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Ultrastructural Observations on *Fasciolopsis buski* and its Alterations Caused by Shoot Extract of *Alpinia nigra*

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KEY WORDS ultrastructure; *Fasciolopsis buski*; anthelmintic; *Alpinia nigra*

ABSTRACT The ultrastructural alterations in the tegument of *Fasciolopsis buski* in response to incubation in the alcoholic extract of *Alpinia nigra* were evaluated using transmission electron microscopy. The body tegument of the trematode is composed of an external syncytial layer, musculature, and an inner layer containing tegumental cells. The syncytium comprises various organelles like mitochondria, lysosomes, and tegumentary bodies of the type 2 kind with rare sighting of the type 1. Severe distortion and disorganization of the tegument was revealed in the parasite exposed to the *A. nigra* extract in the current study. The extent of vacuolization was such that vacuoles proceeded down to the basal lamina causing the syncytium to separate from the tegument at different places. There was depletion of parenchyma material and loss of connecting tubules running down from the syncytium to the tegumental cells causing the cells to be deprived of any proper boundaries. *Microsc. Res. Tech. 00:000–000, 2008.*

INTRODUCTION

Fasciolopsis buski, the giant intestinal fluke infects humans, pigs, dogs, and rabbits in southern Asia (Roy and Tandon, 2003). In heavy infection, the worms are also found in lower areas of the intestine and in the stomach. The parasite is of considerable importance from medical and veterinary points of view because of its zoonotic nature, particularly in Northeast India where high prevalence and abundance of the fluke is recorded during late summer among pigs, which act as reservoir host for human infection (Buckley, 1939; Roy and Tandon, 1992a,b). Apart from the negative, undesired effect on the host recorded for many synthetic drugs, repeated use of the same drug to control worm infection leads to development of resistance against the prescribed dosages in a number of parasite species (Coles and Bruce, 1990; Greets and Gryseels, 2001; Martin and Mckenzie, 1990; Prichard, 1994; Sangster and Bjorn, 1995; Scott and Armour, 1991).

Use of plants as a source of medicine has been inherited and is an important component of the health care system in rural India (Roy and Tandon, 1996; Roy et al., 2007, 2008). *Alpinia nigra* (Family: Zingiberaceae) is one such medicinal plant, in which the aqueous extract of fresh shoots is consumed against intestinal worms by the natives of Tripura state (Northeast India) residing in the remote countryside. Previous studies on the anthelmintic efficacy of the plant extract have revealed that it has a destructive action on the fine surface topography of *F. buski* (Roy and Tandon, 1999). As a sequel, the purpose of the present study was thus to unveil the detailed ultrastructural characteristics of *F. buski* and to evaluate the alterations brought about in the tegument and underlying structures of the fluke, by the action of the plant-derived chemicals.

MATERIALS AND METHODS

Preparation of Extracts

Fresh shoots of *A. nigra* were collected from the rural areas of Tripura state. They were washed gently with water and shade-dried. After grinding, the material was placed in a reflux flask having rectified spirit (100 g/L) and refluxed for 8 h at 60°C. The cooled suspension was then filtered and distilled for removal of solvent. The dried paste thus obtained was stored at 4°C till further use.

Experiment

Live adult *F. buski* were collected in 0.9% PBS from freshly slaughtered pigs at local abattoirs. The flukes were then incubated singly at 37 ± 1°C with varying concentrations of the crude plant extract, viz., 5, 10, and 20 mg /mL of PBS (three replicates for each concentration) in 1% dimethylsulfoxide (DMSO). Control incubation consisted of flukes in PBS with 1% DMSO only. Time taken for complete inactivation of the flukes was recorded, and death was confirmed by dipping such worms in slightly warm water. The parasites incubated in 20 mg of extract per mL of PBS were selected for ultrastructural observations because of the early lethal effect of the dose when compared with the other low concentrations.

