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Anthelmintic efficacy of genistein, the active principle of *Flemingia vestita* (Fabaceae): Alterations in the activity of the enzymes associated with the tegumental and gastrodemal interfaces of the trematode, *Fasciolopsis buski*

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Acid phosphatase (AcPase) and alkaline phosphatase (AlkPase) were found widely distributed in tegumental tissues, i.e., tegument, subtegument and somatic musculature as well as in the gastrodermis of the intestinal fluke *Fasciolopsis buski.* Adenosine triphosphatase (ATPase) and 5'-Nucleotidase (5'-Nu), to the contrary, were localized in the tegumental tissues only while protease was associated with the latter-mentioned tissues. After exposure to the various test materials (crude root-peel extract of *Flemingia vestita* 20 mg/ml, its active component genistein 0.5 mg/ml and the reference drug oxyclozanide 20 mg/ml concentration) a significant decline in the visible stain intensity of all these enzymes except protease was observed in the aforementioned regions. Quantitatively, the activities of AcPase, AlkPase, ATPase, and 5'-Nu were found to diminish by 39-42.75%, 37.5-44.23%, 32.9-54.5% and 41.9-51.9%, respectively after treatment. The reference drug showed somewhat similar effect like the crude extract *of F. vestita.*

Keywords: **Acid phosphatase, Adenosine triphosphatase, Alkaline phosphatase, Anthelmintic, *Flemingia vestita,* Genistein, 5'-Nucleotidase, Phytochemicals, Protease, Tegumental enzymes, Trematode**

The presence of several enzymes viz., acid phosphatase (AcPase), alkaline phosphatase (AlkPase), adenosine triphosphatase (ATPase) and 5'-Nucleotidase (5'-Nu) has been detected both histochemically and biochemically in a number of helminth parasites, wherein these enzymes have been found to be closely associated with the tegument or cuticle, subtegument, somatic musculature and gut (Kwak and Kim, 1996; Buchmann, 1998; Fetterer and Rhoads, 2000). AcPase and AlkPase occur widely in the tegument of trematodes, cestodes and acanthocephalans. In adult cestodes AlkPase is usually the most active, whilst AcPase tends to predominate in the trematode tegument (Barrett, 1981). The metacercarial cyst of *Bolbogonotylus corkumi* and adult *Fibricola seoulensis,* an intestinal trematode of human and rodents, showed moderate AcPase activity (Walker and Wittrock, 1992; Huh, 1993). The functional characteristics of the tegument and intestinal wall were studied in mature *Fasciola hepatica* after treatment with luxabendazole, and alterations were noted in the activities of AcPase, ATPase, inosine triphosphatase and succinate dehydrogenase (Gorchilova et.al., 1990). The aqueous extract of some medicinal plants viz., *Butea monosperma, Embelia ribes* and *Roltlesia tinctoria* caused reduction in both AcPase and AlkPase activities of *Paramphistomum cervi* in vitro (Chopra et al., 1991). Praziquantel (PZQ), on the other hand, induced AlkPase activity on the surface of the adult *Schistosoma mansoni* (Fallon et.al., 1994).

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Majority of the tegumental enzymes are believed to be involved in digestion and/or absorptive function in cestodes (Poljakova et al., 1983). McCracken and Taylor (1983) reported biochemical effects of thiabendazole and cambendazole on *Hymenolepis diminuta* in vivo; the failure of glucose uptake could lead to the depletion of parasite AlkPase activity. The AcPase activity in the isolated brush border membrane of *H. diminuta* was inhibited particularly by levamisole, 2-mercaptoethanol and EDTA (Pappas, 1988).

The broad spectrum anthelmintics parbendazole and piperazine adipate showed a marked reduction in the activity of AcPase activity in the nematodes *Ascaridia galli* and *Heterakis gallinae* but the activities of lactate dehydrogenase and AlkPase were not affected significantly (Sharma et al., 1987). Some drugs like phenothiazine, diethylcarbamazine and centperazine altered the metabolism and inhibited the formation of mitochondrial energy, thus depriving the parasite *Setaria cervi* of ATP (Agarwal et al., 1990). Tetramisole and levamisole also caused inhibition of the specific activities of AcPase and Mg^{++}-dependent ATPase in *A. galli,* though AlkPase activity increased in the presence of both these drugs (Aggarwal et al., 1992). Thiabendazole and mebendazole changed the activity of AcPase in *Nippostrongylus brasiliensis* in infected mice (Mishra and Srivastava, 1990). Rafoxamide also affected the key dehydrogenases involved in the carbohydrate metabolism of *Trichuris globulosa* by blocking the glycolytic pathway depriving the parasite of energy (Parveen et.al., 1992).

