their distribution on the scolex and strobila. The entire surface of the scolex,including the tegument of the suckers, is covered with dense microtriches (Figs. 2,3). Those in the neck region are thin and slender and have pointed ends. They are uniformly distributed (Fig.4). The strobilar surface (from immature and mature to gravid proglottides) is covered with thick unidirectional conical microtriches which are wider at the base and slightly tapering towards the tip (Fig.6).

Treated worms- After in-vitro incubation of the parasite in media containing 50 mg/ ml crude extract for 5,.10,15,30,

Figs.7-12: Scanning electron micrographs of *R. echinobothrida* treated with crude root-tuber-peel extract of *F. vestia* at 50 mg/ml.

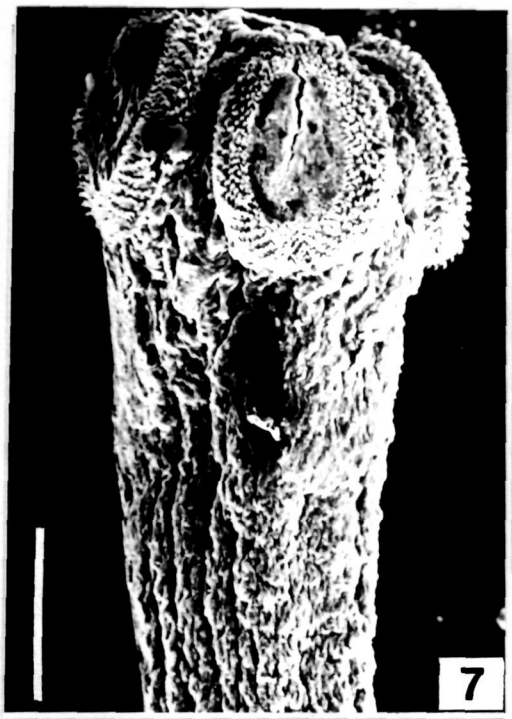
7. Deformity in the anterior region. The scolex and neck region are evident. (Scale bar= 100 μm)

8. Close view of the scolex, showing cracks in the tegument of the sucker. (Scale bar= 10 μm)

9. Tegumental surface of the gravid proglottid after exposure. (Scale bar= 100 μm)

10. Surface of the mature proglottid. Microtriches are conspicuously clumped. (Scale bar= 1 μm)

11,12: Tegumental surface of the immature and mature region after treatment with genistein at 0.5 mg/ml. A Change in the contour of microtriches (in comparison with controls) is noticeable. (Scale bar = 1 μm)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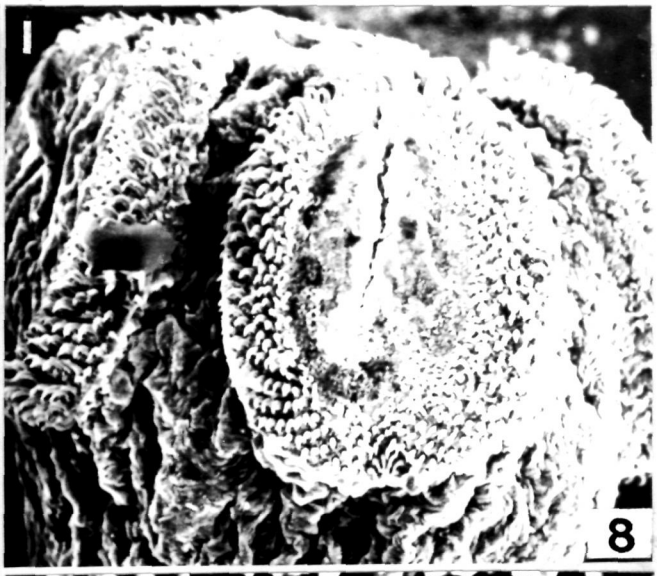
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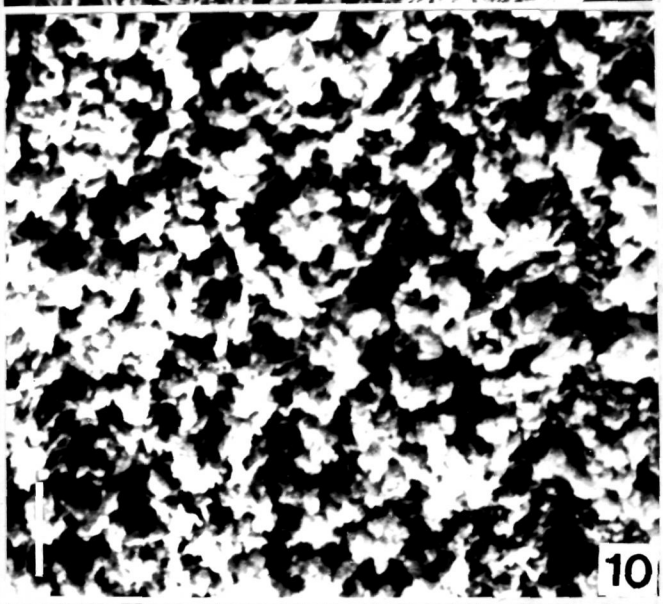
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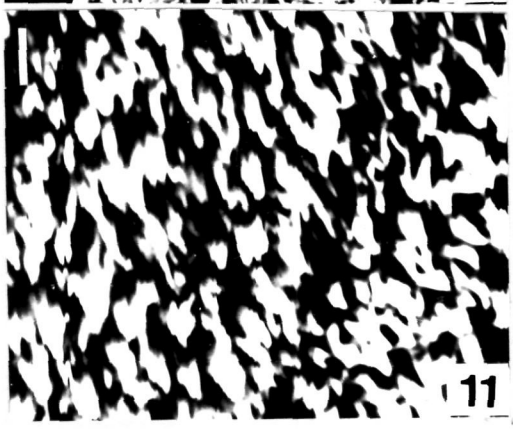
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12

and 60 min, a distinct damage in the tegument of the worm was noticable. The surface became shrunk within 20 min at 37 ± 1°C. The tegumental damage/ change was apparent in the whole worm (Figs. 7-10). While the surface of the suckers revealed cracks, the general tegument appeared distorted (Figs.8.9). The microtriches all through the body exhibited a clumpy appearance with precipitate-like lumps covering them (Fig.10).

Tegumental change in the parasite could also be seen after treatment with genistein at a concentration of 0.5 mg/ ml in the PBS medium; the tips of the microtriches appeared somewhat swollen and stouter than control and showed blunt tips (Figs.11,12).

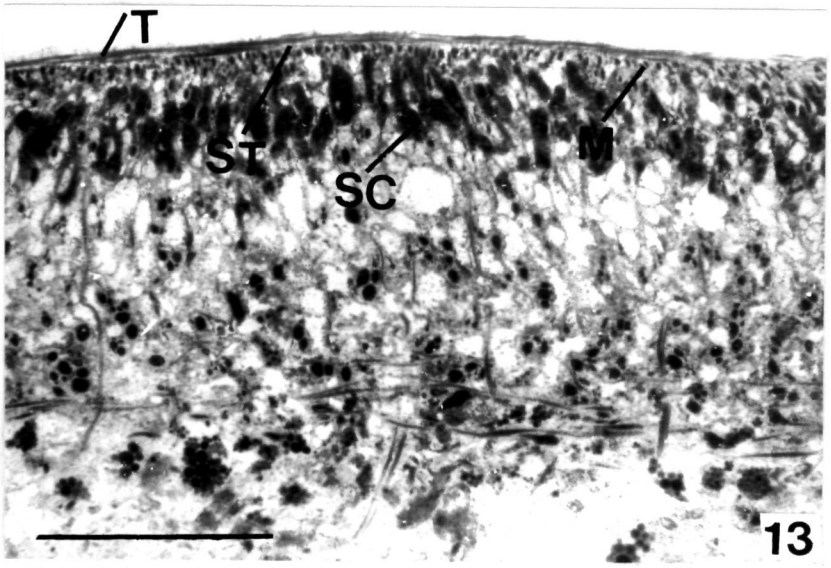
TEM Observations

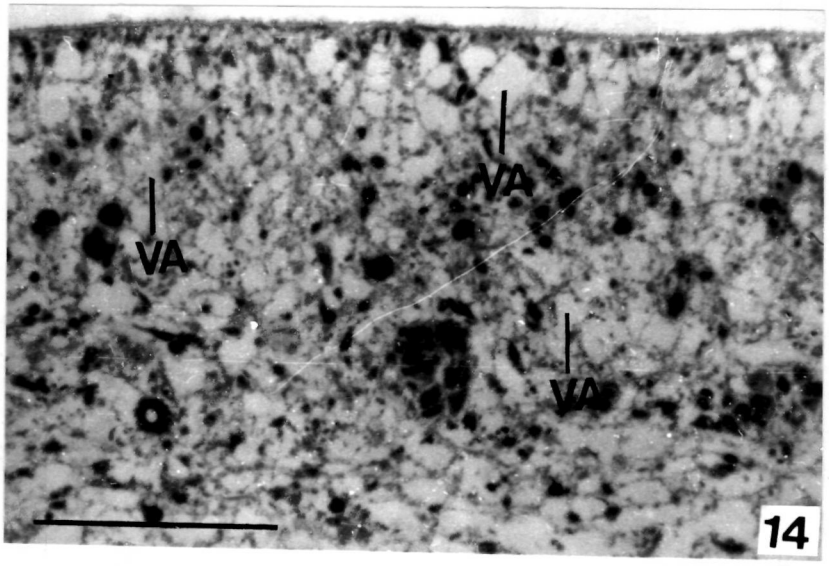
Control- Histologically the body of *R. echinobothrida* is covered with a thin tegument (Figs. 13,14). Ultrastructurally, the body surface is elaborated by the presence of a cytoplasmic zone which is bordered externally by an outer plasma membrane and internally by an inner plasma membrane (Figs. 15,16). The outer plasma membrane

Figs.13,14 : *R. echinobothrida* - Semithin sections, light microscopy.

13. Section of mature proglottid (control), showing normal tegumental and subtegumental organization. Densely staining muscular components (M) in the subtegument (ST) and subtegumental cells (SC) are abundant near the surface. (Scale bar = 0.05 mm)

14. Section of a mature proglottid after incubation with crude peel extract at 50 mg/ml. Note the occurrence of numerous vacuoles (VA) in the tegument and the loss of densely staining components near the surface.(Scale bar= 0.05mm)


T
ST
SC
M
13

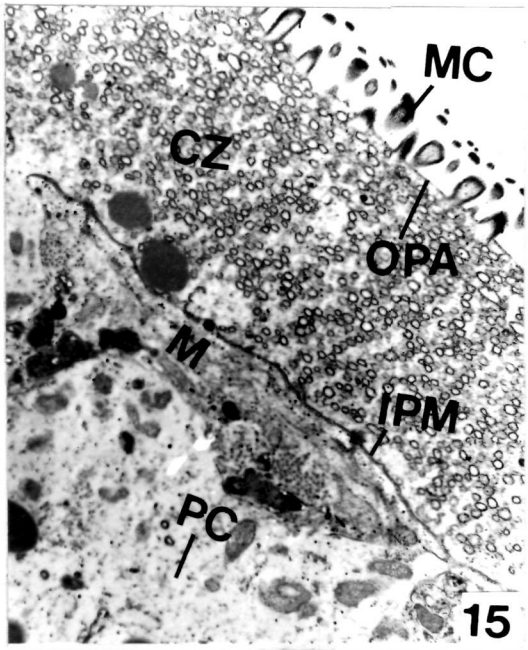

VA
VA
VA
14

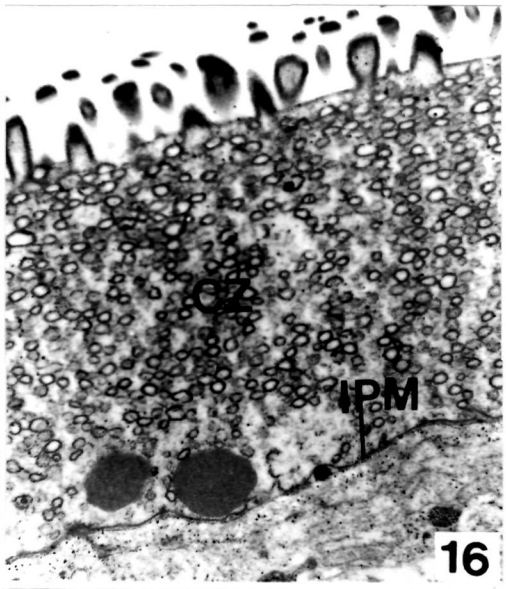
Figs. 15-18: Transmiission electron micrographs of *R. echinobothrida* (Control).

