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ORIGINAL ARTICLE

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Anthelmintic efficacy of ethanolic shoot extract of *Alpinia nigra* on tegumental enzymes of *Fasciolopsis buski*, a giant intestinal parasite

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Abstract Ethanolic shoot extract of *Alpinia nigra*, a traditionally used anthelmintic medicinal plant among the Tripuri tribes of north-east India, was tested *in vitro* to determine its anthelmintic efficacy in gastrointestinal trematode *Fasciolopsis buski*, using alterations in the activity of vital tegumental enzymes viz. acid phosphatase (AcPase), alkaline phosphatase (AlkPase) and adenosine triphosphatase (ATPase). Live adult *F. buski* treated *in vitro* with different concentrations of the plant extract showed significant decline in the visible stain histochemically and enzyme activities. Quantitatively, the total enzyme activity of AcPase, AlkPase and ATPase was found to be reduced by 45, 41 and 43%, respectively compared to the control. The reference drug, praziquantel also showed more or less similar effect like that of the plant extract. The results suggest that the tegumental enzymes of the parasite may be an important target of action for active component(s) of *A. nigra*, which appears to act transtegumentally.

Keywords *Alpinia nigra,* Anthelmintic, *Fasciolopsis buski,* Tegumental enzymes

Introduction

Alpinia nigra (Gaertn) Burtt (Family Zingiberaceae) is a medicinal plant, the shoot of which is considered to have anthelmintic properties and hence aqueous crude juice is consumed by the natives of the Hill states of north-east India, Tripura in particular as a popular cure against intestinal worm infections. The crude shoot extract of this plant has been reported to cause destruction of surface tegument leading to paralysis and death of intestinal parasite (Roy and Tandon 1999); the plant extract is also responsible for distortion and disorganization of cytoplasmic organelles and vacuolization of tegument in parasitic worms (Roy et al. 2009).

The presence of several vital enzymes viz. acid phosphatase (AcPase), alkaline phosphatase (AlkPase), adenosine triphosphatase (ATPase), 5′-nucleotidase (5′-Nu) has been reported in the body tegument, and the various organs/tissues of many flat worms (Kwak and Kim 1996; Pal and Tandon 1998; Kar and Tandon 2004; Lalchhandama et al. 2008). All these enzymes which are involved in various metabolic processes, are also believed to be involved in absorption and/or digestion in the parasite (Roy 1982; Poljakoiva-Krustena et al. 1983). Many commercial anthelmintics like parbendazole, piperazine adipate, phenothiazine, diethiazine, diethylcarbamazine, centperzine, tetramisole and levamisole alter the metabolism and disrupt mitochondrial energy formation results in decrease in ATP levels (Agarwal et al. 1990; Aggarwal et al. 1992; Vinaud et al. 2009). Activities of many of these enzymes are reported to be altered by crude ethanolic extract of traditionally used medicinal plants too, like *Flemingia vestita, Acacia oxyphylla, Millettia pachycarpa* (Tandon and Das 2007; Lalchhandama et al. 2007; Lalchhandama et al. 2008). Aqueous extract of *Butea monosperma*, *Embelia ribes* and *Roltleria tinctoria* causes reduction in both

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AcPase and AlkPase activity in *Paramphistomum cervi in vitro* (Chopra et al. 1991).

In view of the functional significance of tegumental enzymes in digestion or absorption in trematodes, the present investigation was aimed to study the effect of *A. nigra* ethanolic extract on the tegument of *F. buski* and enzymes viz. AcPase, AlkPase and ATPase.

Materials and methods

Preparation of the plant crude extract

The edible fresh shoots of *A. nigra* were collected from different places (villages) of Tripura, India. After washing gently with water the shoots were oven dried, grounded by motor-driven grinder into powder form, and refluxed in 90% alcohol for 12 hours at 60°C. After reflux the solution was filtered through Whatman filter paper No. 1 and the collected solution evaporated to dryness at 50°C to recover dry powder, which was stored at 4°C till further use.