Proteases are known to play an important role in the pathogenesis of several parasitic infections; besides general catabolic functions, these proteolytic enzymes may be significant in parasite immunoevasion, excystment/evagination/encystment, and cell and tissue invasion (Park et al., 2001; Sajid and Mckerrow, 2002). Proteases represent a very heterogeneous group of proteins among parasites (Wijffels et al., 1994) and have been isolated and characterized from the excretory/secretory products or tissue extracts of several helminth species including the liver/intestinal/blood/lung flukes, haematophagous and tissue dwelling nematodes, and cestodes (review-Trap and Boireau, 2000). In view of the differences in the inhibition properties from their host counterparts and specific capacities for immunomodulation the parasite proteases are currently being proposed as major immuno- and chemotherapeutic targets (Hawthorne et al., 2000; Hartmann and Lucius, 2003) and their potential in the development of vaccines is being exploited (Dalton et al., 2003; Williams et al., 2003).

In a previous study, genistein, the active principle of the root-peel of *Flemingia vestita,* a leguminous plant with edible tuberous roots, was shown to induce alterations in the activity of tegumental enzymes in cestodes (Pal and Tandon, 1998). In view of the putative transtegumental functioning of this phytochemical, we extended the study to trematode worms in order to ascertain if the plant-derived components influence the activity of enzymes associated with the tegumental and gastrodermal interfaces of these parasites.

MATERIALS AND METHODS

Parasites and test materials; Mature *Fasciolopsis buski* were collected from the intestine of swine *(Sus scrofa)* obtained from the local abattoirs in 0.9% Phosphate Buffered Saline (PBS, pH 7.2). The crude root-peel extract of *F. vestita* was prepared as described earlier (Tandon et al., 1997). The parasites were incubated with the crude extract (20 mg/ml), genistein (0.5 mg/ml) and oxyclozanide, the reference drug (20 mg/ml) concentration (all prepared in 0.9% PBS with 1% DMSO). The aforementioned concentrations were chosen since at these dosages the paralytic effect of the worm was attained within a shorter time frame as compared to lower concentrations (Roy and Tandon, 1996). Three replicates for each set of incubation medium were used. On attaining the paralytic state after treatment the trematodes were further processed for histochemical and biochemical studies, along with

one set of control specimens (maintained in 1% DMSO in PBS).

Histochemical localization:

AcPase: The AcPase activity was demonstrated following the modified lead nitrate method of Takeuchi and Tanoue as described by Pearse (1968), wherein β-glycerophosphate was used as the substrate. A brownish precipitate indicates sites of AcPase.

AlkPase: A modified coupling azo-dye method described by Pearse (1968) was used for the determination of AlkPase activity using Fast violet-B. The sites of AlkPase activity are coloured brown with salts, nuclei dark blue.

ATPase: For the demonstration of ATPase activity, calcium method using Na-ATP as the substrate, after Maengwyn-Davies et al., as described by Pearse (1968) was followed. The activity of ATPase shows as a blackish brown deposit.

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. Yellow deposits of lead sulphide indicate sites of 5'-Nu enzyme activity.

Protease: For the demonstration of protease the gelatin method of Adams and Tuqan (1961) as described by Pearse (1968) was followed.

[The specimens were fixed in cold (4°C) 4% formaldehyde-saline for 24 h. They were washed briefly in water and 10-15 μm cryostat sections were cut. Sections were mounted on to a blackened photographic plate, cut to a suitable size. The plate was prepared by exposing rapid panchromatic quarter-plate to daylight for 10-15 min, developed in the usual way and hardened in a thiosulphate-metabisulphite bath before drying. After mounting, the plates were allowed to become dry and then the sections and the surrounding gelatin were lightly dampened with 0.15 M phosphate buffer (pH 7.6). The sections were then incubated for 30-60 min at 37°C in a petri dish containing moist filter paper. At 5 min intervals the sections were inspected to make sure that they were not too dry, in which case further buffer must be added. After incubation the preparation is dried without washing and subsequently dehydrated in alcohol, cleared in xylene and mounted in a synthetic medium.]