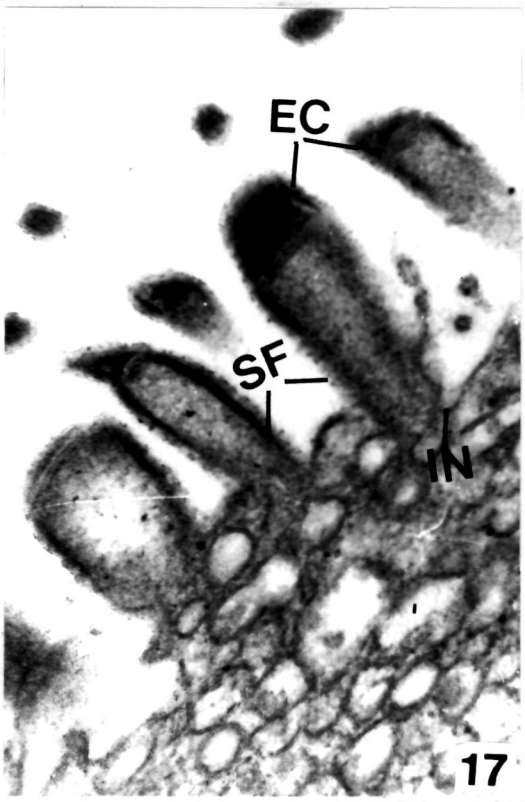
15,16. Tegument in ultrathin section, showing microtriches (MC), outer plasma membrane (OPM), cytoplasmic zone (CZ), inner plasma membrane (IPM), musclecomponents (M) and parenchymal cell (PC). X 7,800 and 12,500, respectively

17. Microtriches as seen at higher resolution. The electron dense cap (EC), the shaft (SF) and invagination (IN) of outer plasma membrane are clearly seen. X 33,750

18. Muscular components, at higher resolution. x 50,000


MC
CZ
OPA
M
IPM
PC
15


CZ
IPM
16


EC
SF
IN
17

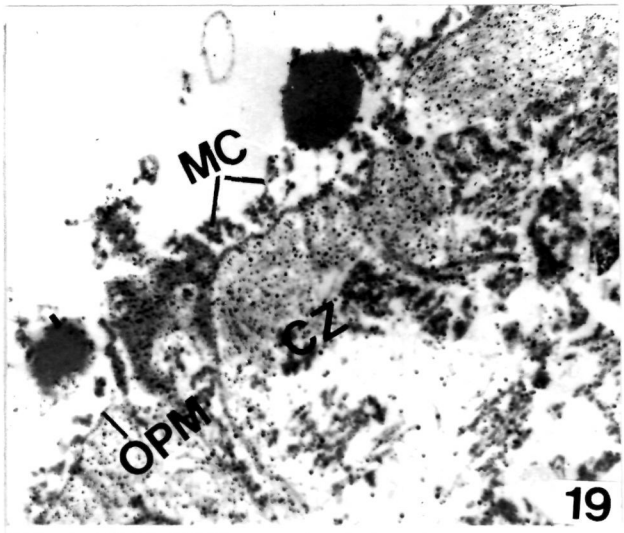

M
18

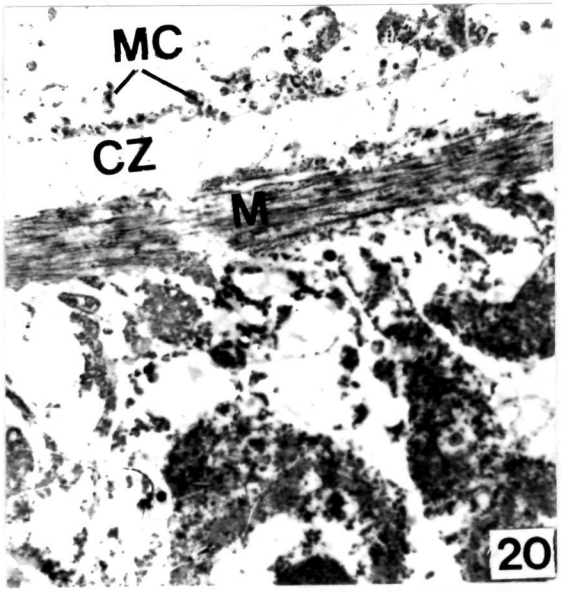
Figs. 19-22. *R. echinobothrida* - Transmission electron micrographs exposed to test materials.

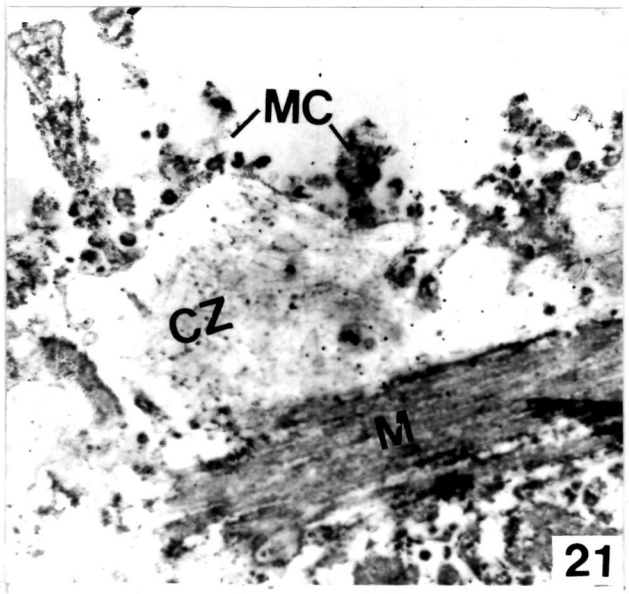
19,20. Section through the tegument of the parasite after treatment with crude tuber peei extract (50mg/ml) of *F.vestita*. Complete disorganization of the microtriches, cytoplasmic zone and muscular components are evident. x 7,800 and 12,5000, respectively

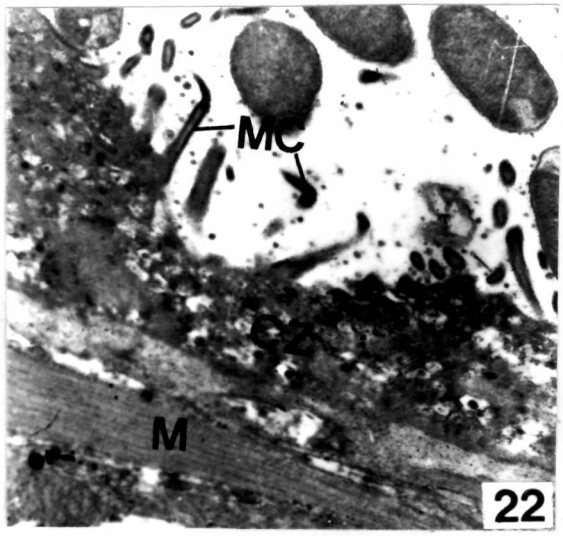
21. Section through the tegument of the parasite after treatment with genistein(0,.5mg/ml). The tegument showed severe distortion with disorganization of the tegumental musculature. x 17,500

22. Same as Fig. 21, after treatment with praziquantel x 12,500


MC
CZ
OPM
19


MC
CZ
M
20


MC
CZ
M
21


MC
M
22

has got inpushings towards the inner side in the form of small invaginations; it is also in continuity with the outer covering of the microtriches (Figs. 15-17). Each microthrix is elongated and distinguished into two parts, an electron - dense cap and a shaft. The electron - dense cap is very dark in appearance, while the shaft has an electron - lucent cap (Fig.17).The cytoplasmic zone is followed by a muscular zone where muscle cells are observed (Fig. 18).

Treated worms - At the histological and ultrastructural levels, changes were noticeable 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. 14,19,20)and genistein (0.5mg/ml), respectively (Fig. 21). Vacuolization became more pronounced after 60 min of incubation, indicating a time-dependent effect. The subtegumental region showed severe distortion with disorganization of the microtriches, cytoplasmic zone and tegumental musculature (Figs. 19-24). Changes were also visible in the praziquentel- treated parasite (Fig.22).

Figs. 23-27. Nervous system in *R. echinobothrida* (photomicrographs, Bromoindoxyl acetate method).

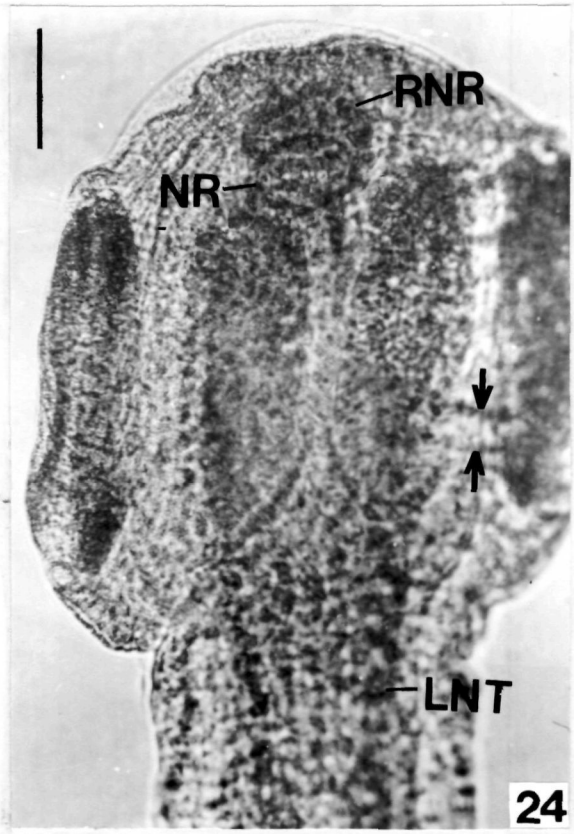
23. Scolex and neck region, showing an intense NSE reactivity revealing the nervous system. (Scale bar = 0.2mm)

24. 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=0.1mm)

25. Innervation in the region of immature proglottides; two prominent lateral nerve trunks (LLNT) are conspicuous.(Scale bar= 0.5 mm)

26,27. 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= 0.05 mm and 0.2 mm, respectively)

23



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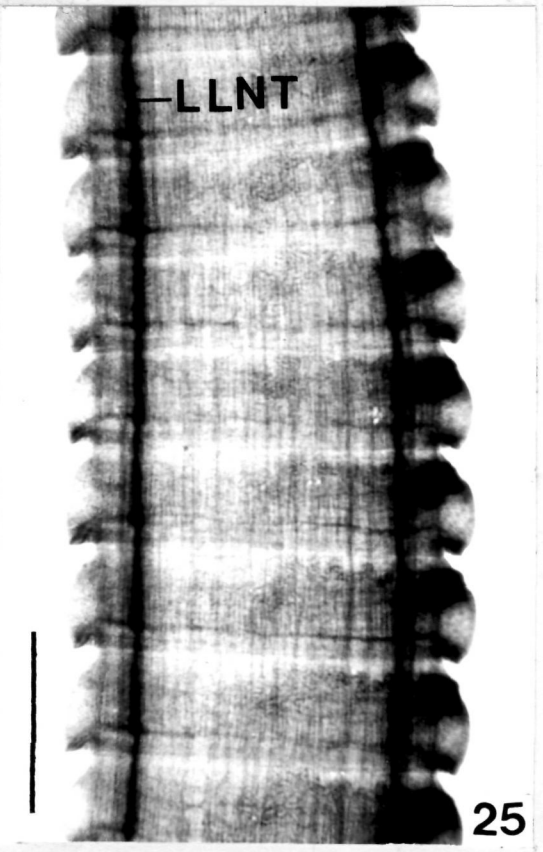

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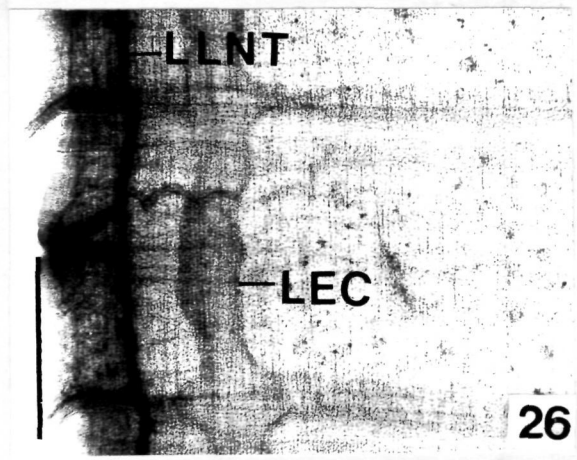

