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ORIGINAL ARTICLE

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In vitro anthelmintic efficacy of *Carex baccans* (Cyperaceae): ultrastructural, histochemical and biochemical alterations in the cestode, *Raillietina echinobothrida*

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Abstract The aqueous juice of the root extract of *Carex baccans* (Family: Cyperaceae) is used as an anthelmintic in Meghalaya, India. The present investigation was carried out to evaluate the extent of ultrastructural, histochemical and biochemical alterations caused by the plant derived component(s) on *Raillietina echinobothrida,* a cestode parasite of domestic fowl. Live tapeworms, collected from the freshly slaughtered host, were exposed to different concentrations of the crude ethanolic root extract of *C. baccans* for varying time durations. The treated parasites revealed complete inactivation and flaccid paralysis leading to death; they were processed for ultrastructural, histochemical and biochemical observations, as soon as paralysis set in. Compared to controls, the treated parasites showed extensive distortion and destruction of the surface fine topography of the tegument, erosion of microtriches, disruption of muscle layers, intense vacuolization of tegumental and subtegumental layers, swelling and vacuolization of mitochondria and a significantly reduced activity of tegumental enzymes like AcPase and AlkPase. Phytochemicals from the root of *C. baccans* seem to be effective against soft-bodied cestode parasites and need to be characterized and identified.

Keywords *Carex baccans* · Cestocide · Enzyme activity · Ultrastructure

Introduction

Parasitic diseases remain a major constraint to livestock production across all agro-ecological zones and production systems of India. Worm infections in small ruminants, in particular, causes slow growth rate, poor reproductive performance and death (Coop and Kyriazakis 2001). The main way of controlling helminth parasites of live stock has been with the use of synthetic anthelmintics. However, repeated use of the same medicine for long duration resulted in developing resistance against most of the currently available anthelmintics. Besides, most of these synthetic drugs are highly toxic and exhibit undesired side effects in host animals (Singh and Ngachi 1999). In this context it is observed that several wild plants/plant parts are used in different parts of India to combat intestinal helminth infections (Sinha Babu 2005).

Carex baccans, locally known as "Kre", is one such traditionally used medicinal plant of Meghalaya, where villagers use the crude aqueous juice of the plant roots to get rid of intestinal helminth infection. The present in vitro investigation was, therefore, carried out to evaluate anthelmintic properties of the plant using the cestode *Raillietina echinobothrida* as the test parasite and taking ultrastructural, histochemical and biochemical changes in the tegument as parameters of the study.

Materials and methods

Preparation of extract

The plant, *C. baccans* (Nees) (Family: Cyperaceae) was collected from rural areas of Jowai, in the state of Meghalaya, India, and identified with the help of a taxonomist in Botanical Survey of India, Eastern Circle, Shillong. The Root tubers of the plant were washed in deionized water and shade dried; about 200 g of dried plant material was cut into small pieces, put in 1,000 ml reflux

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flask having 400 ml of rectified spirit and refluxed for 10–12 h at 60°C and processed to recover the crude extract as described earlier (Challam et al. 2010).

Recovery and in vitro treatment of parasites

The model test parasites, *Raillietina echinobothrida* were collected from the intestine of domestic fowl (*Gallus gallus domesticus*) and maintained at 37 ± 1°C in PBS having 0.1% dimethyl sulfoxide (DMSO) as control. Three to five parasites were incubated in the medium containing the plant extract at varying concentration like 5, 10, 20, 30 and 50 mg per ml of PBS, having 0.1% DMSO. Efficacy of the plant extract was monitored through observation on the motility of the parasites, broad-spectrum cestocide Praziquantel (PQZ) was used as the reference drug. Control worms were maintained in PBS having 0.1% DMSO only.

Electron microscopy

Immediately after the paralysis time set in, the control and treated parasites were fixed in 4% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.2) at 4°C for 4 h, followed by secondary fixation in 1% OsO_4 in the same buffer for 1 h at 4°C and then dehydrated in graded series of acetone. For scanning electron microscopy (SEM), specimens were dried in Tetramethylsilane, gold coated and viewed as per the protocol followed by Roy and Tandon (1991). For transmission electron microscopy (TEM), samples were processed following standard protocol as described earlier (Roy et al. 2008).

Histochemical and biochemical assays

Histochemical localization of Acid phosphatase (AcPase) 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 precipitates indicates sites of AcPase.

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

Enzyme assay

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.