The protease activity is shown as a clear area where the gelatin film and its contained granules have disappeared. Histological, but not intracellular, definition is obtained.

Biochemical assays:

AcPase and AlkPase : AcPase and AlkPase activities were 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. One unit of AcPase or AlkPase activity is defined as that amount which catalyzed the formation of 1 mM of p-nitrophenol/h at 37°C.

ATPase: ATPase activity was assayed by estimating the free phosphate released, following the method of Kaplan (1957) with Na-ATP as the substrate. One unit of ATPase is defined as the amount which catalyzed the release of 1 μmole of phosphate / h at 37°C from ATP.

5'-Nu: The enzyme activity was assayed by estimating the free phosphate released following the method of Bunitian (1970) using AMP as the substrate. One unit of 5'-Nu activity is defined as that amount which catalyzed the release of 1 μmole of phosphate/h at 37°C from AMP.

Protein: The protein content was estimated following the method of Lowry et al. (1951) using bovine serum albumin as a standard.

All the chemicals used in the present study were procured from Sigma Chemicals, USA or SRL, India.

RESULTS

Controls: Intense AcPase and AlkPase activities were observed in the tegument and gastrodermis. Other regions viz., the subtegument and somatic musculature displayed staining intensity lesser than

Table I: Activities of AcPase, Alkpase, ATPase and 5'Nu in various structures of *Fasciolopsis buski* in vitro: histochemical localization

Treatment	AcPase				AlkPase				ATPase				5'-Nu			
(mg/ml)	T	ST	SM	IN	T	ST	SM	In	T	ST	SM	IN	T	ST	SM	IN
Control (0.9% PBS)	+++	+++	+++	+++	+++	-	+++	+++	+++	+++	+++	++	+++	+++	+++	++
F. vestita crude root peel extract (20)	++	+	-	-	+	-	++	++	-	-	-	-	-	-	+	-
Genistein (0.5)	++	++	-	-	+	-	++	+	-	-	-	-	-	-	+	-
Oxyclozanide	++	++	-	-	+	-	++	+	-	-	-	-	-	-	-	-

T- tegument; ST - Subtegument; SM- somatic musculature; IN- intestine
+++ Very strong activity, ++ Strong activity, + Medium activiy, + les activity, N activity

Table II: Biochemical effects of the various plant-derived test materials on *F. buski*

Treatment (mg/ml)	Enzyme activity (total[a]/specific[b])				%decrease*			
	AcPase	AlkPase	ATPase	5'-Nu	AcPase	AlkPase	ATPase	5'-Nu
Control (0.9% PBS)	11.3 ±0.26/ 1.48 ± 0.03	29.7 ± 3.34/ 3.9 ± 0.44	509.35 ± 85.9/ 66.67 ± 11.25	43.3 ± 2.37 5.67 ± 0.31				
Crude extract of *F. vestita* (20)	6.47 ± 0.26/ 0.98 ± 0.04	16.2 ± 1.6/ 2.47 ± 0.25	303.72 ± 44.1/ 46.22 ± 6.72	22.4 ± 2.14/ 3.41 ± 0.3	42.75	44.23	39.85	47.8
Genistein (0.5)	6.54 ± 0.26/ 1.47 ± 0.06	17.3 ± 2.44/ 3.9 ± 0.54	213.9 ± 18.15/ 48.06 ± .08	20.6 ± 1.87/ 4.6 ± 0.42	42	42.31	54.5	51.9
Oxyclozanide (20)	6.6 ± 0.25/ 1.2 ± 0.05	17.46 ± 2.8/ 3.18 ± 0.51	308 ± 27.3/ 56 ± 5	24.84 ± 2.05/ 4.52 ± 0.4	41.6	42.21	36.3	42.8

Values are given as mean (± SEM) from three to five replicate assays (5' Nu, n=4; ATPase, n=3; AlkPase, n=4; AcPase, n=5)

[a] One unit of enzyme activity is defined as that amount of enzyme which consumes 1.0 µmol substrate/g wet wt tissue/h

[b] Specific activity expressed as unit/mg protein

* $P > 0.05$

the tegument (Table I; Figs. 1, 2, 4). Not much of activity was observed in the general parenchyma or the structures related to the excretory or reproductive systems.