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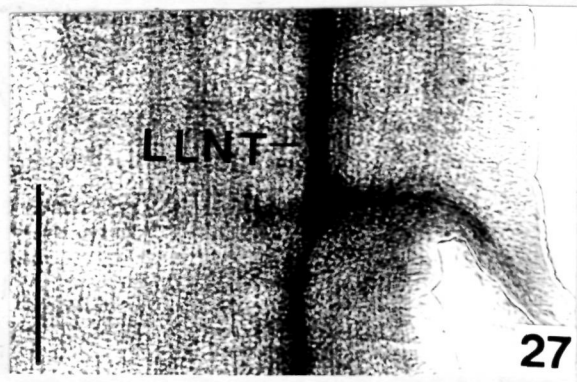


27

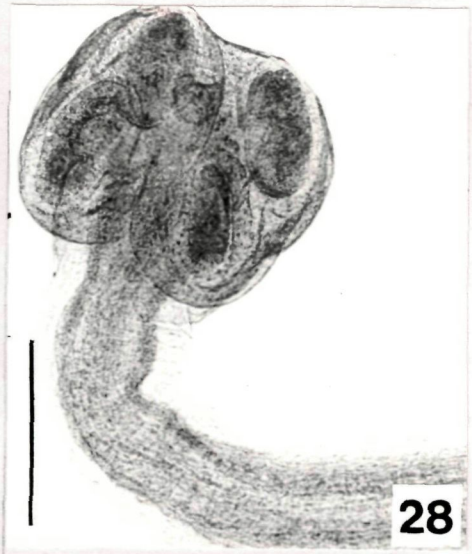
Figs. 28-32: Nervous system in *R. echinobothrida* (photomicrographs, Acetylthiocholine iodide method).

28. Scolex region, showing ChE activity.(Scale bar = 0.2mm)

29,30. Immature proglottides showing the LLNT and other longitudinal nerves; several (10-12) transverse commissures (TRC) joining these nerves form a fine nerve net. (Scale bar= 0.5 mm)

31. A magnified view of the mature proglottides, showing the fine nerve arrangement comprising the LLNT, LNS and transverse commissures. (Scale bar = 0.5mm)

32. Terminal proglottides, showing all the longitudinal nerves joining together at the extreme posterior end. (Scale bar= 0.5 mm)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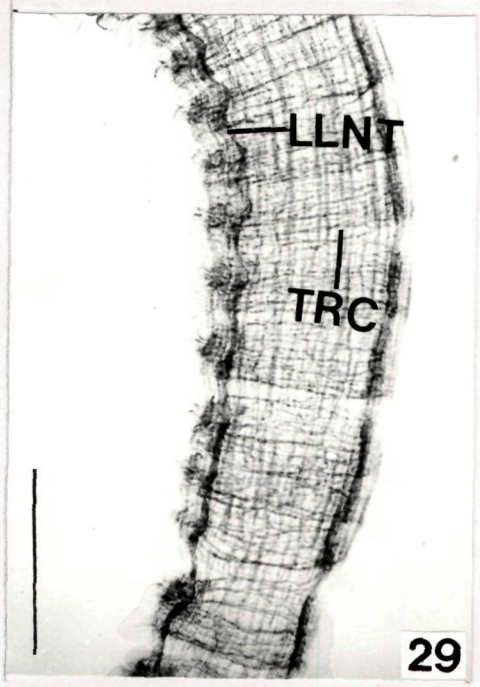
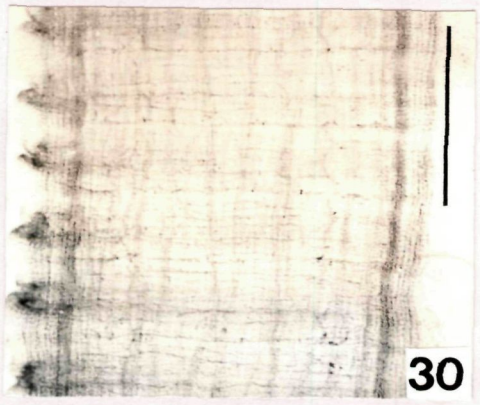

LLNT
TRC
29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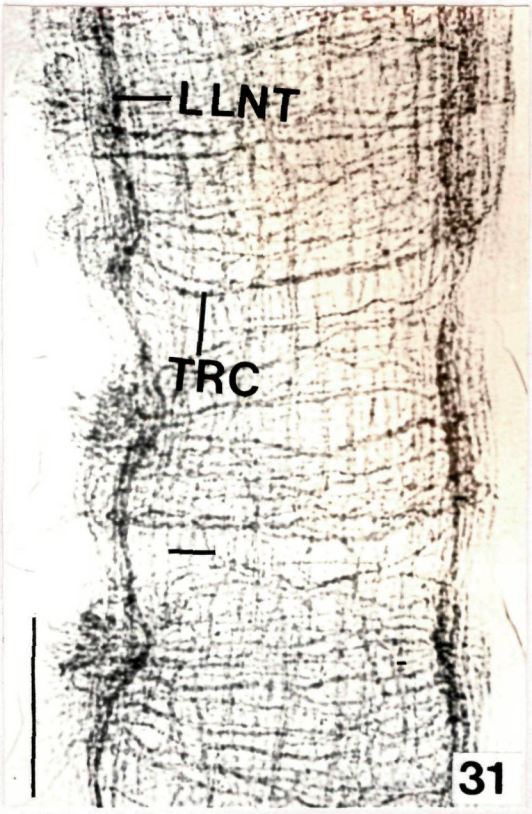
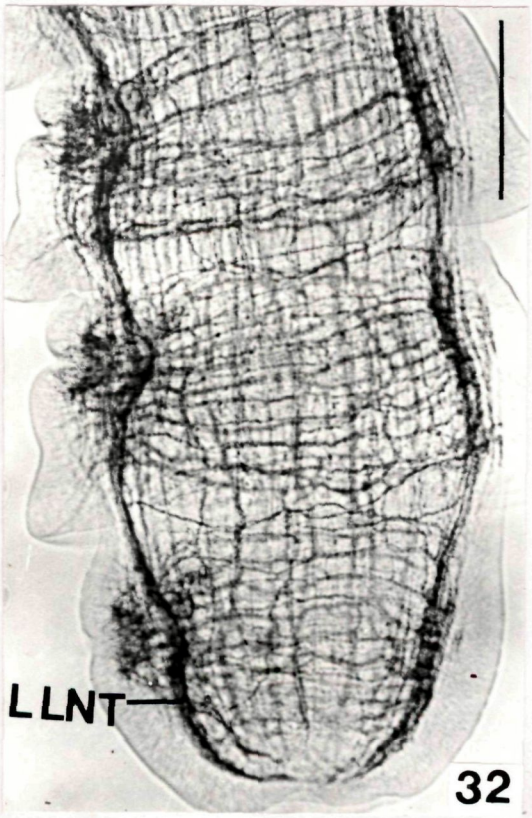

LLNT
TRC
31


LLNT
32

Collagen

Histochemically, no collagen was detectable in the tegument or any other tissue/structures of the parasite.

ENZYMATIC STUDIES

(i) NSE and ChE

Control - With the demonstration of deep indigo blue or brown staining revealing the presence of NSE and ChE activity, the entire arrangement of the nervous system in *R. echinobothrida* could be visualized.

The nervous system of *R.echinobothrida* consists of a central nerve ring below the rostellum, enlarged bilateral extensions of this ring consisting of two cerebral ganglia located in the scolex region. Several transverse small nerves originating from the cerebral ganglia run anteriad and join to form a small rostellar ring. Four longitudinal nerves are seen originating from the cerebral ganglia and run posteriad through the length of the neck. In the scolex region several small branches are given out from each longitudinal nerve which innervate the individual sucker

(Figs. 23,24). In the main strobilar region there are more than four longitudinal nerves detectable. Of these nerves, while most of them are thin nerves comprising one or a couple of nerve fibers each, one pair is very prominent with each nerve trunk consisting of several closely clusterd nerves and running parallel to the lateral margins of the body and lying external to the longitudinal excretory canals (Figs.25-27).These are the lateral longtudinal nerve trunks (LLNT).They are relatively uniform in thickness throughout the germinative and mature regions of the strobila. All the longitudinal nerves are connected by several (10-12) transverse ring commissures (Figs.29-32). The latter lie about equidistance from each other in the proglottid. However, in the region of the velum or interproglottidal zone there is a clear gap in between the transverse commissures near the margins of the adjacent proglottides. At the intersections of each transverse nerve and the main LLNT, there is a small ganglion from which small nerves branch out irregularly towards the lateral margins of the proglottid and terminate in the subtegumental layer. Numerous fine connectives joining the transverse commissures and the fine longitudinal nerves form an intricate

Figs. 33-36: AChE activity in *R. echinobothrida*, cryostat sections.

33,34. Control.

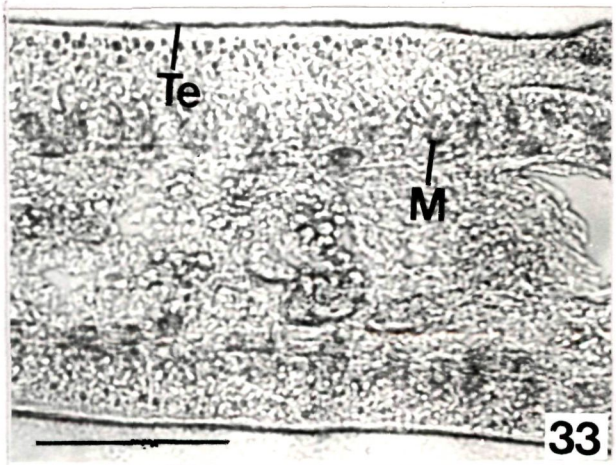
33. Showing intense AChE activity in the tegument (T), subtegument (ST) and muscle elements (M). (Scale bar= 0.2 mm)

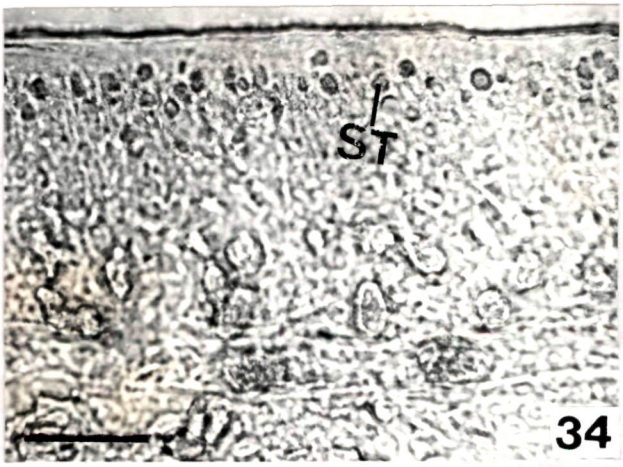
34. A magnified view of a portion ofthe same.(Scale bar = 0.1mm)

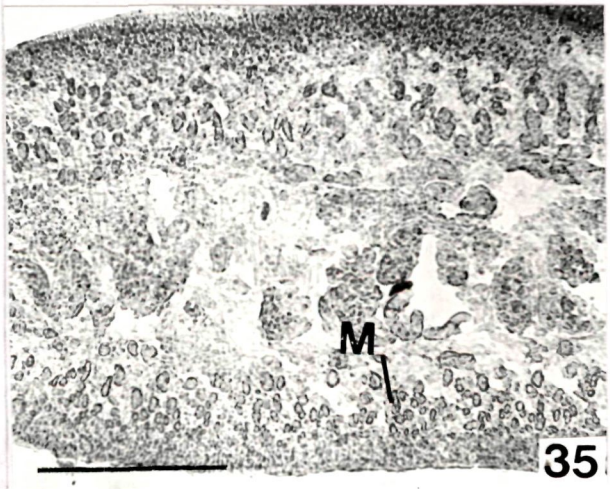
35,36. Treatment with crude root-tuber peel extract of *F. vestita*.