The AlkPase activity was estimated following the same method as for the AcPase activity, except the buffer which is sodium glycine buffer (pH 10.5).The decrease or increase in the optical density was measured at 420 nm using a UV–Vis spectrophotometer (Varian 100).

Results

The effect of different concentrations of ethanolic root tuber extract of *C. baccans* and PZQ on *R. echinobothrida* is depicted in Table 1. A dose-dependent onset of paralysis and death were observed in the parasite treated with the crude extract. The cestodes incubated in the test medium having 5, 10, 20, 30 and 50 mg of the plant extract per ml of PBS became paralysed at 8.02 ± 0.06, 6.03 ± 0.06, 5.06 ± 0.02, 4.68 ± 0.06, and 3.59 ± 0.02 h of incubation, respectively. The control parasites showed physical activity for 72.0 ± 0.06 h, following which they became immobilized.

The parasites incubated with 50 mg of the plant extract per ml of PBS were selected for ultrastructural, histochemical and biochemical studies because of early lethal effects of the dose compared with other low concentrations.

Surface topographical observations

Scanning electron microscopy revealed a normal contour of the parasites body, with suckers in the scolex typically marked with rows of short but thick pointed hooklets or spines; densely packed microtriches throughout the body surface gives it a velvety appearance (Fig. 1a). The cestode treated with the plant extract showed irrevocable destruction throughout the general topography of the body; the scolex appeared greatly distorted with suckers extensively shrunken and the spines round the suckers were sharply crooked (Fig. 1b).

Table 1 In vitro effect of different concentration of alcoholic extract of *Carex baccans* (root tuber) and praziquantel on the survival of *Raillietina echinobothrida*

Control/plant/praziquantel	Concentration of crude extract/drug (mg ml^{-1})	Time taken in hours	
		Paralysis	Death
Control	0	–	72.0 ± 0.06
C. baccans	5	8.02 ± 0.06	8.49 ± 0.05
	10	6.03 ± 0.06	6.41 ± 0.05
	20	5.06 ± 0.02	5.98 ± 0.06
	30	4.68 ± 0.06	5.23 ± 0.07
	50	3.59 ± 0.04	4.13 ± 0.06
Praziquantel	0.01	3.12 ± 0.56	5.05 ± 0.49

Values are expressed as mean ± SEM ($n = 5$); $P < 0.05$; control versus treated

Table 2 Biochemical effects of root tuber extract of *C. baccans* and praziquantel on *R. echinobothrida*

Treatment	Enzyme activity (total[a]/specific[b])		% changed after treatment	
	AcPase	AlkPase	AcPase	AlkPase
Control	9.36 ± 0.20/0.94 ± 0.02	37.48 ± 0.8/3.76 ± 0.09	–	–
Plant extract	1.24 ± 0.08/0.16 ± 0.02	2.11 ± 0.04/0.20 ± 0.00	86.75	94.30
Praziquantel	0.38 ± 0.01/0.25 ± 0.00	0.63 ± 0.01/0.29 ± 0.01	95.94	98.31

Values are given mean (±SE) from four replicates assays

[a] Total activity is the formation of 1 μmol of product/h/g of wet tissue

[b] Specific activity is activity/mg protein

Table 3 Histochemical localization of AcPase and AlkPase in various body parts of *R. echinobothrida*

Treatment (mg/ml)	Distribution and enzyme intensity							
	AcPase				AlkPase			
	TG	ST	M	T/O	TG	ST	M	T/O
Control (0.9% PBS)	++++	++++	++	+++	++++	++++	++	++++
C. baccans (20 mg)	+	+++	–	+++	++	+++	–	++++
Praziquantel (0.01 mg)	–	–	–	++	++	–	–	++

TG tegument, *ST* subtegument, *M* muscle, *T/O* testes/ovary

++++, Very intense activity; +++, intense activity; ++, moderate activity; +, mild activity