Electron Microscopy

Soon after paralysis, the treated material along with one set of control was fixed in 2.5% gluteraldehyde

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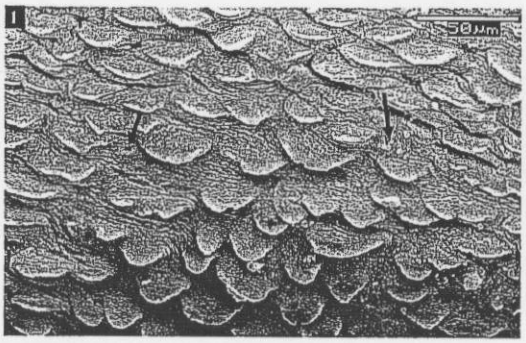

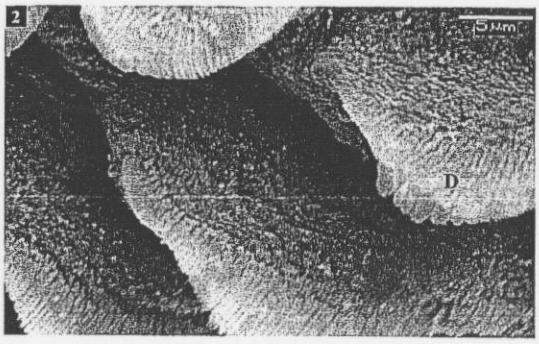

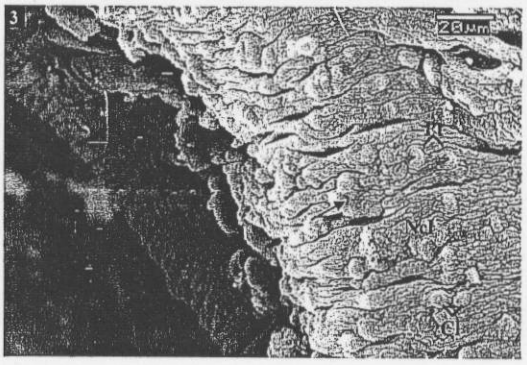

Figs. 1–3. Surface topography (SEM) of *F. buski*—Control. **1.** Tegument covered with posteriorly-directed scale-like papillae along with pits at many places. **2.** Magnified view of the scales with denticulate projections (D) at the edges. **3.** View of the oral sucker showing pitted (Pt), ciliated (Cl) and non-ciliated (Ncl) papillae. Arrow points out a cilium within a ciliated papilla.

buffered with 0.1 M sodium cacodylate (pH 7.2) at 4°C for 4 h, postfixed in OsO_4, dehydrated in graded acetone, and embedded in araldite. Ultrathin sections were double-stained with uranyl acetate and lead citrate and viewed in a JEOL-JSM-100CXII transmission electron microscope (TEM). For scanning electron microscopy (SEM), the material fixed in 4% neutral buffered formalin (NBF) was processed following the standard procedure as described earlier (Dey et al., 1989; Roy and Tandon, 1991). The gold-coated specimens were viewed in a JEOL scanning electron microscope at an electron accelerating voltage of 10–15 kV.

RESULTS

Control

The untreated control *F. buski* reveals normal body contour with scale-like papillae on the ventral surface. Surface invaginations forming deep pits are present throughout the body surface, including the surface of scales. The rim of acetabulum is radially corrugated and provided with domed ciliated and nonciliated papillae. Pitted papillae are also abundant in between the radial corrugations (Figs. 1–3). F1-F3

The tegument has a deeply invaginated apex and is outwardly covered by 54–72 nm thick glycocalyx layer followed by distal cytoplasm and subtegumental layer (Fig. 4). The distal and even the proximal cytoplasm is densely packed with innumerous biconcave discoid inclusions (T2 secretory bodies) measuring 72–108 nm in length, a few rod-shaped bodies, mitochondria of different size, food bodies, and rarely vesicular inclusions. F4

Distal anuclear syncytium is followed by a 300–500 nm thick fibrous basal lamina. Tegumental cells lying beneath the subtegumental muscle blocks maintain good connections with each other and with the surrounding parenchyma as well (Fig. 5). These cells as well as the muscle cells have abundant cell organelles including mitochondria which have prominent cristae and dense matrix (Fig. 6). Granular endoplasmic reticulum (GER) is abundant with no dilation of cisternae and is well-spaced out in the cytoplasm, which has plenty of free ribosomes. Nucleus in the cytons is rounded and has double-layered nuclear membrane (with no swelling of the perinuclear space), granular nucleolus, nucleoplasm, and chromatin material (Fig. 5). Golgi complexes contain dense flocculent material and are composed of stacked concave cisternae and a few vesicles (Fig. 7).