Test parasites

Mature *F. buski* were collected from the intestine of freshly slaughtered pigs from the local abattoir in 0.9% phosphate buffer saline (PBS, pH 7.2). After washing in PBS, the flukes were incubated at 37°C ± 1°C with different concentrations of the plant extract viz. 5, 10 and 20 mg/ml in PBS. Praziquantel, a broad-spectrum anthelmintic drug, was used as the reference drug at similar concentrations. Control flukes were incubated in PBS alone. Three replicates for each set of incubation medium were used and time taken for attaining paralytic state and death was recorded as described earlier (Roy et al. 2009). Immediately after attained paralytic state, the parasites incubated in 20 mg/ml of PBS were selected for histochemical and biochemical studies because of the early effect of the dose when compared with other low concentrations.

Histochemical localization of enzymes

The following enzymes were investigated histochemically using duly processed frozen sections cut at a thickness of 10–15 μm in a Leica CM 1850 cryostat.

AcPase activity was detected in cold formol-calcium-fixed specimen following the modified Lead nitrate method (Pearse, 1968), using sodium β-phosphoglycerate as the substrate. A brownish precipitate indicates the sites of AcPase. A modified coupling azo-dye method as described by Pearse (1968) was used for the detection of AlkPase activity at room temperature (17–20°C). The brown color was observed with fast violet B. For the localization of ATPase activity, the calcium method of Pearse (1968) was followed; ATP was used as the substrate and the enzyme activity was determined through observation of blackish brown deposit.

Biochemical assays

Activities of AcPase and AlkPase were measured by estimating the p-nitrophenol formation following the method of Plummer (1988) with some modifications. A 10% (w/v) tissue homogenate was prepared in sodium acetate buffer (125 mM, pH 4.5) and sodium glycine buffer (pH 10.5) for AcPase and AlkPase, respectively and centrifuged at 5,000 rpm for 20 minutes at 0 ± 2°C. The supernatant was taken for the enzyme study. Using p-nitrophenyl phosphate as substrate the enzyme activity was measured by increase or decrease in the optical density at 412 nm in a double beam UV-visible spectrophotometer (Beckman Model-26).

Table 1 Histochemical localization of AcPase, AlkPase and ATPase in various body parts of *Fasciolopsis buski*

Treatment mg/ml	AcPase				AlkPase				ATPase			
	T	St	Sm	In	T	St	Sm	In	T	St	Sm	In
Control (0.9% PBS)	++	++++	++++	+++	++++	++++	+++	+++	++++	+++	+++	+++
A. nigra (20)	++	++	-	+	++	++	+	++	++	++	+	+
Praziquantel (20)	+	+	+	+	-	+	+	+	+	+	+	+

T - Tegument; St - Sub-tegument; Sm - Somatic musculature; In - Intestine
++++ very intense activity, +++ intense activity, ++ moderate activity, + mild activity, - no activity.

Table 2 Biochemical effects of shoot-extract of *A. nigra* and praziquantel on *F. buski*

Treatment mg/ml	Enzyme activities *(Total and specific)*			% decrease		
	AcPase	AlkPase	ATPase	AcPase	AlkPase	ATPase
Control (0.9% PBS)	17.30 ± 0.26 *1.70 ± 0.04	37.40 ± 0.54 *3.93 ± 0.09	86.28 ± 0.54 *8.25 ± 0.06			
A. nigra (20)	9.44 ± 0.29 *0.97 ± 0.04	22.09 ± 0.28 *2.53 ± 0.07	49.17 ± 0.53 *5.06 ± 0.09	45.43	40.93	43.01
Praziquantel (20)	7.33 ± 0.26 *0.83 ± 0.02	22.04 ± 0.27 *2.55 ± 0.04	44.85 ± 0.27 *5.19 ± 0.04	57.63	41.07	48.01

Values are given as mean (±SE) from four replicates (n = 4).
Total activity = formation of 1 µmol of product/h/g of wet tissue.
*Specific activity = Activity/mg protein.