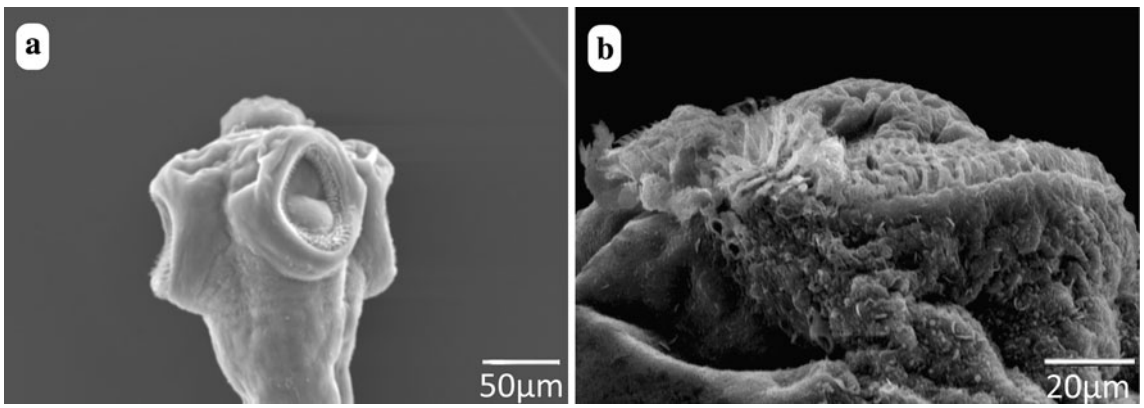

Fig. 1 Scanning electron micrographs of *Raillietina echinobothrida.* **a** Control cestode showing normal contour of scolex having circular rows of hooklets in the suckers. **b** Parasites treated with *C. baccans* revealed shrunken scolex and deformed suckers (*scale bar* = 20 μm)

Ultrastructural observations

The TEM observations revealed the presence of a microtriches-laden layer on the surface tegument giving the appearance of a brush border. Conforming to the typical ultra structure of the tegument, the microtriches are covered with a fuzzy layer of glycocalyx, and are followed by distal cytoplasm having a syncytial mass, basal lamina and muscle fibers; rounded vesicles are seen to pack the distal cytoplasm and extend into the base of microtriches; many of these vesicles contain small electron-dense aggregates (Fig. 2a); mitochondria are abundant in the distal as well as proximal cytoplasm and having double membrane with intact cristae (Fig. 2b).

The parasite treated with 50 mg of the ethanol extract per ml of PBS revealed more electronlucent distal cytoplasm as compared to the untreated control. The other architectural changes included deformation, destruction and erosion of microtriches; vacuolization of distal and proximal cytoplasm throughout, and loss of distinct basal lamina, circular and longitudinal muscle fibers. Scars and pits were evident throughout the tegument surface

