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Short communication

Surface topographical and ultrastructural alterations of *Raillietina echinobothrida* and *Ascaridia galli* induced by a compound isolated from *Acacia oxyphylla*

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ABSTRACT

The stem bark of *Acacia oxyphylla* Graham ex Bentham is used as an anthelmintic by the natives of Mizoram (North-East India). Therefore, the aim of the study was to assess the effect of the active compound isolated from *A. oxyphylla* on the tegument of adult *Raillietina echinobothrida* and *Ascaridia galli*. The test parasites *R. echinobothrida* and *A. galli* were incubated in physiological buffered saline containing 0.0005, 0.001, 0.05, 0.1 and 1 mg/ml of the isolated compound. The alterations in the tegument of the parasites post paralysis were examined using electron microscopes. The compound reduced the cestode's motility soon after incubation, but did not induce paralysis in the nematodes till about 11–14 h at highest concentration. The compound caused extensive digestion of cestode tegument as evident by electron microscopy. Disorganization of muscle bundles, loss of cell–cell contact, extreme vacuolization and oedema were some of the changes observed. Loss of cellular organelles combined with distortion of those present was markedly noted throughout the parasite tissue. Deformation and disorganization of epicuticle, disruption of mitochondrial and nuclear membrane were also observed in nematode exposed to the active compound of the plant. Substantial structural deformities in the treated parasites are indicative of an efficient vermicidal activity of the isolated compound against cestodes and nematodes.

1. Introduction

A number of plants have been tested for their anthelmintic potential (Stepek et al., 2007; Roy et al., 2009). In Northeast India, the natives consume the aqueous concoction of the stem bark of *Acacia oxyphylla* to cure intestinal worm infections. Earlier reports validating the anthelmintic potential of the plant have been provided by Dasgupta et al. (2010), where treatment with the ethanol extract of the plant was seen to cause severe alterations of the tegument of the test parasite *Raillietina echinobothrida* along with disappearance of cellular organelles. In India, poultry meat and egg production has been the fastest growing in agricultural or livestock production, with an average growth of 8% per annum. The tapeworms belonging to the genus *Raillietina* are the most prevalent avian helminth parasites in North-East India, followed by the nematode *Ascaridia galli*, which are responsible for malnutrition, retardation of growth of young chicken, emaciation of the adult, and decreased egg production of the hen (Kumar et al., 2007). As the anthelmintic effect of all plants is the result of their active principles, the active component of *A. oxyphylla* was isolated and identified. The active compound was tested against *R. echinobothrida* and *A. galli* to establish it as the factor promoting the anthelmintic effect of the plant. The aim of

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the present work was to study the surface architecture and ultrastructural details of the parasites post treatment with the isolated active compound.

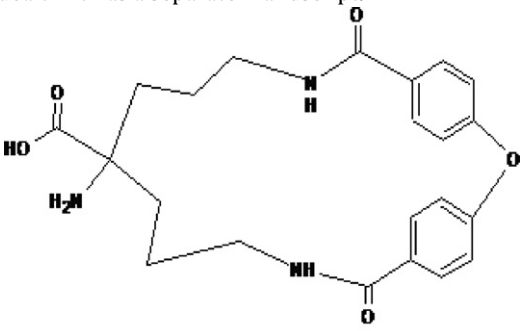
2. Materials and methods

2.1. Extract from A. oxyphylla

The fresh stem bark of *A. oxyphylla* (voucher number PUC-BOT-A012, deposited at Department of Botany, Pachhunga University College, Mizoram University) was collected from Mizoram. The crude ethanol extract was made following standard protocol as described by Roy et al. (2008). About 2.0 g crude ethanol extract was obtained from 100 g dried material.

2.2. Isolation and identification of active compound

The crude alcohol extract (2.0 g) of the plant material was mixed with 40% methanol and stirred on a rotary shaker for 24 h after which the resulting supernatant solution was decanted and filtered. This filtrate was then subjected to reverse phase chromatographic column (Waters Delta Pak 15 μm, C18, 300 Å, 19 mm × 300 mm), eluted with a mobile phase composed of methanol–water solution (40:60, v/v, also containing 0.1% formic acid) with a flow rate of 8 ml/min and detected at 220 nm using Agilent 1100 HPLC, that afforded five fractions, F1–F5 (yield: F1: 1400 mg, F2: 178 mg, F3: 27 mg, F4: 50 mg, F5: 310 mg). Testing of these subfractions against the parasites, indicated fractions F1 and F5 to be very promising, with the latter exhibiting higher anthelmintic potential. Further purification was done through reverse-phase chromatography of F5 (SUPELCOGEL ODP-50, 5 μm, 4 mm × 150 mm); a mobile phase composed of methanol–water solution (40:60, v/v, also containing 0.1% formic acid) with a flow rate of 1 ml/min and detected at 220 nm using Agilent 1100 HPLC yielded two pure compounds, F5-1d (160 mg) and F5-2d (16 mg). Further *in vitro* testing of these two compounds indicated F5-2d to be the most potent anthelmintic. Structure of F5-2d was further elucidated with the help of spectral data including NMR, IR and MS ($m/z = 411$) to be 12-amino-7,17-dioxo-2-oxa-8,16-diazatricylo [14.2.2.2 3, 6] tetraicosa-1 (20),3,5,18,21,23-hexaene-12-carboxylic acid (given below). Detailed chemical characterization will be dealt with as a separate manuscript.