The ATPase activity was assayed by estimating the free phosphate released, following the method of Kaplan (1957) with ATP-Na_2 as a substrate with little modification, following Zaidi et al. (1981). A 10% tissue homogenate was prepared in 0.1 M Tris-HCl buffer (pH 7.5). In a final volume of 1 ml reaction mixture containing 0.1 M Tris-HCl buffer, 3 mM ATP-Na_2, 30 mM $MgCl_2$, 100 mM NaCl and 20 mM KCl, 0.2 ml of tissue homogenate was added and the mixture incubated for 1 hour. After incubation 1 ml 10% TCA was added. The mixture was centrifuged for 10 minutes at a speed of 3,000–4,000 rpm in room temperature. Using the supernatant inorganic phosphate was determined by the method of Fiske and Subba Row (1925). Similarly, blank tissue was prepared following the same method except the TCA was added before the addition of tissue homogenate.

One unit of enzyme activity is defined as the amount of product formed per h per g tissue. Specific activity is expressed as units/mg protein. The protein content of all the tissue was estimated following the method of Lowry et al. (1951) using bovine serum albumin as a standard protein.

Results

The distribution and intensity of AcPase, AlkPase and ATPase in *F. buski* maintained as control, and treated with the ethanolic crude extracts of *A. nigra* and praziquantel are presented in Table 1 and Figures 1–3. Quantitative estimation of enzyme activity is depicted in Table 2.

In the control fluke intense staining activities of AcPase, AlkPase and ATPase were observed in the tegument, sub-tegument, somatic musculature as well as intestine (Figs. 1a, b; 2a, b and 3a, b). For all the enzymes highest intensity were observed mainly in tegument and sub-tegument regions. In other regions like somatic musculature and intestine also, high staining was noticed, though not similar to that in tegumental and sub-tegumental regions.

On exposure to the crude shoot extract of *A. nigra* and praziquantel, reduced activities of AcPase, AlkPase and ATPase were observed (Table 1). The AcPase activity of the somatic musculature and intestine were diminished, while mild visible staining was observed in the tegument and sub-tegument regions of the plant extract-treated fluke (Fig. 1c, d). Almost all the regions of the praziquantel-treated flukes showed totally diminished staining activity for AcPase (Fig. 1e, f). Reduction in the activity of AlkPase was also observed in the tegument, sub-tegument, somatic musculature and intestine of the crude plant extract- and praziquantel-treated flukes (Fig. 2 c, d, e, f); compared to other regions, less staining activity was observed in somatic musculature and intestinal regions. The ATPase activity was also found to be reduced in all the regions of the *Alpinia*- and praziquantel-treated parasites as compared to the controls (Fig. 3 c, d, e, f).

In biochemical analysis also, all the enzymes in the treated parasites showed highly reduced activity. AcPase, AlkPase and ATPase were found to be reduced by 45.43, 40.93 and 43.01%, respectively in the parasite treated with shoot-extract of the plant. Decreased enzyme activities were also recorded in the flukes treated with praziquantel

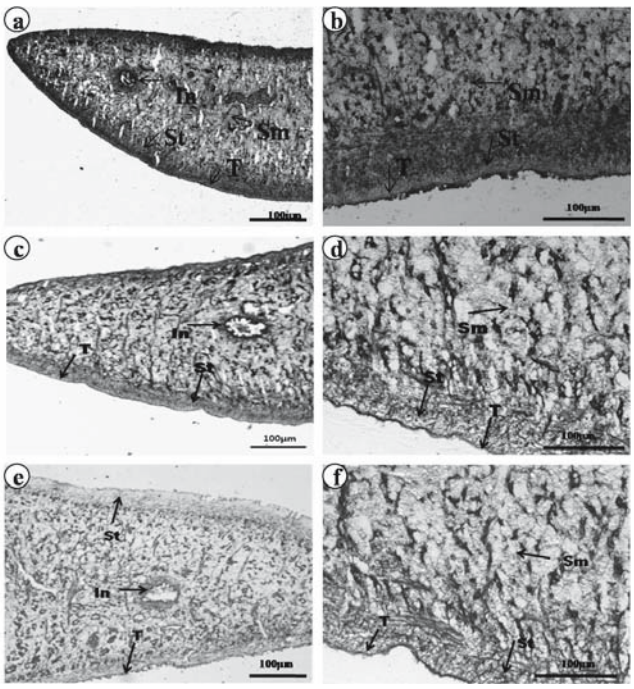

Fig. 1 Acid phosphatase activity in *F. buski*, photographs of fresh frozen sections
a. Control (25×)
b. Enlarged view of control (100×)
c. Shoot extract of *A. nigra* treated section (25×)
d. Enlarged view of *A. nigra* treated section (100×)
e. Praziquantel treated section (25×)
f. Enlarged view of praziquantel treated section (100×)