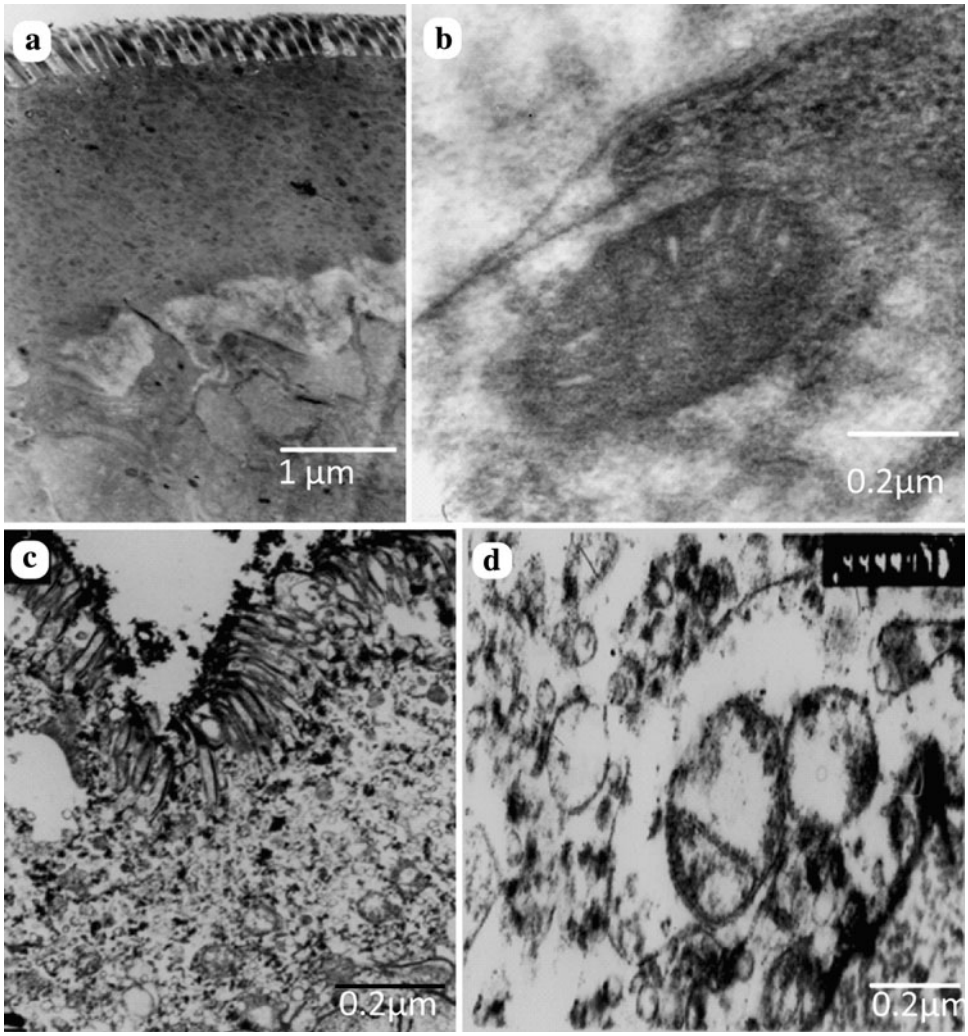

Fig. 2 Transmission electron micrographs of *R. echinobothrida*. **a** Untreated control cestode showing normal tegument having smooth microtriches followed by plasma membrane, syncytium and cell organelles. **b** Enlarged view of control mitochondria. **c** *C. bacans* treated tegumental layer showing deformed microtriches and vacuolization of tegument. **d** Enlarged view of vacuolated and acristate mitochondria (treated)

(Fig. 2c). Mitochondria of the distal and proximal cytoplasm showed an increase in size, dilation and vesiculization of their cristae, rupturing of the mitochondrial membrane and releasing their contents to the surrounding cytoplasm (Fig. 2d).

Histochemical and biochemical observations

Intense AcPase and AlkPase activities were observed in the tegument, sub-tegument, muscles, testes and ovary in untreated parasites (Fig. 3a, c). Parasites treated with the crude plant extract, retained mild AcPase activity in testes and ovary and to some extent in tegument and sub-tegument. Other regions like somatic musculature and the general parenchyma showed very low enzyme activity (Fig. 3b). Mild AlkPase activity was also exhibited in the tegument and sub-tegument, though with very low intensity in testes and ovary (Fig. 3d). Compared to AlkPase, AcPase showed much less stain intensity in treated worms (Table 3). In the control parasites the biochemical investigation revealed high activity of both AcPase and AlkPase, as $9.36 \pm 0.20/0.94 \pm 0.02$ and $37.48 \pm 0.80/3.76 \pm 0.09$ total/specific activities, respectively as compared to $1.24 \pm 0.08/0.16 \pm 0.02$ and $2.11 \pm 0.04/0.20 \pm 0.00$, respectively in the treated ones. The results also indicated that between the two enzymes, AlkPase exhibited higher enzyme activity in comparison to that of AcPase in both control and treated *R. echinobothrida* (Tables 2).

Discussion

Raillietina echinobothrida exposed to different concentrations of root peel extract of *C. baccans* revealed a dose-dependent paralysis and death. A similar kind of dose-dependent motility was also recorded among trematode and cestode parasites, treated in vitro with the crude extract of several plants like *Flemingia vestita, Alpinia nigra, Millettia pachycarpa, Accacia oxyphylla* and *Lysimachia*