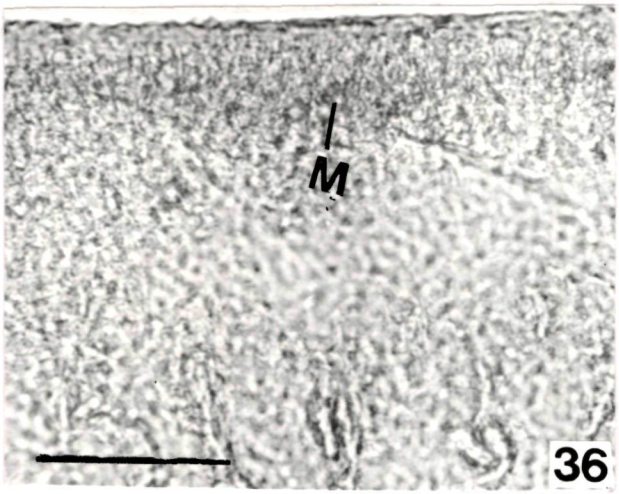
35. Mild AChE activity revealed in the subtegument and muscle; the tegument shows no activity. (Scale bar = 0.2 mm)

36. A portion of Fig. 35 in a closer view.(Scale bar = 0.05 mm)


Te
M
33

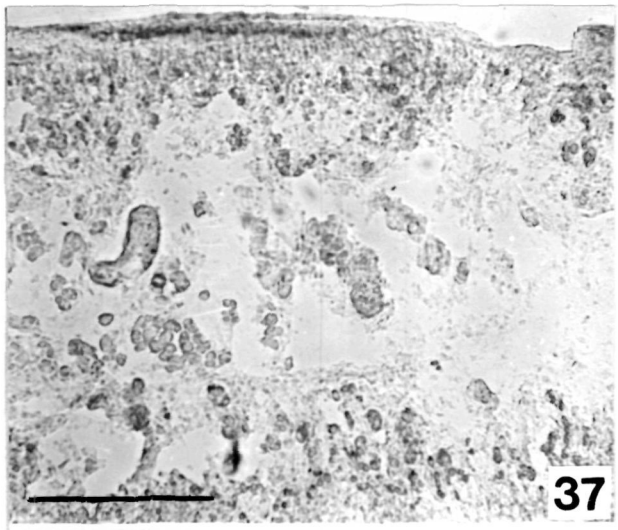

ST
34


M
35


M
36

Figs. 37,38 : Treatment with genistein (0.05mg/ml). Loss of AChE activity is revealed in the mentioned tissues compared to controls. (Scale bar = 0.2mm and 0.1 mm, respectively)

Figs. 39,40 : Treatment with praziquantel (0.01 mg/ml). Loss of AChE activity, as with genistein treatment, in the tissue is evident.(Scale bar = 0.2 mmand 0.1mm, respectively)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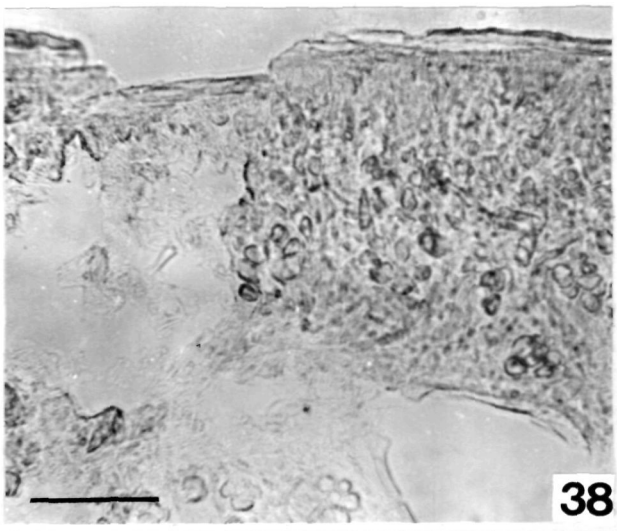
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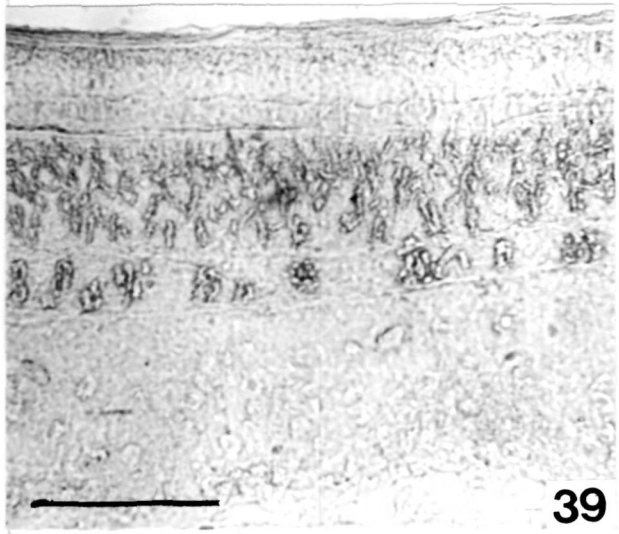
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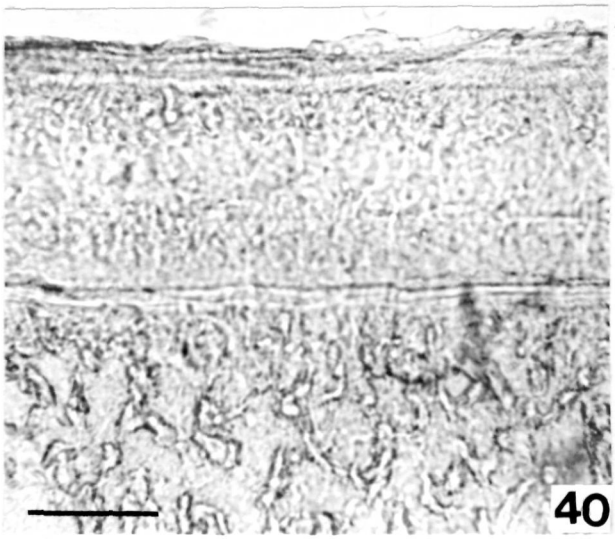
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subsurficial network across the length and breadth of the proglottides. Posteriorly, at the terminal proglottid all the longitudinal nerves join together (Fig. 32).

The 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 (Fig.26). The intense staining reaction with acetylcholine iodide used as a substrate indicated that the cholinergic component in the nervous components is AChE.

Histochemically, AChE was found to be present in the tegument, subtegument and muscle layers, with intense activity restricted mainly to the tegument (Figs. 33,34); in the whole tissue homogenate high AChE activity was measureable (Table 2).

The total and specific levels of AChE activity, determined in freshly prepared homogenates of the adult cestode, are listed in Table 2.

Treatment- After exposure of *R. echinobothrida* to the crude peel extract of *F. vestita* and its purified active component,i.e., genistein, in the paralyzed worm there was a pronounced decline in the visible stain intensity of the

Table 2. AChE activity in the various tissues of *R. echinobothrida* in vitro

Treatment (mg/ml)	Longevity of the parasite (in hours)	Enzyme activity and intensity (Histochemical localization)* Tegument	subtegument	Muscle	Testis/Ovary	Total activity **	Specific activity***	% change after treatment
1. Control (in PBS)	72 ± 0.05	++++	++	+++	+/+	2.45 ± 0.011	0.013	
2.Crude extract (50.0)	P=0.3 ± 0.01 D=6.5 ± 0.4	-	+	+	-	1.25 ± 0 12	0.01	49.07
3.Genistein (0.5)	P=4.4 ± 0.07 D=19.08 ± 0.02	-	-	-	-	1.06 ± 0 02	0.007	56.77
4 Praziquantel (0 01)	P=0.47 ± 0 07 D=6.1 ± 0.34	-	-	-	-	1 08 ± 0.19	0.004	55.84

*++++ = very intense activity; +++ = intense activity; ++ = moderate activity;

+ = mild activity; - = no activity.

Values are the mean (± SE) from three replicates' assays. ** Enzyme activity

expressed as a specific unit which consumes 1.0 μ mol substrate/g wet wt tissue/h

*** Specific activity expressed as μ mol/mg protein/h

nervous components, indicating very less or no activity of NSE and ChE in the parasite; no activity was observable in the tegument (Figs. 35-38).

Quantitatively also, the AChE activity was reduced by 49.07% and 56.77% following treatment with the crude extract and genistein, respectively (Table 2). Changes were also noticeable in the praziquantel-treated worm (Figs. 39,40; Table 2); the reduction in the AChE activity was somewhat at par with that of the genistein- treated worms when compared to control.

(ii) Acid phosphatase (AcPase)

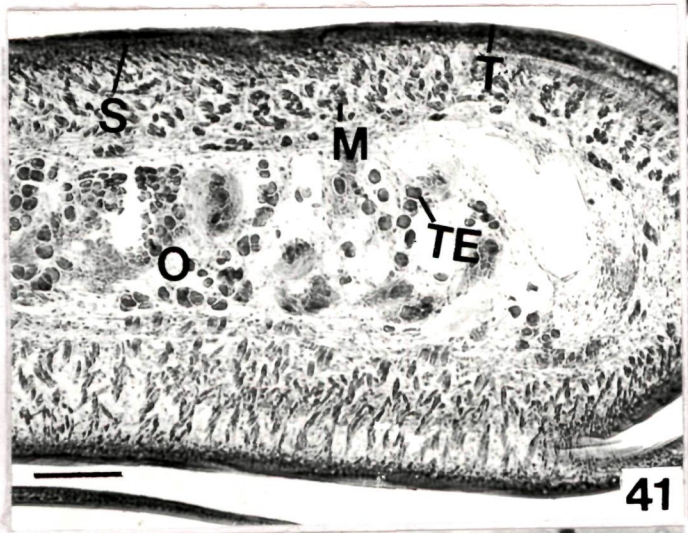
Controls - The most intense AcPase activity was observed in the tegument; the other regions, viz., the subtegument, somatic musculature, testes and ovary displayed moderate reactions (Table.3). No activity was observed for AcPase in either the parenchyma or the excretory canal (Figs.41,42). Quantitatively, the enzyme activity was higher in the supernatant, about 4 times higher as compared with the genistein - treated worm (Table 4).

Figs. 41-44: AcPase activity in *R. echinobothrida*, cryostat sections.

Figs. 41,42. Cross section showing very intense activity in the tegument (T), subtegument (ST), somatic muscle layer (M), testes (TE) and ovary (O). (control) (Scale bar = 0.2 mm and 0.1 mm, respectively)

43. Transverse 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)

44. Transverse section, showing no AcPase activity in the tegument after treatment with praziquantel. (Scale bar= 0.1 mm)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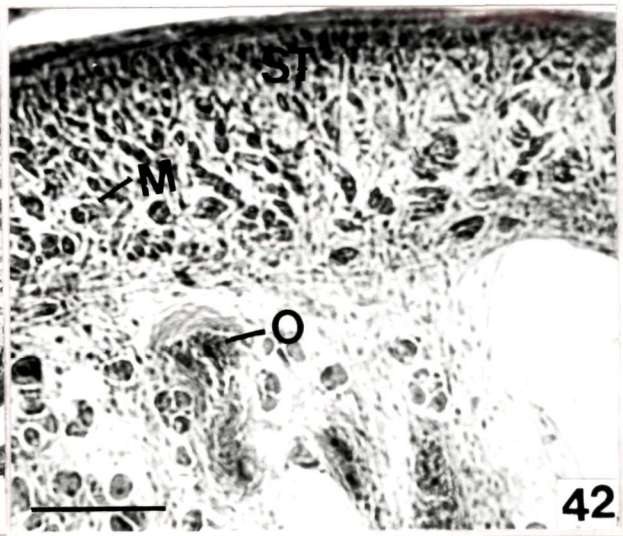
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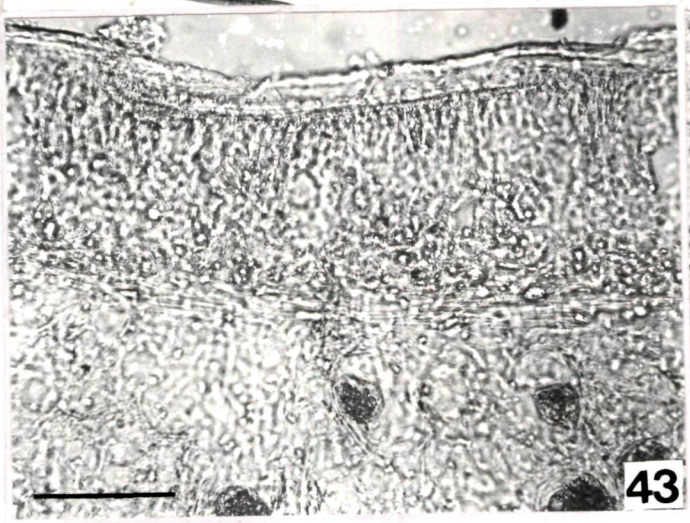
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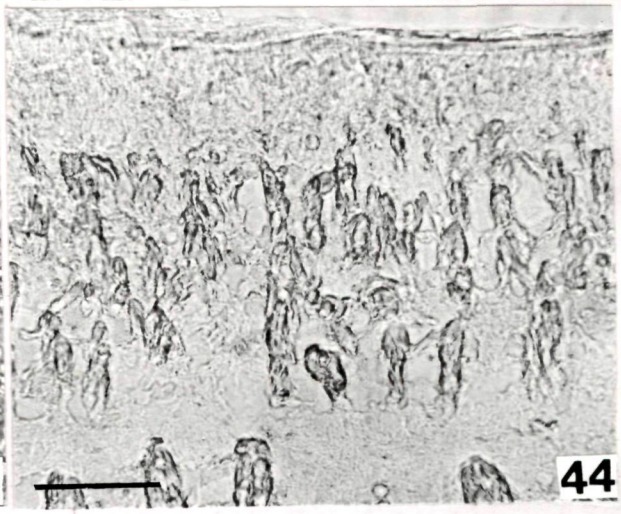
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Treatment - The activity of AcPase in the tegument and subtegument was completely inhibited after treatment with the crude peel extract of *F. vestita* and genistein but partial inhibition was observable in the somatic musculature (Fig. 43). Moderate to weak reaction was sometimes noticeable in the subtegument and strong activity persisted in the gonads (testes) following treatment with genistein . Almost all the structures of the parasite were invariably negative for staining with the modified lead nitrate method after exposure to the genistein -containing medium (Fig. 43, Table 3). Similar result was observed after treatment with the reference drug praziquantel (Fig. 44, Table 3).