The ATPase activity was observed mainly in the tegument of the fluke. In the subtegument, somatic musculature and intestinal caeca moderate staining was demonstrated. Among the structures that were studied tegument, subtegument, somatic musculature and gastrodermis showed an equally strong activity of the 5'-Nu enzyme. The stain intensity was much less in the muscle layer (Figs. 7, 8, 10, 11).

Quantitatively the control parasites showed more enzyme activities than the treated ones with respect to all the four enzymes that were tested.

Treatment: The activities of AcPase, AlkPase, ATPase and 5'Nu in the tegument and gastrodermis were inhibited after treatment with the plant test

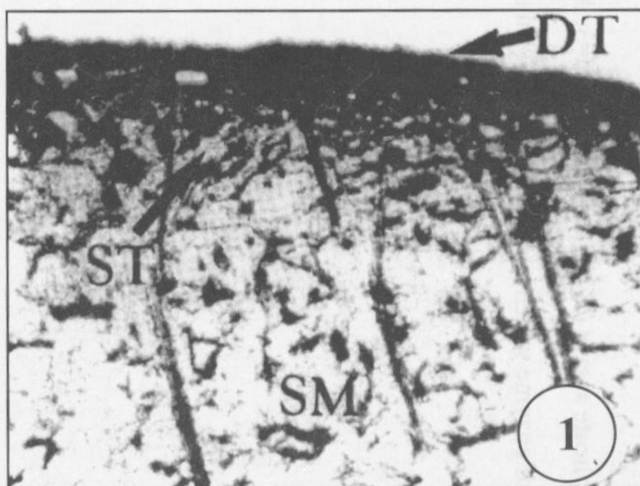


Fig. 1: AcPase activity in the cryostat transverse sections: Control - Intense enzyme activity in the dorsal tegument (DT), subtegument (ST) and somatic musculature (SM) (120x).

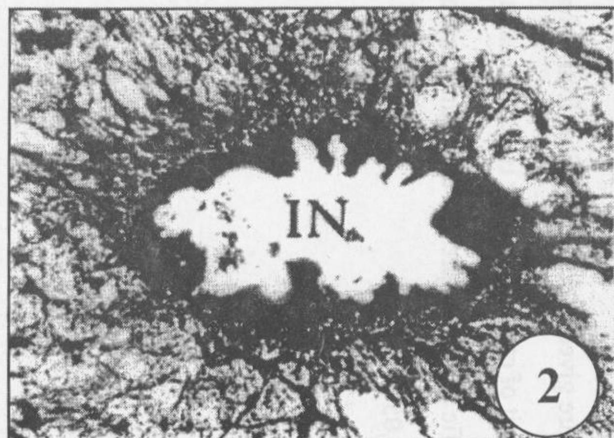


Fig. 2: AcPase activity in the cryostat transverse sections: Control- strong enzyme activity in the intestinal caecum (IN) (120x)

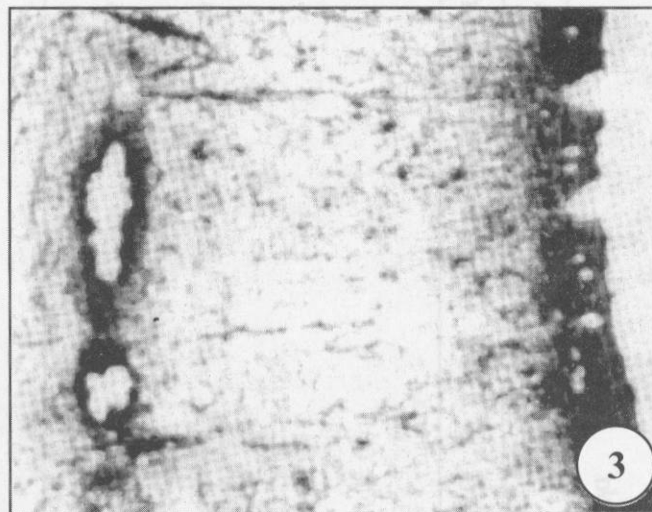


Fig. 3: AcPase activity in the cryostat transverse sections: Reduced enzyme activity in the dorsal and ventral tegument, subtegument, somatic musculature and intestinal caecum after treatment with the root tuber peel extract of *F. vestita* (48 x).