The study reveals the presence of lamellar processes at the apex of the gastrodermal cells in the intestinal caecum of the fluke. These processes are densely packed, elongated, and round-ended measuring 540–1,440 nm in length and 36 nm in width, having a dense central core and are extended into the lumen of the caecum. Within the gastrodermal cells, there are a large number of mitochondria, secretory bodies, and autophagic vacuoles (Figs. 8, 9).

Treatment

Within a few minutes of incubation in the crude alcoholic extract of *A. nigra*, the flukes were seen to first contract sharply and then relax and continued being in

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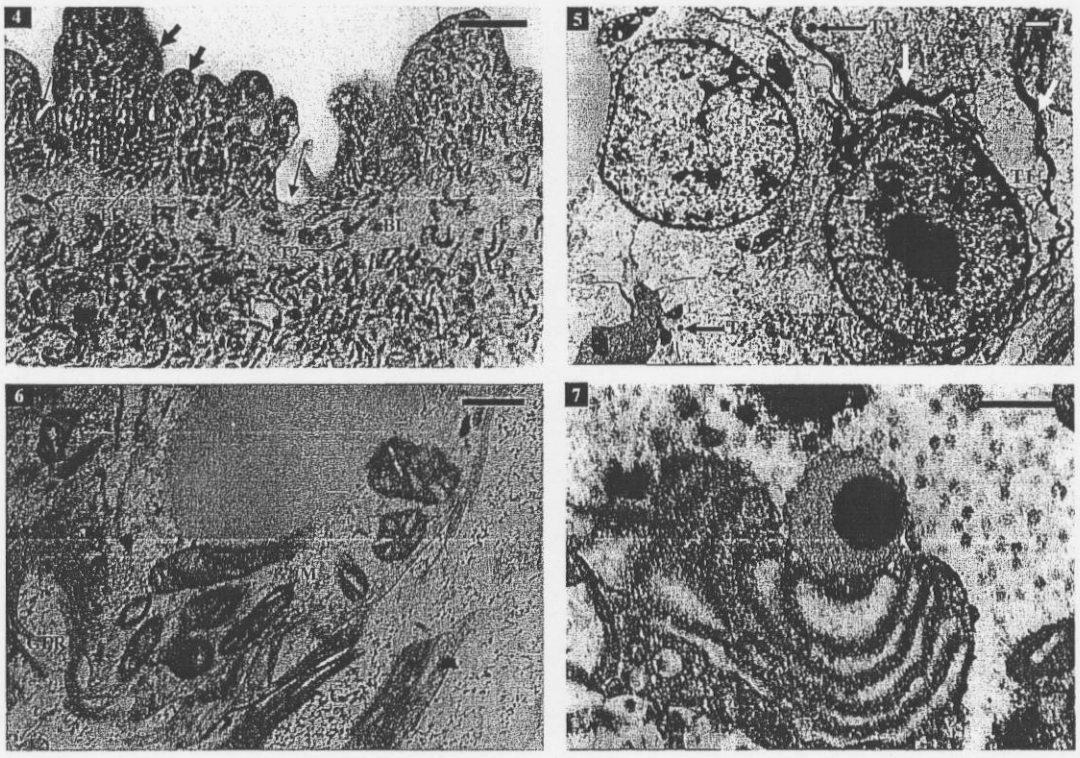