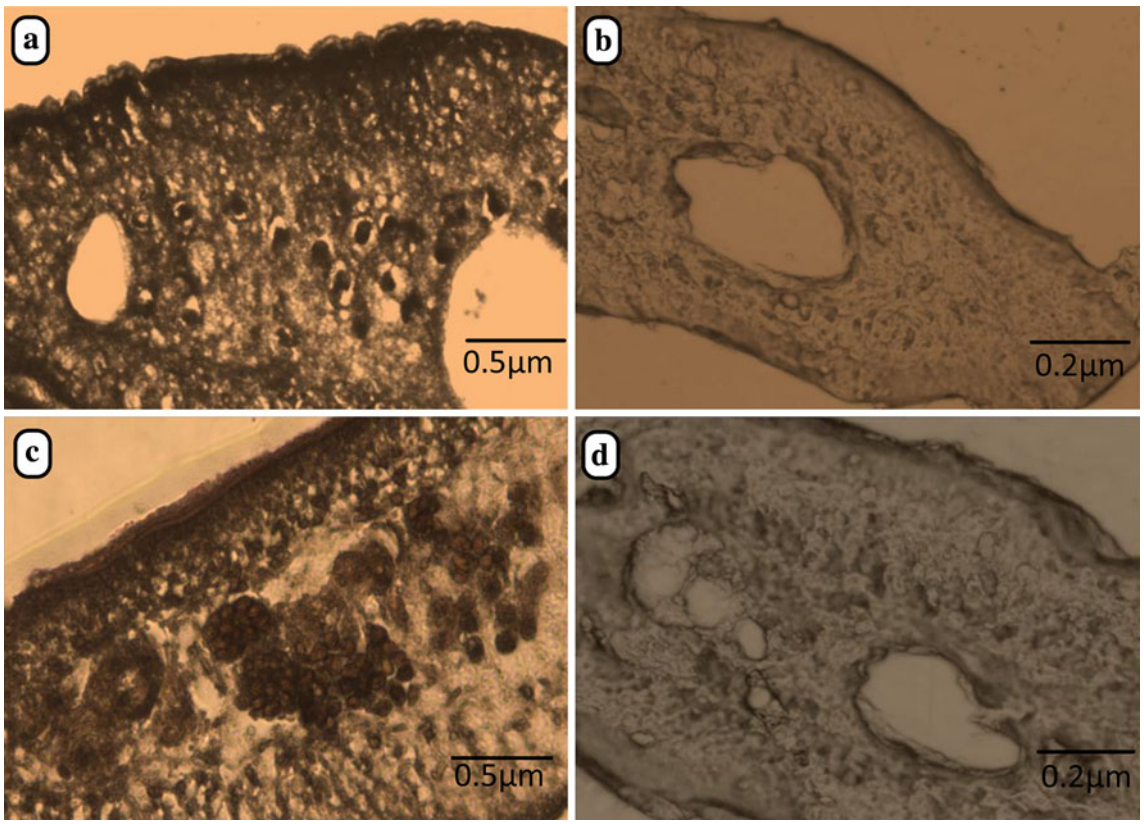

Fig. 3 Cryotome section of *R. echinobothrida* showing histochemical localization of acid phosphatase (**a, b**) and alkaline phosphatase (**c, d**). **a** Control tegument showing high intensity of AcPase. **b** Parasites treated with 50 mg of plant extract showing reduced intensity of AcPase. **c** Control tegument showing high intensity of AlkPase. **d** Parasites treated with 50 mg of plant extract showing reduced intensity of AlkPase

ramosa (Roy and Tandon 1996, 1999; Tandon et al. 1997; Dasgupta et al. 2010; Challam et al. 2010).

In the present investigation extensive erosion of microtriches, vacuolization in the tegument and sub-tegument and scar formation in the surface tegument was evident. Since cestodes are devoid of a structural digestive system, it is the microtriches that perform the function of nutrient absorption, apart from osmoregulation, immune protection and sensation (Kuperman 1988; Halton 1997; Jones 2000). Any alteration (disruption, disintegration), therefore, in the microtriches would lead the worms to starve and also vulnerable to host's immune attack (Roy et al. 2008). Deformation of microtriches, tegument and subtegument layer were also observed in the cestode treated with the extracts of several plants, though the form and extent of damage were different (Roy et al. 2008, 2009; Challam et al. 2010; Dasgupta et al. 2010).

Two vital tegumental enzymes, viz. AcPase and AlkPase are reported to be involved in digestion and/or absorption of nutrients and modulation of host-parasite interactions (Polijakova et al. 1983). In the present investigation both these enzymes showed a decline to the extent of 86.75% (AcPase) and 94.30% (AlkPase) in the plant extract treated parasites. These diminished activities of the enzymes might probably be associated with inhibition or reduced uptake of glucose in *R. echinobothrida,* leading to a loss of motor activity due to deprivation of energy source and thus culminating in paralysis. Similar results were recorded by Chopra et al. (1991), who found that the extracts of *Butea monosperma, Embelia ribes* and *Roltlesia tinctoria* drastically decreased the activities of AcPase and AlkPase in the trematode *Paramphistomum cervi,* a parasite of ruminant hosts.

Active anthelmintic components of *C. baccans* are not known; however, another species of the genus, i.e., *C. felia* is reported to contain high amount resveratrol, a phytoalexin of the family phenylpropanoids. Resveratrol is highly antioxidant (Ken et al. 1987; Jang et al. 1997; Baur and Sinclair 2006), and also anticancer, and antiblood sugar properties and is known to reduce plaque formation in animal brain, which is a component of Alzheimer's and other neurodegenerative diseases (Karuppzagounder et al. 2008; Elliott and Jirousek 2008). The phytochemical(s) of *C. baccans*, thus, may act as anthelmintic and they need to

be isolated and identified to ascertain the precise mode of action.

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