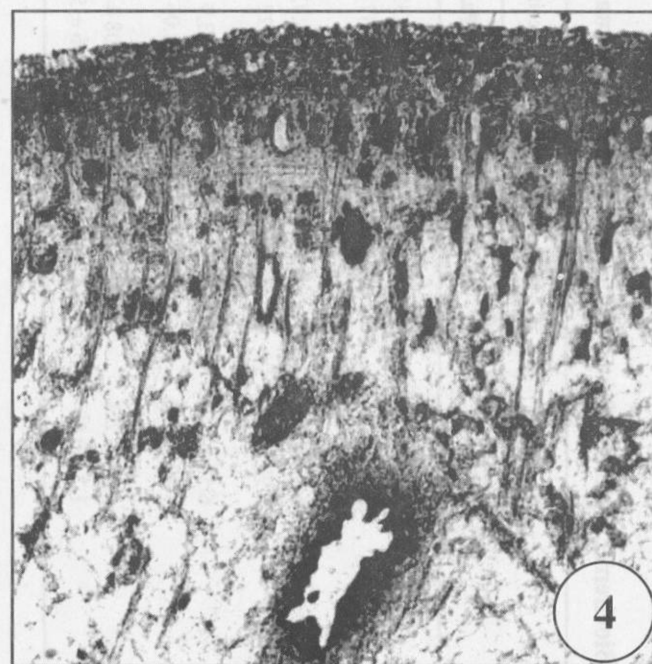


Fig. 4: AlkPase activity in the cryostat transverse sections: Intense enzyme activity in the dorsal tegument, subtegument, somatic musculature and gastrodermal lining of control flukes (48x).

Figs. 5-6: AlkPase activity in the cryostat transverse sections

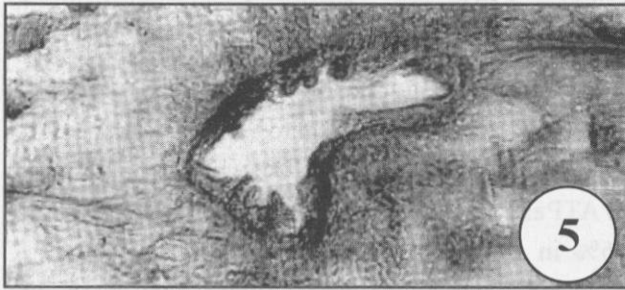


Fig 5: Enzyme activity in the intestinal caecum after treatment with the active component of *F. vestita,* genistein (120x).

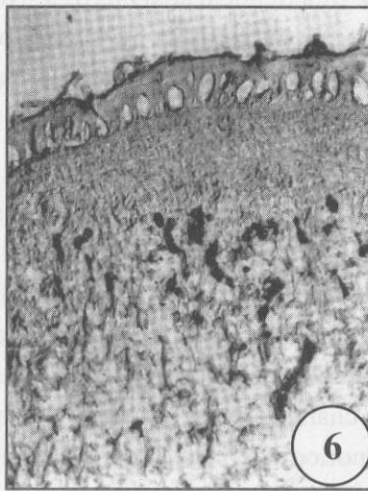


Fig 6: Reduced enzyme activity in the ventral tegument, subtegument and somatic musculature after genistein treatment (48x).

Figs. 7-8: 5'-Nu activity in the cryostat transverse sections of *F. buski*

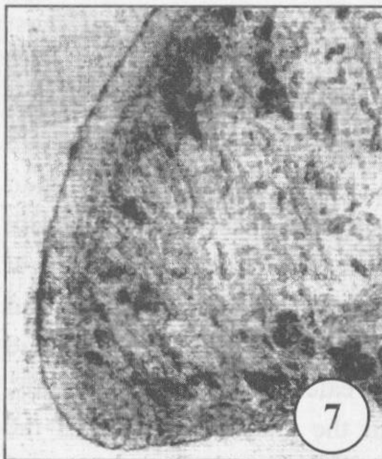


Fig.7: Control- section showing intense enzymatic activity in the tegument, subtegument and somatic musculature (48x)

Fig. 8: Control- sections showing enzyme activity in the intestinal caecum and (48x).