Figs. 4–7. Transmission elctron micrographs of *F. buski*—Control. (Scale bar = 0.5 μm). **4.** Tegument with glycocalyx layer (thick arrows); distal cytoplasm (DC) electron-dense with T2 and T1 tegumental discs, mitochondria, and a non-disrupted basal lamina (BL). Thin arrows show the invaginations in the apical plasma membrane. **5.** Two T1 tegumental cytons lying beside each other. A few T1 bodies, intact double-membraned nuclei, abundant cell inclusions, mitochondria (M) and autophagic vacuole (A) are seen. Cytoplasmic tubules (*) running down from the syncytium connect the cytons; interstitial material between the cells can also be seen (white arrows). **6.** Part of a parenchymal cell with mitochondria, GER and glycogen deposits (G). **7.** Golgi complex at high magnification.

that state till they attained flaccid paralysis that took 6.0–7.2 h, 4.0–4.8 h, and 2.5–3.0 h to set in for treatments with 5 mg, 10 mg, and 20 mg concentration of plant extract per mL of PBS, respectively. The treated flukes showed a deformed body with shrunken and wrinkled tegumental surface that showed extensive pit formations and scarring due to sloughing off of the scale-like spines (Figs. 10, 11). Ultrastructurally, the tegument appeared shredded and at many places there was severe vacuolization and release of internal tissue materials to the exterior. In the tegumental syncytium, no discs were evident, and the vacuoles in the distal cytoplasmic region were observed to stream down and flood the basal plasma membrane, which showed signs of separation from the basal lamina (Figs. 12, 13). In the subtegumental region, there was a downright decline in the number of tegumental cytons, which had highly deformed and swollen-up nuclei, and the cytoplasmic tubules connecting them with the syncytium were not prominent. The whole cytoplasmic mass was encroached upon with big vacuoles and the cytons had no defined boundaries (Fig. 14). Although some treated cells contained normal nuclei, most had lost their double-layered membrane and were edematous; many had no nucleolus and had big patches of heterochromatin. Very few mitochondria were found, which did not have prominent membranes and showed electron-lucent matrix due to loss of cristae. An increased number of autophagic vacuoles were observed in the subtegumental cells as well as in the surrounding parenchyma (Fig. 14).

DISCUSSION

The control fluke exhibited normal surface fine topography with the pits and scale-like spines covering most of the tegument in conformity with earlier observations (Roy and Tandon, 1999). Ultrastructurally also, the tegumental architecture of *F. buski* conformed to generalized structure of digenean trematodes (Threadgold, 1963). In the liver fluke *Fasciola hepatica*, two distinct types of tegumental cells are present that produce two different types of inclusions: type 1 cells with T1 bodies that are characterized by dense vesicular inclusions and are more in number, and type 2 cells with rather rare T2 bodies having biconcave discoid electron lucent inclusions (Smyth, 1996). In the present study, T2 bodies were observed to be present more predominantly than T1 bodies. In conformity with the observa-

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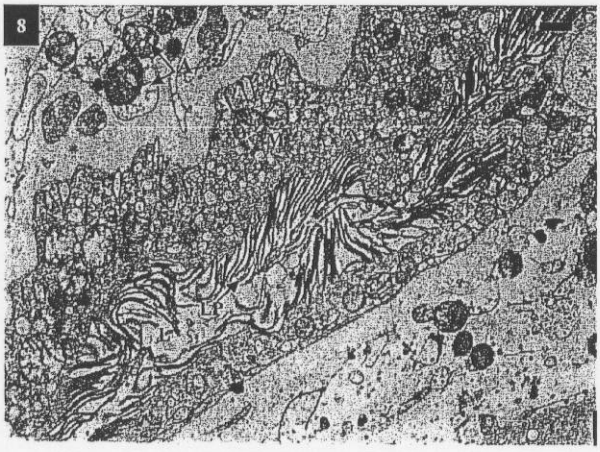
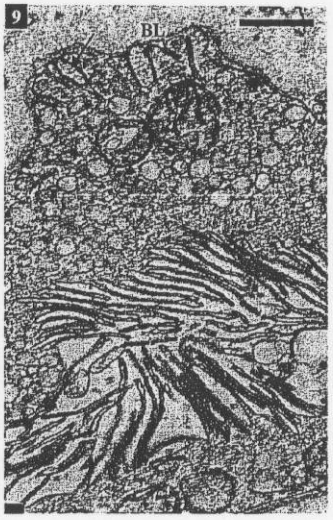
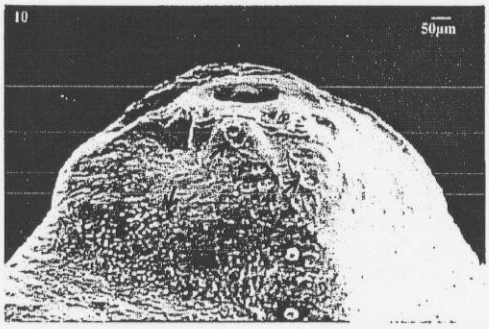
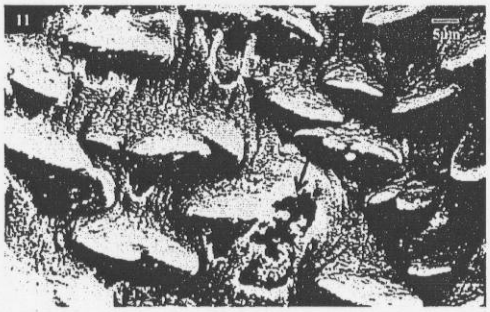