The results of the AcPase enzyme assay are summarized in Table 3. The AcPase activity of *R. echinobothrida* was inhibited by 96% and 97% following treatment with the crude extract and genistein, respectively (Table 4). The inhibition for the praziquantel- treated worms was 92% (Table 4).

(iii) Alkaline phosphatase (AlkPase)

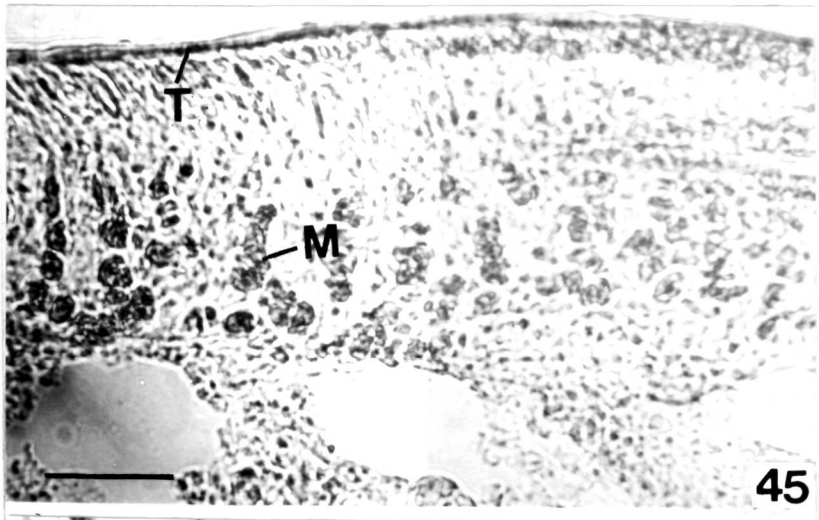
Control- An intense AlkPase activity was observed restricted

Figs. 45-47 : AlkPase activity in *R. echinobothrida*, cryostat transverse sections.

45. Control, strong activity in the tegument and somatic muscle layer. (Scale bar= 0.1 mm)

46. AlkPase activity restricted in the tegument, subtegument, somatic muscle layer and testes after treatment with the crude root peel extract. (Scale bar = 0.1 mm)

47. Mild AlkPase activity in the tegument, subtegument and testes after treatment with genistein. (Scale bar= 0.1 mm)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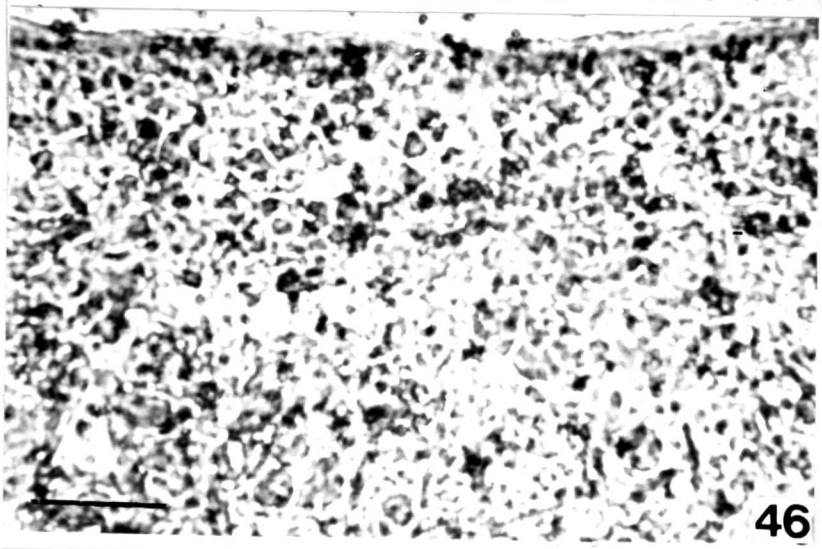
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mainly to the tegument and somatic muscle layer. In the subtegument zone the enzyme activity was lower (Fig.45). Quantitatively, AlkPase showed a high activity in the supernatant fraction assayed in the nontreated parasite (Table 4).

Treatment- After in-vitro exposure of the worm in the incubation medium containing the crude peel extract, and genistein, considerable reduction in the stain intensity from the control parasite was detectable histochemically in the tegument of the treated parasite (Figs. 46,47; Table 3).

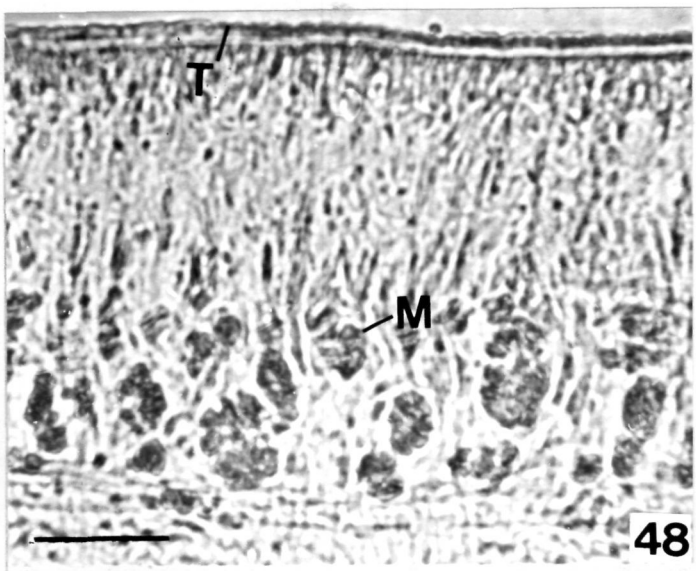
At biochemical level, varying degree of inhibition of the AlkPase activity was observed after treatment with the plant- derived components. The relative activities of AlkPase from the supernatant fractions of *R. echinobothrida* are summarized in Table 4. After exposure to the crude extract and genistein, the AlkPase activity was reduced by 86% and 95% , respectively and with praziquantel treatment, the enzyme activity was inhibited by 97%. The total activity was lower after treatment with genistein than that of control (Table 4).

Figs. 48-51 : ATPase activity in *R. echinobothrida*, cryostat sections.

48. Control, intense enzyme activity shown in the somatic musculature.(Scale bar= 0.1 mm)

49. Mild ATPase activity, after treatment with the crude root-peel extract. (Scale bar= 0.1 mm)

50,51. Absolutely negative ATPase activity in the tegument after treatment with genistein and praziquantel, respectively. (Scale bar= 0.1 mm)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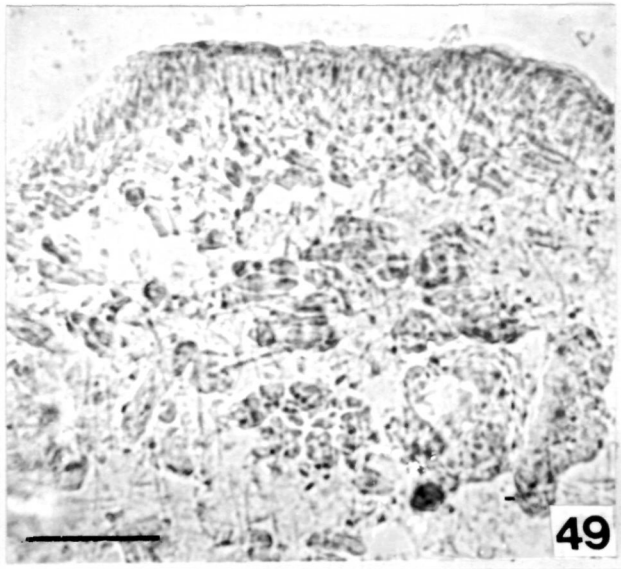
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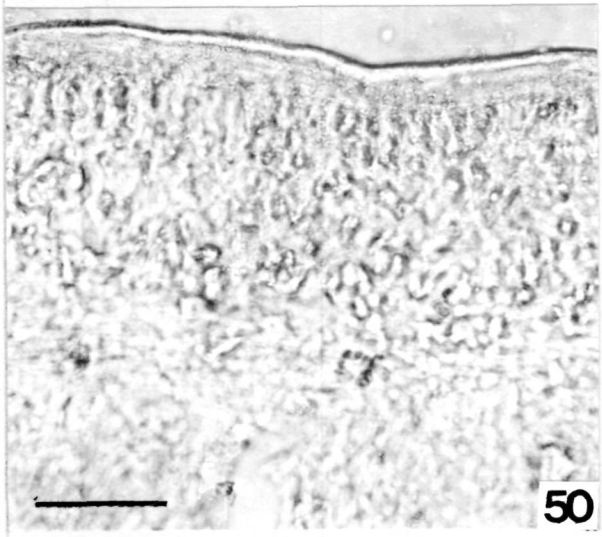
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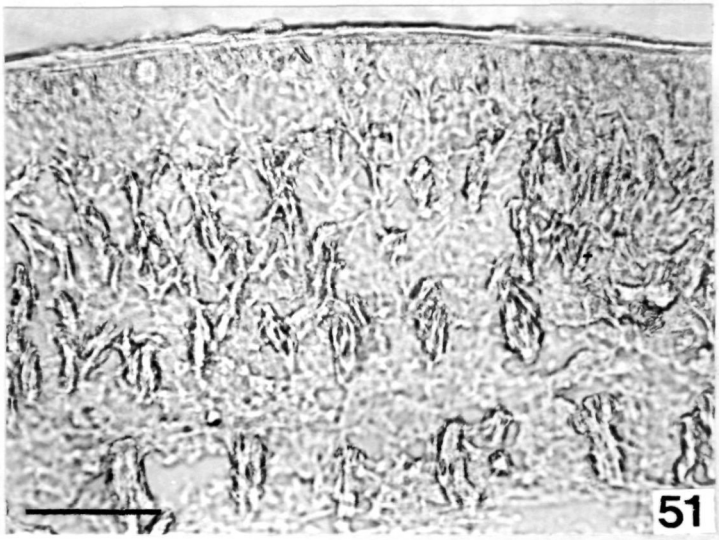
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(iv) Adenosine triphophatase (ATPase)

Control- The ATPase activity was noted mainly in the tegument and musculature of *R. echinobothrida* where intense to moderate enzyme staining was demonstrated (Table 3). Positive activity was also observed to be associated with the subtegument and gonads (Fig. 48).

Quantitatively, the ATPase activity was high in the whole tissue homogenate (Table 4).