Structure of F5-2d

2.3. Experiment

Live *R. echinobothrida* and *A. galli* collected from the intestine of freshly sacrificed fowl were incubated in 0.05, 0.1 and 1 mg of F5-2d/ml of PBS at 39 ± 1 °C. Dimethylsulphoxide (DMSO) was added to each concentration to give a final solvent concentration of 0.1%. Controls were prepared by incubating the worms in the culture medium (PBS) with 0.1% DMSO only. Praziquantel (PZQ) and albendazole were used as the reference drugs for cestode and nematode at a concentration of 0.01 and 0.05 mg/ml of PBS. Each incubation medium consisted of six worms and repeated for five times. Total loss of detectable movements in the parasite was taken as a sign of paralysis, and death was ascertained by dipping the paralysed worms in warm PBS, which evoked movements in the live worms.

2.4. Scanning electron microscopy

Worms treated with the active compound, PZQ, albendazole, and one set of control were fixed in neutral buffered formalin at 4 °C for 4 h. The fixed specimens were dehydrated through ascending grades of acetone and then air-dried in tetramethylsilane as described earlier (Challam et al., 2010). After gold coating, the surface architecture of the parasites was studied using a JEOL JSM 6360 scanning electron microscope operated at 15 kV.

2.5. Transmission electron microscopy

Control worms and worms exposed to the active compound albendazole and PZQ were fixed in modified Karnovsky's fixative, postfixed in 2% OsO_4 buffered with 0.2 M sodium cacodylate for 1 h, dehydrated through graded acetone and embedded in araldite. Ultrathin sections were stained with uranyl acetate and lead citrate and viewed in a JEM 100 CXII (JEOL) transmission electron microscope operated at 80 kV.

3. Results and discussion

When *R. echinobothrida* incubated in F5-2d and PZQ and *A. galli* incubated with F5-2d and albendazole, there was a decline in the motility of the worms resulting eventually in their death (Table 1). The control parasites continued to show physical activity for an average timing of 72 h and 86 h for cestode and nematode, respectively, following which they became immobilized. At 1 mg F5-2d/ml of PBS, spontaneous movements ceased at about 0.5 h and 11.72 h in the cestodes and nematodes, respectively. The observations noted in Table 1 indicated a significant difference (at $p < 0.05$) between the active principle-treated group and the control group, except the group treated with a concentration 0.0005 mg.

Control cestodes revealed characteristic morphology portraying a round scolex followed by a neck and a long string of proglottids. The scolex had a retractable rostellum surrounded by spines. Four suckers were arranged around the scolex, each having a circle of hooks. The tegument showed layers of tiny projections, called microtriches that gently sloped downwards (Challam et al., 2010).

Table 1
In vitro efficacy of active principle F5-2d and reference drug praziquantel and albendazole on *R. echinobothrida* and *A. galli* respectively (number of worms in each test medium, $n = 6$).

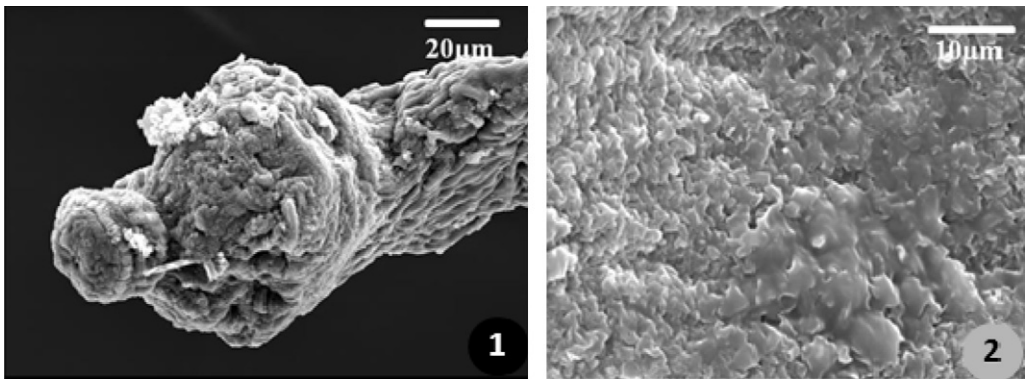
Control/treated cestode	Concentration (mg/ml)	Paralysis (h)	Death (h)
Control	–	–	72.0 ± 0.06
F5-2d	1	0.5 ± 0.2	5.75 ± 0.10
	0.1	5.25 ± 0.14	7.89 ± 0.21
	0.05	9.31 ± 0.11	11.44 ± 0.09
	0.001	61.11 ± 0.58	64.70 ± 0.14
	0.0005	68.32 ± 0.21	71.76 ± 0.12
PZQ	0.05	0.38 ± 0.11	5.32 ± 0.03
	0.01	0.5 ± 0.01	7.3 ± 0.15
Nematode			
Control	–	–	86.21 ± 0.12
F5-2d	1	11.72 ± 0.12	14.84 ± 0.16
	0.1	20.76 ± 0.10	22.52 ± 0.12
	0.05	28.64 ± 0.15	30.65 ± 0.18
	0.001	66.56 ± 0.16	69.14 ± 0.14
	0.0005	83.60 ± 0.21	85.93 ± 0.15
Alb	0.05	52.24 ± 0.79	55.17 ± 0.89
	0.01	64.1 ± 0.13	67.78 ± 0.41