Figs. 8, 9. 8. Ultrastructure of the intestinal caecum showing gastrodermal cells with their lamellar processes (LP) extending into the lumen (L); abundant mitochondria (M), autophagic vacuoles (A) and lipid droplets (*) can also be seen. 9. A part of a gastrodermal cell where the gap junction (arrow) near the basal lamina (BL) of the cell can be made out.





Figs. 10, 11. SEM micrographs of *F. buski* treated with crude extract of *A. nigra*. 10. Tegument in the body, showing a wrinkled appearance along with scars (arrows) and pits (arrowheads). 11. A portion of the ventral surface under higher magnification showing scars of the sloughed scales (arrows).

tions in other digenea (e.g., *F. hepatica* and *Paragonimus ohirai*), gastrodermal lamellae having rounded tips were also observed (Fujino and Ishii, 1978).

In trematodes, the general body surface acts as a vital structure in terms of various functions such as attachment to the host surface, nutrient uptake, immunoprotection, osmoregulation, and sensation (Meaney et al., 2001). In this study, the tegumental surface of the test plant-treated parasite was found to be affected along with severe disruption of the underlying structures. Similar to the present observations, destruction of absorptive surface was also caused by other drugs like praziquantel, oxyclozanide, and alcoholic extract of several botanicals in trematodes and cestodes (Mehlhorn et al., 1983; Roy, 2001, 2003; Roy et al., 2008; Tandon et al., 1997). The tegumental changes followed a steady progression with time and were also observed to be dose-dependent (Anderson and Fairweather, 1995; Keiser et al., 2006). Onset of flaccid paralysis, disintegration of the surface tegument and necrosis of the worm tissue, occurring under the influence of anthelmintics, have been correlated with inhibition of neuromuscular activity, disturbance in ion flux across the membrane and altered membrane transport, and osmoregulation together with accelerated myelin degeneration, and autophagy by host immune system (Brindley et al., 1989; Gorchilova and Spaldonova, 1988; Harnet and Kusel, 1986; Pax et al., 1996; Prescott et al., 2000; Ribeiro et al., 2005; Webster, 2001; William et al., 2001).

The plant-derived chemicals also caused extensive vacuolization in the parasite's tegument, which led to the release of tissue material to the exterior. This vacuolization with early signs of separation of the syncytium from the basal lamina probably corresponds to