Treatment- All structures were almost invariably negative for staining with the calcium method for ATPase activity after treatment with the plant - ' derived components (Figs.49,50). The enzyme activity was reduced by 71% and 88% after treatment with the crude peel extract and genistein, respectively. There was a decrease in the ATPase activity of the genistein- treated worms; changes were also noticeable in the praziquantel- treated parasite (Fig. 51, Table 4).

(v) 5'- Nucleotidase (5'-Nu)

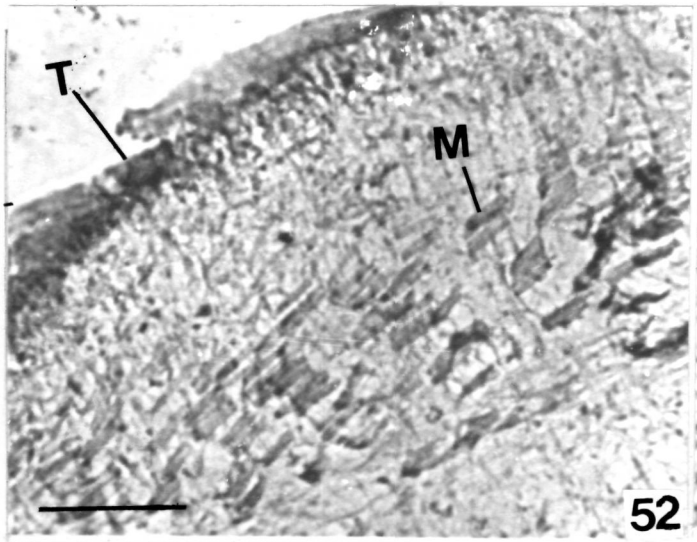
Control - Of all the structures studied (viz.,tegument,

Figs. 52-55 : 5'-Nu activity in *R. echinobothrida*, cryostat sections.

52. Control, transverse section, showing pronounced enzymatic activity in the tegument, subtegument and somatic muscle layer. (Scale bar= 0.1 mm)

53. Mild 5'-Nu activity observed after treatment with the crude root- tuber peel extract in the tegument, subtegument and somatic muscle layer. (Scale bar= 0.1 mm)

54,55. Absence of 5'- Nu reaction in the tegument after treatment with genistein and praziquantel, respectively. (Scale bar = 0.1 mm)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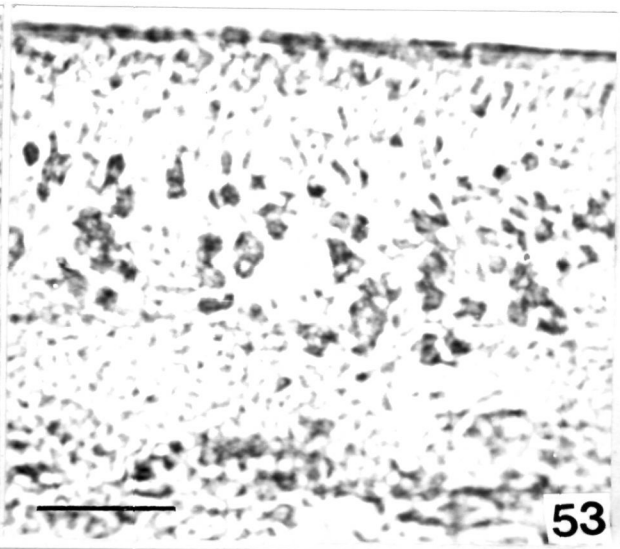
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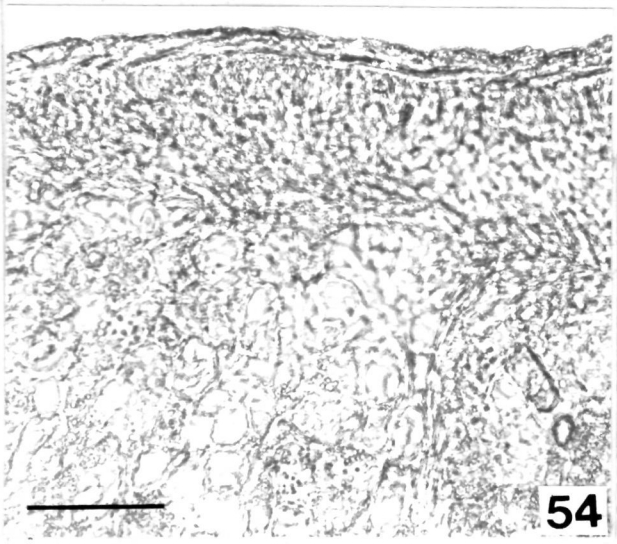
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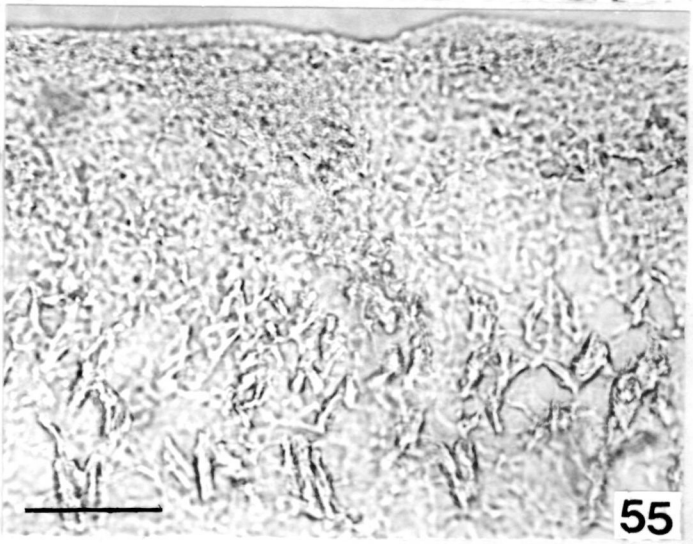
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Table 3. Activities of AcPase, AlkPase, ATPase and 5'- Nu in the various structures of *R. echinobothrida* in vitro: histochemical localization

Treatment (mg/ml)	Distribution and enzyme intensity															
	AcPase				AlkPase				ATPase				5' - Nu			
	Tg	ST	M	T/O	Tg	ST	M	T/O	Tg	ST	M	T/O	Tg	ST	M	T/O
1.Control (in 0.9% PBS)	++++	+++	+++	++/++	+++	+	++	+/-	++++	++	+++	+/+	++++	+++	++	+/+
2.Crude extract (50.0)	-	-	-	-	-	++	++	-	+	-	+	+/+	+	+	++	-/-
3 Genistein (0.5)	-	-	-	+/-	+	++	-	+/+	-	-	-	-	-	-	-	-
4.Praziquantel (0 01)	-	-	+	-	+	-	-	-	+	-	-	-	-	-	-	-

++++ = Very intense activity; Tg = Tegument;

+++ = Intense activity; ST = Subtegument;

++ = Moderate activity; M = Muscle layer;

\+ = Mild activity; T/O = Testis/Ovary

\- = No activity;

Table 4. 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*/specific acitivity**)				% 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	86%	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 mean (± SE) from three replicates assays.

* Enzyme activity expressed as a specific unit which consumes 1.0 μ mol substrate/g wet wt tissue/h;

** Specific activity expressed as unit/mg protein/h

subtegument, gland cells, somatic musculature, testes and ovary),the tegument showed a relatively strong positive staining reaction to the 5'- Nu activity (Fig. 52, Table 3). A fairly less intensity of reaction was observed in the muscle layer. The biochemical assay showed high enzyme activity in the tissue homogenate (Table 4) of the cestode.

Treatment - The 5' - Nu activity in the tegument and subtegument was partially inhibited by the crude extract and genistein treatments (Figs. 53,54); the enzyme activity was reduced by 37% and 28%,after exposure to these test materials, respectively (Table 4).

No stain or enzyme activities were observed in the tissues histochemically or biochemically,respectively when frozen sections or tissue homogenate were incubated without the respective substrate solution.

FREE AMINO ACID (FAA) AND AMMONIA ANALYSIS

Control - The amino acid compositiion of *R. echinobothrida* was demonstrated using HPLC, on a lithium column. As given in Table 5, the free amino acid pool of the control worm included phophoserine (phser), taurine (tau), phosphoamine

Table 5. Qualitative analysis of free amino acid composition of *R. echinobothrida* in vitro, determined by HPLC

No.	Control (0.9% PBS in 1% DMSO)	Crude extract (50mg/ml)	Genistein (0.5mg/ml)	Praziquantel (0.01 mg/ml)
1.	Phser	Phser	Phser	Phser
2.	Tau	Tau	Tau	Tau
3.	PhNH2	PhNH2	BLD	BLD
4.	Thr	Thr	Thr	Thr
5.	Ser	Ser	Ser	Ser
6.	Glu	Glu	Glu	Glu
7.	Pro	Pro	Pro	Pro
8.	Gly	Gly	Gly	Gly
9	Ala	Ala	Ala	Ala
10.	Cit	BLD	BLD	BLD
11.	Val	Val	Val	Val
12.	Met	Met	Met	Met
13.	Ile	Ile	Ile	Ile
14.	Leu	Leu	Leu	Leu
15.	Tyr	Tyr	Tyr	Tyr
16.	Phe	Phe	Phe	BLD
17.	β-Ala	β-Ala	β-Ala	BLD
18.	α-AIBA	α-AIBA	α-AIBA	α-AIBA
19.	GABA	GABA	GABA	GABA
20.	Trp	Trp	Trp	Trp
21.	His	His	His	His
22.	Orn	Orn	BLD	Orn
23.	Arg	Arg	Arg	Arg
24.	Ammonia	Ammonia	Ammonia	Ammonia

BLD = Below the level of detection

($phNH_2$), threonine (thr), serine (ser), glutamic acid (glu), proline (pro), glycine (gly), alanine (ala), citrulline (cit), valine (val), methionine (met), isoleucine (ile), leucine (leu), tyrosine (tyr), phenylalanine (phe), β-alanine (β-ala), α-aminobutyric acid (α-AIBA), γ-aminobutyric acid (GABA), tryptophan (trp), histdine (his), ornithine (orn), arginine (arg) and ammonia. The quantitative analysis of amino acids is also presented in Table 6 . Out of the amino acids detected, ala was found to be at the maximum level, followed by val, tau, pro, tyr, ser, α-AIBA, leu, glu and phe, respectively, in a decreasing order. Other amino acids, as mentioned above, were at comparatively lower levels. A low level of ammonia was also detected in the worm.

Amino acids which were excreted by *R. echinobothrida* in the incubation medium include phser, glu, val, met, ile, leu, tyr, phe, β-ala, α-AIBA, GABA, his, orn and arg (Table 7). Significant level of ammonia was also excreted by the parasite which could be detected both by HPLC and enzymatic methods (Table 7, 8).

Treatment - After treatment with the plant-derived products of *F. vestita* and also the reference drug, the free amino acids of *R.echinobothrida* showed qualitatively close similarity with those of the control maintained in 0.9% PBS. As shown in Table 7, the level of different FAAs and ammonia in the parasite was significantly affected by the treatment with crude root peel extract of *F. vestita*, genistein and also by praziquantel. When the parasite was treated with crude extract, most of the amino acids were markedly decreased except glu, pro, ile, trp and orn, the levels of which either did not change much or increased in certain cases (met, ile and trp). Almost similar decrease of FAA levels was also noticed in the parasite treated with either genistein or praziquantel except trp, the level of which decreased markedly in the case of praziquantel. The levels of glu, pro, met and ile were not affected much by either of these treatments. Interestingly, however, the ammonia level in the parasite was increased by 373% , 128% and 52% following treatment with the crude extract, genistein and praziquantel, respectively. Amino acids excreted by *R. echinobothrida* and detected in the effluent are listed in Table 7.