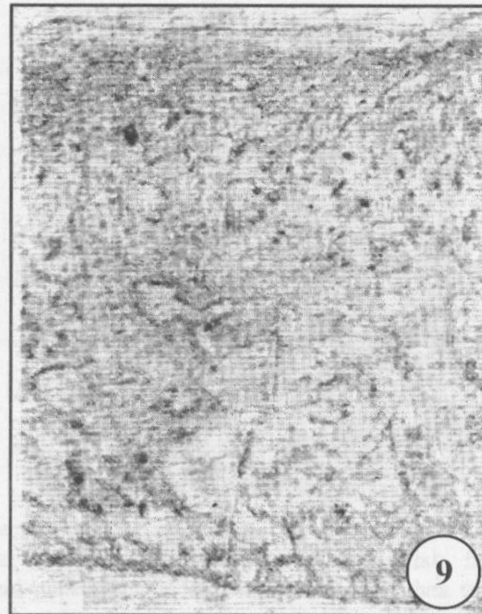


Fig. 9: 5'-Nu activity in the cryostat transverse sections of *F. buski,* Mild enzyme activity detectable in sections after treatment with root tuber peel extract of *F. vestita* (48x).

Figs 10-11: ATPase activity in the transverse cryostat sections.

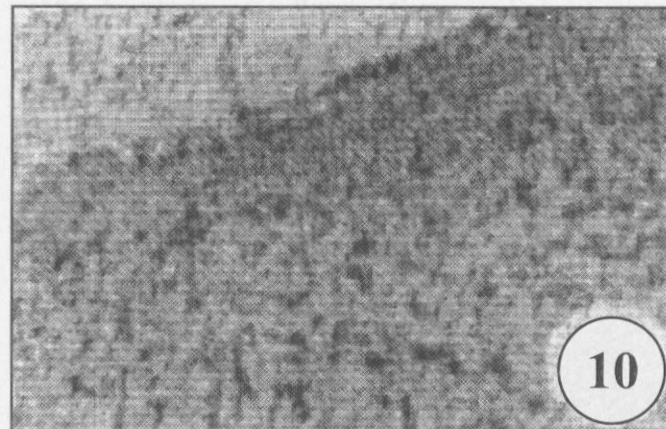


Fig. 10: Control- enzyme activity demonstrated in the dorsal tegument along with somatic musculature (48x).

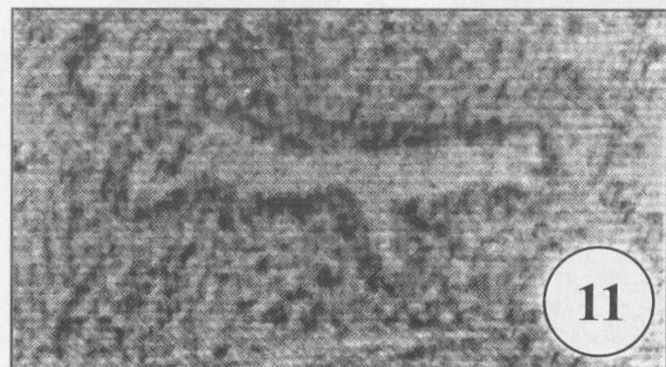


Fig. 11: Control- enzyme activity in the intestinal caecum (120x).