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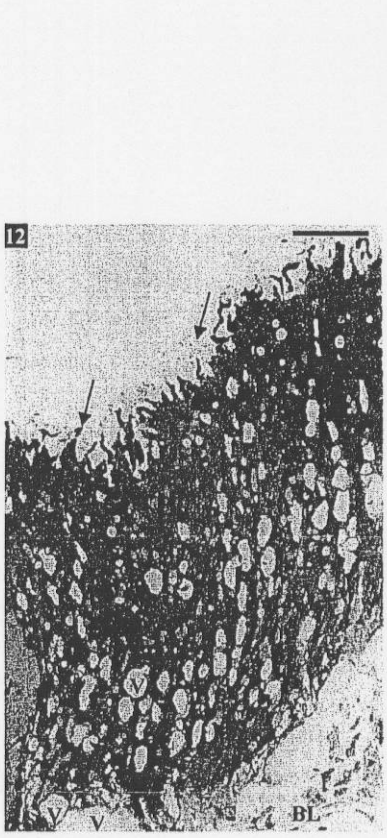

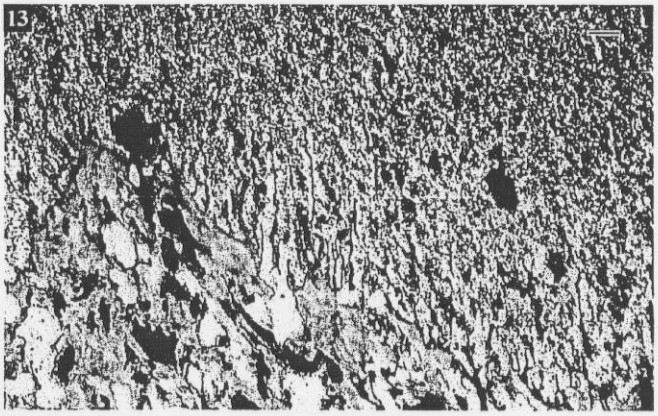

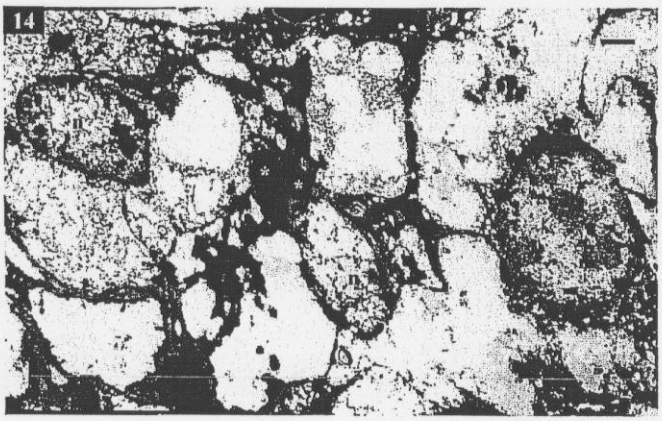

Figs. 12–14. TEM pictures of treated *F. buski*. (Scale bar = 2 μm). **12.** Shredded tegument with intensely vacuolated (V) distal cytoplasm; arrows show the materials being released from the syncytium to the exterior. Tearing off of the syncytium from the basal lamina is also observable. **13.** Electron-lucent region with intense vacuolization beneath the tegumental layer. **14.** Three tegumental cytons with deformed nuclei. The cytons are not connected by cytoplasmic tubules and the interstitial spaces have been taken over by autophagic vacuoles (*).

the scar formation on the surface of the fluke along with the deformity of the spines (Roy and Tandon, 1999). A pronounced damage was observed in the mitochondrial membranes and cristae in the present study, which might be due to a general stress brought by the effect of the plant-derived components (Anderson and Fairweather, 1995).

The severe deleterious effects brought about in the tegument of the fluke may account for the loss of movement, which ultimately led to death through a preceding paralytic state. The tegumental interface of *F. buski* seems to be a target organ for the active components of *A. nigra* to exert its vermifugal or vermicidal effect. Several species of *Alpinia* viz. *A. officinarum, A. tonkinensis*, and *A. jianganfeng* are known to have different chemical constituents like galangin, ombuine, kaempferol, and β-sitosterol (Heo et al., 2001; Qiao et al., 2002; Zhang et al., 2003), though much information on the chemical constituents of *A. nigra* is not yet available. However, in view of the potential anthelmintic efficacy of the plant-derived components, active principles of *A. nigra* need to be isolated and identified to understand their mode of action.

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