Table 6. Levels of different free amino acids and ammonia (n mol/g wet wt) in *R. echinobothrida*

Amino acid	Control (0.9 % PBS in 1% DMSO	Crude extract (50 mg/ml)	Genistein (0.5 mg/ml)	Praziquantel (0.01 mg/ml)
1 Phser	254	130 (-49)	165 (-35)	185 (-27)
2. Tau	2334	1088 (-53)	1733 (-26)	1442 (-38)
3. $PhNH_2$	251	66 (-76)	BLD	BLD
4. Thr	206	138 (-33)	195 (-5)	116 (-44)
5. Ser	922	385 (-58)	859 (-7)	344 (-63)
6. Glu	667	739 (+10)	856 (+29)	953 (+43)
7. Pro	1756	1930 (+10)	1988 (+ 3)	2225 (+27)
8. Gly	3802	1001 (-74)	2517 (-34)	2641 (-19)
9. Ala	6001	3560 (-41)	5342 (-11)	1168 (-81)
10. Cit	11	BLD	BLD	5 (-54)
11. Val	4584	455 (-90)	467 (-90)	1424 (-68)
12. Met	116	223 (+92)	143 (+23)	136 (+17)
13. Ile	348	554 (+59)	358 (+3)	1535 (+54)
14. Leu	756	364 (-52)	141 (-81)	153 (-80)
15. Tyr	1486	734 (-51)	671 (-54)	1047 (-30)
16. Phe	555	284 (-49)	449 (-19)	173 (-69)
17 β- Ala	619	257 (-58)	548 (-11)	266 (-57)
18. α- AIBA	769	484 (-37)	556 (-28)	901 (-17)
19. GABA	167	110 (-34)	105 (-37)	60 (-64)
20. Trp	138	202 (+46)	135 (-2)	426 (-208)
21. His	289	106 (-63)	187 (-35)	554 (-92)
22. Orn	1070	1217 (+14)	BLD	1085 (-11)
23. Arg	435	183 (-58)	154 (-64)	161 (-63)
24. Ammonia	467	2208 (+373)	1064 (+128)	709 (+52)
Total aa =	27536	14062 (-53)	17369 (-50)	17025 (-49)

% increase (+) or decrease (-) of FAA levels are given in parentheses .
BLD = Below the level of detection

Table 7. Amino acids excreted in the incubation medium by *R. echinobothrida*

No.	Amino acid	Control	Crude extract	Genistein	Praziquantel
1.	Phser	+	-	-	-
2.	Tau	-	+	-	+
3.	$PhNH_2$	-	-	-	-
4.	Thr	-	+	-	+
5.	Ser	-	+	-	+
6.	Glu	+	+	+	+
7.	Pro	-	-	-	-
8.	Gly	-	+	-	+
9.	Ala	-	+	-	+
10.	Cit	-	-	-	-
11.	Val	+	+	+	+
12.	Met	+	+	-	+
13.	Ile	+	+	+	+
14.	Leu	+	+	+	+
15.	Tyr	+	+	+	+
16.	Phe	+	+	+	+
17.	β- Ala	+	+	+	+
18.	α- AIBA	+	+	+	+
19.	GABA	+	+	+	+
20.	Trp	-	-	-	-
21.	His	+	+	+	-
22.	Orn	+	-	-	-
23.	Arg	+	+	+	+
24.	Ammonia	+	+	+	+

- = BLD
+ = Present

Table 8. Levels of ammonia in *R. echinobothrida* after in vitro treatment

Treatment * (mg/ml)	Ammonia ** in effluent
1. Control	77.3 ± 3.21
2. Crude extract (50.0)	79.2 ± 2.92
3. Genistein (0.5)	76.7 ± 1.19
4. Praziquantel (0.01)	97.33 ± 2.3

* Incubation was continued for 1h

** μ mol /g wet wt tissue

DISCUSSION

(i) The anthelmintic 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; Kasuya et al., 1990; Togo et al., 1992). The present investigation indicates that the cestode, *R. echinobothrida* tested herein is quite sensitive to the root-tuber peel extract of *Flemingia vestita*, since it could survive in the test medium for considerably less period of time. The nematode parasites were found to be less sensitive to this plant product, since they could survive in the test medium for long periods. Whereas the bigger nematode species, *Ascaris suum* and *Ascaridia galli* survived for about 3 days in the test medium, even the small-sized worm, *Heterakis gallinarum* could survive for more than 16 h at the crude peel extract concentration of 50 mg/ ml (Tandon et al., 1997). In a preliminary study, the whole root- tuber ethyl-acetate extract (peel and the fleshy part together) of *F. vestita* (0.36%) was found to be effective against *A. suum*, since a decrease in motility with paralysis occurring 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 possible that some active principles which might be present in the root tuber pulp are lost by extraction procedure. *Artyfechinostomum sufrartyfex* revealed sloughing off, wrinkles and rupture of the general tegument; severe tegumental alterations and deformities were also displayed by *Fasciolopis buski* exposed to 20 mg/ml of crude root peel extract of *F.vestita* (Roy and Tandon, 1996). The root-tuber peel extract of *F.vestita* appeared to be effective also against *Paramphistomum* sp., since in a considerably short period of time complete inactivation and irreversible immobilization were observable in the treated worms, indicating a lethal effect on the parasite (Tandon et al., 1997). However, the present study indicates considerable anticestodicidal activity of the crude root-tuber peel extract of *F. vestita* and its active component- genistein in vitro. The cestode, *R. echinobothrida* was obviously affected by the treatment, since a spontaneous loss of movement or paralysis could ensue soon after incubation in the test medium, even though the death occurred after a long gap of time. The time taken for paralysis and death rate showed a direct correlation with concentration of the test materials. A dose-dependent efficacy of the plant components has been reported in the earlier studies also

(Kalyani et al., 1989; Kasuya et al., 1990). Exposure of the cestode to various concentrations of the crude extract, residue extract (i.e.,crude extract minus genistein) and formononetin and pseudobaptigenin mixture did not cause any mortality for sometime to follow and acquisition of the paralytic state was also not an irreversible change. It may 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 to the other active components, i.e., formononetin-pseudobaptigenin mixture,the genistein component appeared quite efficaceous 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 showed onset of paralytic state only after 29 h. The paralyzing effect of the residue was much less even at very high concentration; a concentration of 50 mg/ml of the residue extract took 2.1 h to bring about paralysis and death occured after 13 h. The paralysing effect of praziquantel was much faster even at very low concentrations; 0.001 mg durg/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.(Tandon et al., 1997).

(ii) In the treated *R. 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 and loss of muscular components in comparision with the control. In *Taenia taeniaeformis* and *H. nana*, Borgers et al. (1975) and Verheyen et al. (1976) reported an increase of undefined secretory substances in the golgi areas. Isatin produced an hypervacualization of the tegumental cytoplasmic syncytium in the secondary cysts of *E. multilocularis* (Delabre - Defayolle et al., 1989) as also observed during treatment with bunamidine on *H. nana* by Hart et at.(1977). Becker et al. (1981) also reported vacuolization in the syncytial zone during studies on the effect of praziquantel on five species of cestodes including *E. multilocularis*. Tegumental alterations and severe vacuolization on exposure to

flukicidal drugs have been observed in several species of trematodes (Schmahl and Taraschewski, 1987; Zheng and Zhang, 1988; Gorchilova et al., 1990; Jiang et al.,1990; Jiang and Xia, 1992; Schmahl, 1993; Stitt and Fairweather, 1993; Xu-lin et al., 1994; Anderson and Fairweather, 1995); the extent of damage induced was reported to increase with the exposure time. Similar changes were also noticed in the tegument of cestode parasites (Imai et al.,1981; Delabre-Debayolle et al., 1989; Perez et al., 1994). In digenetic flukes, the site of origin of vacuoles was found to be basal lamina (Mehlhorn et al., 1983), whereas in the monogenean, *Diclidophora* spp., vacuoles originated from the surface of the tegument (Schmahl and Mehlhorn, 1985). Changes also occurred in the tegumental cells which were indicative of a disruption in the synthesis and release of tegumental secretory bodies; the amount of granular endoplasmic reticulum became reduced, the cisternae became swollen and their ribosomal convering decreased, the golgi complexes disappeared from the cells and the number of secretory bodies in the cells also decreased. The ultrastractural changes in the tegument are linked to a possible mode of action of the drug as an inhibitor of protein synthesis (Anderson and Fairweather, 1995).

Vacuolization and contraction in the parasite body surface are closely related to the levels of Ca^{2+} concentration in the media used (Bricker et al., 1982; Xiao et al., 1984). Disturbances in ion flux across the membrane, leading to changes in the tegumental integrity in different trematodes on treatment with praziquantel, are well established (Bricker et al., 1982; Mehlhorn et al., 1983; Schmahl and Mehlhorn, 1985).Sobhan et al.(1986) showed that treatment of *Opisthorchis viverrini* with amoscanate caused severe swelling and pit formation, leading to total disruption of the surface tegument and suggested that the drug may have caused in imbalance in osmosis, thus resulting in impaired ion transfer. Perhaps the chemical component in the tuber peel extract 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; Bogoyavlenskii et al., 1988; Semenkov and Akilzhanov, 1988; Xiao et al., 1989; An, 1990; Storte et al., 1990; Mackenstedt et al., 1993; Rothwell and Sangster, 1996).

Collagen, one of the abundant structural proteins in animals, is reported to be present in the basal lamina of the cestode tegument (Smyth and McManus, 1989). However, in *R. echinobothrida*, no collagen could be detected as a constituent of the tegument. Non detection of collagen using histochemical method in the present study may perhaps be attributed to the absence of hydroxyproline in the amino acid constituents of the cestodes collagen (Torre- Banco, 1982).

It seems tempting to speculate that the death of the cestode, *R. echinobothrida*, on exposure to the crude plant extract or genistein, is related to the changes in tegumental integrity of the parasite. 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.

(iii)Using indoxyl acetate and acetylthiocholine iodide as substrate, 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; biochemical assay of AChE in the treated worms also indicated a considerable loss of enzyme activity.

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

AChE receptors in platyhelminthes are known to have

pharmacological properties different from their mammalian counterparts (Mellin et al., 1983). Anthelminthcs including organophosphates and praziquantel possibly have a neuromuscular mode of action (Mckay et al., 1989; Cox, 1994) by interfering with transsmission at nerve- nerve synapse or at neuromuscular junction by inhibiting the enzyme (AChE) responsible for inactivating the neurotransmitter at the synpase (Bryant and Behm, 1989; Raether, 1988). Treatment both with the root- peel extract of *F. vestita* and genistein does cause paralysis of the worm 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). ACh 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 to 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).

(iv) Of the four tegumental enzymes studied, AcPase, AlkPase, ATPase and 5' - Nu were clearly detectable in the parasite at several sites such as the tegument, subtegument, somatic musculature and reproductive organs; at no time was there any enzyme activity detectable in the parenchyma cells. The widespread and impressive amounts of certain tegumental enzymes demonstrated in various cestodes suggest that they might play a highly significant role in digestion or absorption in distinctive tissues (Smyth and McManus, 1989).

In helminths, as in mammals, AcPase is usually associated with lysosomes and AlkPase is regarded as indiciative of membrane transport mechanisms. In cestodes like *Hymenolepis diminuta,* the phosphates are thought to be in proximity to the sugar and nucleoside uptake sites (Barrett, 1981). In the present study histochemical and biochemical changes occurred after treatment with the crude root-tuber peel extract of *F. vestita* (50 mg/ml) and its purified active principle- genistein (0.5 mg./ml). The results of the present investigation indicate that the highest activity change was revealed with regard to AcPase (97%) and 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, the AcPase showed much less or negligible staining reaction in the tegument following treatment; genistein showed greater effectiveness to AcPase in comparison with the crude peel extract. A similar observation was made by Gupta and Sharma (1973) in several trematode species exposed to treatment with hexachlorophene. The inhibition of AcPase activity by the plant- derived component observed druing the present study may suggest that the absorption and intracellular digestion of drugs may involve lysosomes (Colam, 1971). A decrease in enzyme activity is probably due to its leakage into the medium as a result of the disruption of the absorptive surface (Borgers et al., 1975; Hart et al., 1977). The occurrence of high concentration of AcPase activity in the tegument of *R. echinobothrida* may well indicate similar roles of the enzyme in metabolism and active transport,not only in normal carbohydrate metabolism (phosphohydrolysis) but also in the active transport of metabolites (transphosphorylases- Lumsden, 1975; Barrett, 1981). The tegument of cestodes represents the major route for nutrient absorption. Electron microscopical observations

on the cuticle and underlying hypodermis of various species of filaroid nematodes have also suggested the presence of an active transport mechanism engaged in the exchange of materials across the cuticle with the external environment (Deas et al., 1974; Vincent et al., 1975; Chen and Howells, 1981; Franz and Biitther, 1983).