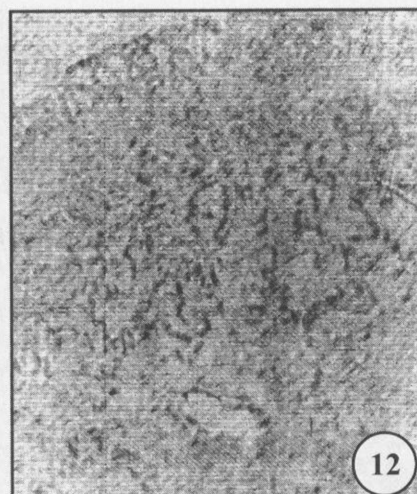
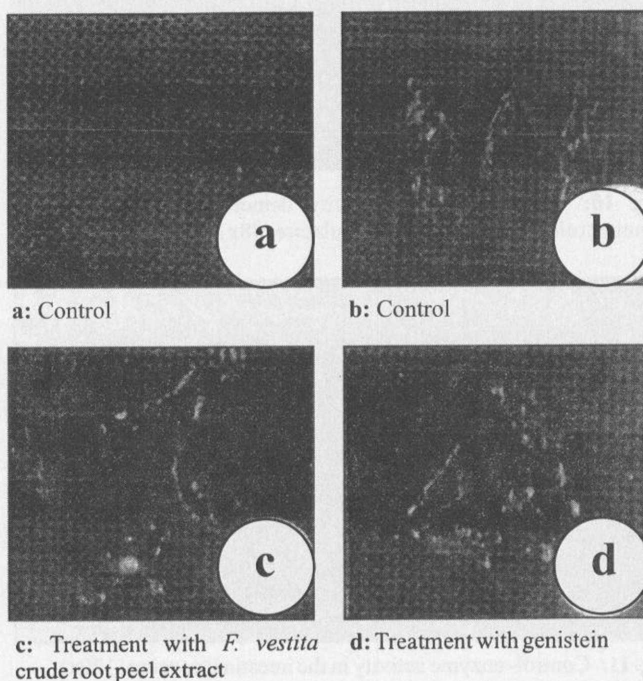


Fig.12: ATPase activity in the transverse cryostat section: Diminished stain intensity in the dorsal tegument, somatic musculature and the intestinal caecum after genistein treatment (48x).

materials, but moderate activity was discernible in the somatic musculature. Almost all the structures of the treated flukes showed decrease in enzyme staining intensity and a similar result was observed after treatment with the reference drug (Figs. 3, 5, 6, 9, 12).

The results of the enzyme assays are described in Table II. The AcPase activity in *F. buski* paralyzed with the *F.*

Fig. 13a- 13d: Protease enzyme activity in *F. buski,* fresh frozen sections (photographs)



a: Control **b:** Control

c: Treatment with *F. vestita* crude root peel extract **d:** Treatment with genistein

vestita crude extract and genistein was inhibited by 42.75 % and 42 %, respectively, whereas oxyclozanide caused a decline by 41.6 % compared to the controls. Varying degree of inhibition of the AlkPase activity (37.5-44.23 %) in comparison to control was observed after treatment with the plant-derived components. The ATPase activity also got reduced by 39.85% and 54.5% in treatments with crude peel extract of *F. vestita* and genistein, respectively, while in the reference drug-treated parasite a decrease by 36.6 % was recorded. Biochemical analysis of 5'-Nu showed a decrease in the enzyme activity by 47.8 % for *F. vestita* and 51.9 % for genistein. The parasites treated with the reference drug showed a decrease by 42.8 % (Table II).

Protease: The proteolytic enzyme activity was demonstrable histochemically in both control as well as the parasites treated with the various test materials, though owing to the thickness of the sections, cellular or subcellular localization of the protease activity was not discernible. At the generalized tissue level, no conspicuous change in the intensity of the enzyme reaction was noticeable in the treated parasite from that of the control (Fig. 13 a-d).

DISCUSSION

Ultrastructural studies have revealed that the tegument in trematodes has an absorptive potential. The processes of secretion and absorption appear to operate concurrently in the gastrodermis of most trematodes, although one or other function may predominate at any given time. In a number of helminth parasites AcPase, AlkPase, ATPase and 5'-Nu have been detected both histochemically and biochemically, and found to be closely associated with the tegument, subtegument, somatic musculature, gut and cuticle (Pappas, 1988; Leon et al., 1989; Kwak and Kim, 1996; Pal and Tandon, 1998; Fetterer and Rhoads, 2000). As revealed in the present study, alterations occurred in the enzyme activity of the parasite after treatment with the crude root tuber peel extract of *F. vestita* and its active component genistein, both showing synchronous effectiveness. Oxyclozanide showed more or less similar effect as the

plant-derived components. After treatment, AcPase showed less stain intensity compared to the controls. Similar observations were made in various trematode species exposed to treatment with hexachlorophene (Gupta and Sharma, 1973). Likewise, thiabendazole and mebendazole changed the activity of AcPase in *N. brasiliensis* (Mishra and Srivastava, 1990). As in mammals, in helminths also, AcPase is usually associated with lysosomes and AlkPase is regarded as indicative of membrane transport mechanisms (Barrett, 1981). The inhibition of AcPase activity by the plant-derived components observed during the present investigation is suggestive of the fact that absorption and intracellular digestion of drugs may involve lysosomes (Colam, 1971). The lessening of enzyme activity in the present study may probably be due to its leakage into the medium as a result of the disruption of the absorptive surface (Hart et al., 1977).