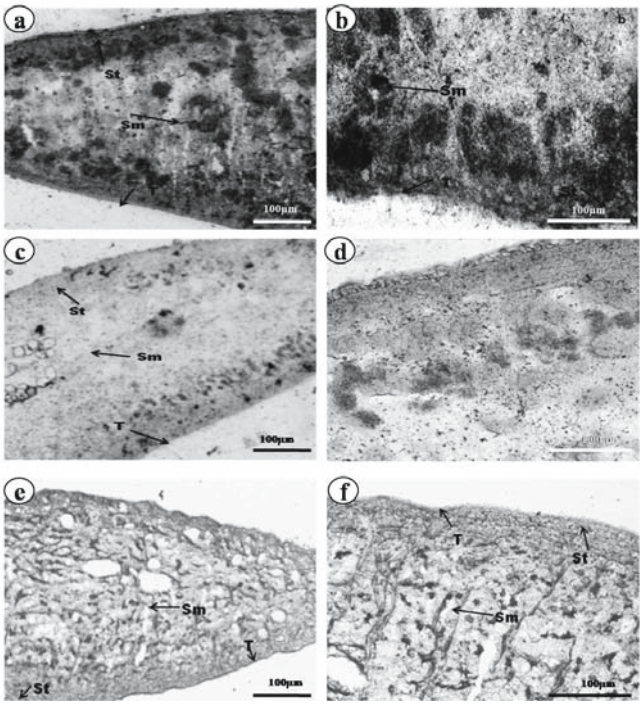

Fig. 2 Alkaline phosphatase activity in *F. buski*, photographs of fresh frozen sections
a. Control (25×)
b. Enlarged view of control (100×)
c. Shoot extract of *A. nigra* treated section (25×)
d. Enlarged view of *A. nigra* treated section (100×)
e. Praziquantel treated section (25×)
f. Enlarged view of praziquantel treated sections (100×)

(7.33 ± 0.26/0.83 ± 0.02, 22.04 ± 0.27/2.55 ± 0.04 and 44.85 ± 0.27/5.19 ± 0.04, respectively). The enzyme activities in the untreated control flukes were observed to be much higher than the treated parasite (Table 2).

Discussion

Anthelmintic drugs are known to enter target parasites by oral ingestion or by tegumental diffusion or sometime by combination of both routes (Thompson et al. 1993; Thompson and Geary 1995). Among trematode parasites the function of nutrient uptake, osmoregulation and digestion is performed by tegument also in addition to intestine (Meaney et al. 2002, 2004).

Consequently, it has been sufficiently accounted that one of the hallmark effects of any anthelmintic is destruction of trematode surface (Roy and Tandon 1996, 1999; McKinstry et al. 2003; Xiao et al. 2004; Rivera et al. 2004).

In the present investigation high abundance and intensity of AcPase, AlkPase and ATPase were observed in the tegument of *F. buski*. Similar to the present observation, in a number of trematode and cestode parasites these phosphatases have been detected both histochemically and biochemically, and found to be closely associated with the tegument, sub-tegument, somatic musculature and gut (Leon et al. 1989; Pappas 1991; Pal and Tandon 1998; Lalchhandama et al. 2007, 2008).