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 the most active, while AcPase tends to predominate in trematodes (Barrett, 1981). AlkPase has been localized on the surface of both male and female *Schistosoma mansoni* worms as a result of praziquantel- induced tegumental damage (Mehlhorn et al., 1981; Modha et al., 1990; Fallon et al., 1994 a).The AlkPase activity was significantly inhibited / reduced by 86% and 95% after treatment with the crude peel extract of *F. vestita* and genistein component, respectively. In *Echinococcus multilocularis* metacestodes, this enzyme is localized in the tegument (Pappas, 1983). Delabre- Debayolle et al. (1989) reported inhibition of AlkPase activity by 23% and complete inhibition of glucose uptake in these metacestodes following treatment with isatin, a known

phosphatase inhibitor in vivo and attributed the depletion in the enzyme activity to the failure of glucose uptake. AlkPase hydrolyzes several phosphoric monoesters and influences glucose uptake (Hart et al., 1977; Pappas, 1983). Chopra et al. (1991) also showed that some plant extracts cause a reduction in both AcPase and AlkPase activity in *Paramphistomum cervi* in vitro. However, Fallon et al. (1994 b) observed more than two fold increase in the AlkPase activity in the praziquantel- treated *S. mansoni* and attributed this increase to drug- induced tegumental damage exposing the normally concealed enzymes on the tegumental surface of the worm.

The present studies revealed that ATPase showed high activiity in the control worm. The ATPase activity is widely distributed in helminths and probably represents a variety of enzymes (Barrett, 1981). Three classes of specific ATPase have been described by Firth (1978): (i) protein -translocating ATPases catalyzing the synthesis of ATP, (ii) other ion - transporting ATPases that normally hydrolyze ATP and use energy released for active ion transport across plasmalemmal surfaces, and (iii) cytoplasmic ATPases associated with contractile systems. The latter group has been demonstrated ultrastructurally in

myofibrils (Somogyi and Sotonyi, 1969). The localization of ATPase in somatic musculature of *R. echinobothrida* indicates that the enzyme occurs in the myobibrils. Since ATP plays a prominent role in muscle contraction (Barrett, 1981), the high concentrations of ATPase detected in association with musculature of *R. echinobothrida* strongly suggest that one of the roles of this enzyme is the hydrolysis of ATP in this tissue. The enzyme is known to be related to energy metabolism, active transport and lipid synthesis (Lehninger, 1975). In helminths muscle tissue usually has enough ATP to sustain contraction for only a fraction of a second (Barrett, 1981); hence high ATPase present in *R. echinobothrida* may be involved in cellular regulation of ATP when there is a sudden energy demand. 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 acrisaline; the ATPase activity reduced to 50-100% in homogenates of isolated scoleces of multilocular hydatids as reported by Benediktov (1980).Some drugs, viz., phenathiazines, chlorimazine, diethylcarbamazine and centperazine caused significant

inhibition of Ca^{+2} ATPase and Na^{+}-K^{+} ATPase of the filarial parasite,*Setaria cervi* indicating thereby that this enzyme system could be a common target for the action of anthelmintic drugs (Agarwal et al., 1990).

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 membranes has been suggested (Mao et al., 1966). In parasitic helminths, 5'-Nu activity may be involved with other enzymes in the hydrolysis of nucleosides to pyrimidine and purine bases (Barrett, 1981). There is also some evidence that this enzyme may be involved in the uptake of nucleosides in the filarial worms, *Onchocerca volvulus* and *Dirofilaria immitis* (Walter and Albiez, 1985).5'- Nu may have an accessory role in the degradative metabolism of purines and pyrimidine nucleotides in the parasite.

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.

(v) The FAAs composition of *R. echinobothrida* detected in the present study includes 23 amino acids. Of these 15 (threonine, serine, glutamic acid, proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, histidine, and arginine) represent common or standard amino acids. Besides, taurine, citrulline, α- aminobutyric acid, γ aminobutyric acid, and ornithine, are non-protein amino acids which could appear as metabolic intermediates or neurotransmitters as reported in other cestodes (Eriksson et al., 1995).

As reported earlier in other cestodes, (Barrett, 1991), alanine, proline and glycine, the three non essential amino acids, were also found to be predominantly present in this parasite. In addition, taurine and valine also constituted a major part of its FAA pool. Relatively high levels of taurine reported in this bird cestode might play a significant role for osmotic balance, since birds predominantly synthesize and excrete uric acid instead of urea as major nitrogenous waste. The size of the total FAA pool in this cestodde was also high (about 2.7 m moles/100g fresh wt) as in the case in most cestodes (Barrett, 1991). The high levels of the FAA pool may be physiologically

significant in more than one ways: (i) it can provide a means to detoxify ammonia, (ii) may play an important role in osmotic balance, and /or (iii) may serve as an alternate source of energy supply. Il'yasov (1978) earlier reported the occurrence of 17 amino acids in *R. echinobothrida*, of which one, i.e., lysine was not detected in the present study. In qualitative analysis, alanine, valine, and ornithine emerged as the major amino acids. There are reports that in *Hymenolepis diminuta*, the major free amino acids is alanine (Daugherty,1952; Foster and Daugherty, 1959; Campbell, 1963; Chappell and Read, 1973; Wack et al., 1983; Webb, 1986).

The levels of most of the amino acids detected in the control parasite decreased markedly (about > 50 to 90%) having a total decrease of about 53% when the parasite was treated with the crude extract of *F. vestita*. A reduction in the FAA pool was also noticed in the parasite treated with genistein and praziquantel, with a total decrease of about 50% in both the cases. This decrease in FAA pool in the treated parasite could be due to an increase in the catabolism rate of different amino acids after exposure to treatment, since this decrease in amino acid levels was accompanied by a marked increase in the level of ammonia

which would be formed as a first end product in amino acid catabolism. Higher accumulation of ammonia in the parasite treated with the crude extract and genestein might be one of the causes of paralysis noticed in the prsent study, as ammonia is known to have a neurotoxic effect in animals (Campbell, 1991).

Of the helminths that have been so far studies, most excrete significant amounts of nitrogen in the form of amino acids, peptides or protein (Barrett, 1991). Amino nitrogen constitutes 28% of the nitrogenous end products of *Hymenolepis diminuta* (Fairbairn et al., 1961). The amino acids excreted by *R. echinobothrida* were glutamic acid, valine, isoleucine, leucine, tyrosine, phenylalanine, α-aminobutyric acid, γ aminobutyric acid, histdine, ornithine, phosphoserine, methonine, β-alanine and arginine and showed a similar trend to that of *H. diminuta* (Fairbairn et al., 1961).The excretion of amino acids can provide a means of detoxyfying ammonia (Barrett, 1991). There was no significant difference in the ammonia levels of the control and the genistein- treated parasite (Table 8). Some amino acids such as alanine and proline may represent true metabolic end products; the two amino acids have been found to be the major component in the protonephridial tissue

fluid of cestode and trematode parasites (Webster and Wilson, 1970; Lutz and Siddiqi, 1971; Zavras and Roberts, 1984). Though not detected in the incubation medium of the control, threonine, and serine were detectable in the effluent of the parasite treated with the crude extract or praziquantel; the effluent of the treated parasite also did not contain ornithine, which was present in control. In many invertebrates, and possibly in helminths as well, amino acids are involved in the regulation of intracellular osmotic pressure; disruption of the osmotic balance when helminths are removed from their hosts and incubated in vitro could lead to amino acid leakage (Barrett, 1991). The differences between the pathways of amino acid metabolism in helminths and mammals, particularly in the metabolism of the sulphur amino acid and arginine and proline (Barrett, 1991), may be exploited in newer drug design.Proline biosynthesis inhibitors have shown promise as potential fasciolicides (Sheers et al., 1982). In the presence of hexachloroethane in the amphistomid fluke, *Fischoederius cobboldi*, valine amino transferase levels increased and leucine and isoleucine amino transferases (LAT and ILAT) were inhibited, whereas rafoxanide and albendazole caused high LAT and ILAT activity (Vanaja and Rao, 1992). Alterations

in the free amino acid content of the cestode on exposure to genistein indicate an antagonistic potential of this phytochemical towards the cestode parasite. In the search for newer target sites for anthelmintics development, amino acid metabolism of the parasite seems to provide a potential tool.

(vi) Genistein is shown in the present study, to bring about considerable change in the activity of tegumental enzymes, particularly of AcPase, AlkPase and ATPase, in the cestode parasite. Several effects of genistein on cellular physiology are now well known e.g., as inhibitor of protein tyrosine kinases (PTKs), hexose and dehydroascorbic acid transport (Vera et al., 1996), and L- type calcium currents (Chiang et al., 1996; Hatakeyama et al., 1996). Genistein which is a naturally occurring dietary PTK inhibitor is hypothesized to be responsible for the lower rate of breast cancer observed in Asian women consuming soyfoods (Peterson and Barmes, 1996). The results of the present study prompt further investigation in ascertaining the role of this phytochemical as anthelmintic.

SUMMARY

The present work incorporates a study on the anthelmintic efficacy of *Flemingia vestita* Benth and Hooker, an indigenous leguminous plant consumed by the natives in North East India. The in-vitro activity of the root- tuber peel extract of this plant and its active chemical principles was tested against the cestode, *Raillietina echinobothrida*. Changes in physical motility, histomorphological alterations, activity of non-specifiic and specific esterases that are associated with the nervous system, activity of some tegumental enzymes and changes in the levels of free amino acid pool and tissue ammonia are the parameters for this study.

(I) Live parasites from domestic fowl were collected in 0.9% physiological buffered saline (PBS) and maintained at 37 ± 1°C. In- vitro treatment of the parasite with the crude extract (50 mg/) in PBS revealed complete immobilization of the cestode in about 20 min. 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 cestodicide.

(II) Stereoscan and transmission electron microscopic observations revealed several tegumental alterations and deformity in the treated worms. Alterations in the contour of microtriches and disorganization of the tegumental region were conspicuously evident; the microtriches showed deformity and clumping in the parasite exposed to the crude peel extract. The tegument, inner subtegumengtal region and muscle layers were the sites predominantly affected by the genistein treatment (0.5 mg/ml); the severe distortion and disorganization occurred in the region of microtriches, and the inner subtegumental region showed pronounced vacuolization in comparison to controls.

(III) With the localization of non-specific esterases and cholinesterases, the organization of cholinergic components of the nervous system in toto could be visualized in the cestode; a pattern in conformity with the general plan of nervous system in cyclophyllidean cestodes was revealed. The cholinesterase in the parasite is acetylcholinesterase. Both non-specific esterases and cholinesterase 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 acetylcholinesterase activity. After exposure to the crude peel extract (50 mg/ml) and genistein (0.5 mg/ml), a pronounced decline in the visible stain intensity in the cholinergic components of the nervous system and tegument was noticeable, indicating extremely reduced activity of nonspecific esterases and cholinesterase in these sites. The total acetylcholinesterase 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 a reduction in the enzyme activity, somewhat at par with the genistein treatment.

Alteration in the acetylcholinesterase activity points towards acetylcholine, an inhibitory neurotransmitter in cestodes, as a potential target of action.

(IV) Acid phophatase, alkaline phosphatase, adenosine triphosphatase and 5'- nucleotidase are predominantly distributed in the tegument, subtegument and somatic musculature. After exposure to the crude extract and genistein, a pronounced decline in the visible stain

intensity was observed indicating very less or no activity in these sites. Quantitatively the activity of AcPase, AlkPase , ATPase and 5'-Nu was found to be supressed by 97%, 95%, 88% and 57%, respectively, following genestin treatment. The reference drug, praziquantel also caused a reduction in the enzyme activity, somewhat similar with the genistein treatment.

(V) Using high performance liquid chromatography phosphoserine, taurine, phosphoamine, threonine, serine, glutamate, proline, glycine, alanine, citrulline, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, β-alanine, α-aminobutyric acid, γ- aminobutyric acid, tryptophan, histidine, ornithine, arginine and ammonia were detected in the tissue homogenate of the parasite both qualitatively and quantitatively. After exposure to the crude extract (50 mg/ml) and genistein (0.5 mg/ml), alterations were noticeable in the free amino acid pool. Following genistein treatment, quantitatively the contents of phosphoserine, taurine, β-alanine,α- aminobutyric acid, tryptophan, histidine and valine were significantly lower and glutamate, methionine, isoleucine, γ- aminobutyric acid and ammonia were slightly higher than those in the control;

phosphoamine, citrulline and ornithine were not detectable. The reference drug, praziquantel (0.01 mg/ml) also caused a quantitative reduction in the free amino acid contents of the parasite, somewhat at par with the genistein treatment.

(VI) The active principle (genistein) of *F. vestita* root peel suggestively acts transtegumentally on the parasite. In view of the known effects of genistein on cellular physiology, anthelmintic role of this chemical of plant origin needs to be further ascertained.

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