The major AcPase activity in trematodes is localised at the luminal surface of the intestinal caeca and is associated with the plasma membrane of the lamellar folds or the microvilli (Threadgold and Brennen, 1978). It is possible that the activity at these sites is related to digestion of phosphate esters at membrane level and in common with the phosphatases of the mammalian intestinal brush border yields a kinetic advantage in the subsequent absorption of the digestive products.

In *F. buski* though histochemically AcPase showed higher stain intensity compared to AlkPase, biochemical results showed an increased AlkPase activity in the tissues compared to AcPase. In the present study AlkPase activity decreased by 37.5 to 44.23% after treatment with the various test materials, and in histochemical localization also it showed decreased stain intensity. AlkPase has been localized on the surface of both male and female *Schistosoma mansoni* as a result of praziquantel-induced tegumental damage (Mehlhorn et al., 1981; Modha et al., 1990). Any direct involvement of phosphatases in absorption, as suggested by their association with absorptive structures in trematodes, seems unlikely since studies on several epithelial cell types of mammals indicate that the transport systems and sites of phosphatase activity are separate (Lumsden, 1975). Non-specific AlkPase activity was localised at the tegumental plasma membrane of *Cyathocotyle,* and in the surface channels of the tegument *of Schistosoma* (Ernst, 1976). These findings (of AcPase and AlkPase in tegument and gastrodermis) raise the possibility that the tegument in some trematodes may function in a digestive-absorptive capacity, analogous to that of the mucosal lining of the vertebrate intestine. Delabre-Defayolle et al. (1989) reported inhibition of AlkPase activity by 23% and complete inhibition of glucose uptake in *Echinococcus multilocularis* 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 (Pappas, 1983). Some plant extracts also showed a decrease in both AcPase and AlkPase activities in *Paramphistomum cervi* in vitro (Chopra et al., 1991), but Fallon et al. (1994) showed more than two-fold increase in AlkPase activity in the PZQ-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 investigation revealed a high ATPase activity in the control worm. A tegumental involvement in ionic and water regulation in *F. hepatica* was indicated by the demonstration at the tegumental plasma membranes of Na^+/K^+-dependent ATPase activity (Threadgold and Brennen, 1978). The localization of ATPase in the somatic musculature of *F. buski* indicates that the enzyme occurs in the myofibrils. The ATPase activity was inhibited by 36.6-54.5% in the flukes treated with all the test materials. Similar observations were also made for *E. multilocularis* isolated scolices of hydatids after exposure to mebendazole, thiabendazole, levamisole and acrisaline (Benediktov, 1980). Drugs like phenathiazines, chlorimazine, diethylcarbamazine and centperazine caused significant inhibition of 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).

In the present study the 5'-Nu activity decreased by about 42-52% following treatment with genistein or other test materials in *F. buski.* 5'-Nu is supposed to have an active role in transport across plasma membrane or may be involved with other enzymes in the hydrolysis of nucleosides to pyrimidine and purine bases in parasitic nematodes (Barrett, 1981; Walter and Albeiz, 1985).

In the present investigation protease activity was demonstrated histochemically in *F. buski,* both control and treated and there was no discernible change in the enzyme activity in the parasite exposed to the various treatments. The protease activity in *Schistosoma* is reduced by specific trypsin inhibitors (Zussman and Bauman, 1971). As demonstrated with histochemical and immunocytochemical techniques a major source of proteolytic activity is the intestinal caeca and protonephridia in trematodes (Howell, 1973; Rotmans, 1977; Skelly and Shoemaker, 2001).

The alterations in the activity of the tegumental gastrodermal enzymes after treatment with the various plant-derived components suggest that these enzymes (excluding protease) may be a potential target of action for genistein, which, besides acting transtegumentally, may as well affect through the gastrodermal interface.

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