AcPase and AlkPase are found to be involved in the uptake of certain nutrients, glycogen and lipoprotein in various helminth parasites (Sharma 1976). Differences in the activity of these phosphatases have been detected in a large number of helminth parasites in relation to their difference in pH values (Park and Seo 1968). Following the treatment of *Genistein*, the active component of *Flemingia vestita*, the tegumental enzymes like AcPase, AlkPase, ATPase and 5′-nucleotidase of *Raillietina echinobothrida* were found to be decreased many fold (Pal and Tandon 1998). Changes in the aforementioned enzymes have also been observed in *F. buski* following the treatment with genistein

(Kar and Tandon 2004). Inhibition of AcPase activity by the plant extract as observed in the present investigation may suggest that the absorption and intracellular digestion of drugs may involve lysosomes (Colam 1971). Ultrastructural observations carried out on the surface tegument of *F. buski* also confirmed its secretory and absorptive functions, a decrease in the enzyme activity is probably due to its leakage into the medium as a result of the disruption of the absorptive surface (Roy et al. 2009).

In the present investigation both AcPase and AlkPase were found to be equally active in control *F. buski*. However, in the plant extract-treated fluke, the AlkPase activity was revealed to be higher in the somatic musculature and intestine compared to AcPase, total activity of the enzyme was reduced by 40.93% after exposure to plant extract. A similar amount of reduction was also noticed in praziquantel-treated fluke. In metacestodes of *Echinococcus multilocularis*, inhibition of the AlkPase activity by 23% and complete inhibition of glucose uptake were reported following treatment *in vivo* with isatin, a known phosphatase inhibitor, and the depletion in the enzyme activity was attributed to the failure of glucose uptake (Delabre-Defayolle et al. 1989). However, more than 2-fold increase in the AlkPase activity, observed in the praziquantel-treated *Schistosoma mansoni* was attributed to the drug-induced teguemntal damage exposing the normally concealed enzymes of the tegumental surface of the worm (Fallon et al. 1994).

In the present study, high ATPase activity was observed in the tegument, sub-tegument, muscle layer and intestine of the parasite. The ATPase activity was reduced by 43.01% in the flukes treated with the crude extract of *A. nigra*. A similar type of observation was recorded in *F. buski* treated with crude extract of *Flemingia vestita* and its active principle, genistein (Kar and Tandon 2004).

The alteration observed in the activity of the phosphatase enzymes of the shoot extract of *A. nigra* suggest that the tegumental enzymes of the parasite may be a plausible target of action for the plant product, which appears to act transtegumentally.

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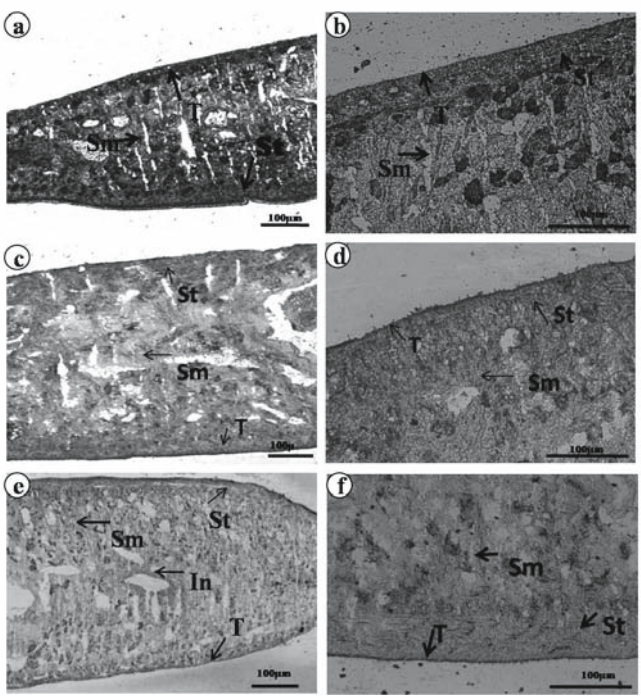

Fig. 3 Adenosine triphosphatase activity in *F. buski*, photographs of fresh frozen sections
a. Control (25×)
b. Enlarged view of control (100×)
c. Shoot extract of *A. nigra* treated sections (25×)
d. Enlarged view of *A. nigra* treated sections (100×)
e. Praziquantel treated sections (25×)
f. Enlarged view of praziquantel treated sections (100×)

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