F5-2d: 12-amino-7,17-dioxo-2-oxa-8,16-diazatricylo [14.2.2.2 3, 6] tetraicosa-1 (20),3,5,18,21,23-hexaene-12-carboxylic acid. Values given as mean ± SE from 5 replicate assays. $p < 0.05$ *vs*. control value, Student's *t*-test.

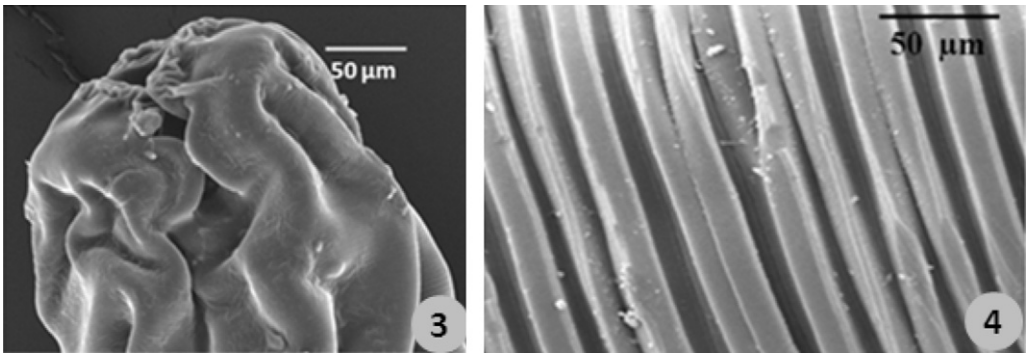
Treatment with F5-2d of *A. oxyphylla* led to total depletion of microtriches which fused together to form masses in the super-contorted proglottides and the suckers in the scolex collapsed (Figs. 1 and 2). PZQ treatment led to great contortion of the parasite scolex, wrinkling of the proglottide and intense clumping of microtriches (Challam et al., 2010).

Control *A. galli* showed normal contour of body having triangular mouth with three conspicuous denticulate lips. Labial papillae are distinct on the lips. Cuticle is smooth with series of continuous transverse annulations (Hassanain et al., 2009). Nematodes treated with the active component of the plant (F5-2d) showed deformed contour with collapsed lips, irregular wrinkles of the cuticular surface (Figs. 3 and 4). Distortion and deformation also observed in the sensory papillae of the mouth. Worms treated with reference drug albendazole also showed considerable deformation and disorganization of the cuticular surface and mouth parts (Lalchhandama et al., 2009).

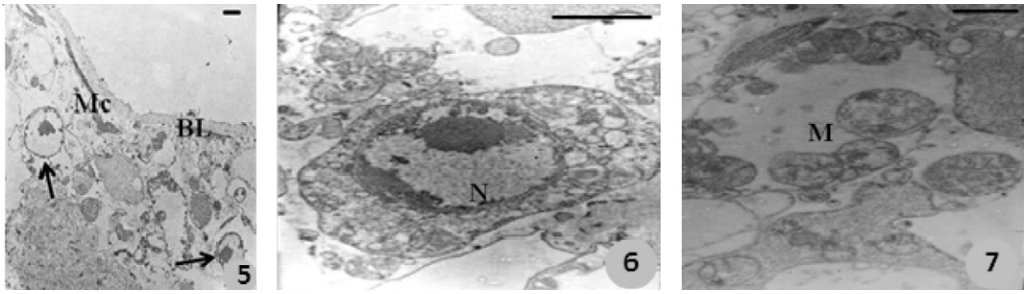
Transmission electron microscopic observations revealed a layer of long shaft-like microtriches covered with a glycocalyx coat in control cestode. Underneath this layer lies the distal cytoplasm, followed by basal lamina, muscle bundles and the subtegumental cell bodies. Abundant cristate mitochondria were found within the cytons and muscle fibres (Dasgupta et al., 2010). F5-2d treatment caused the tegument to strip off and the nuclei of subtegumental cytons to swell up severely. The muscle bundles had fewer muscle filaments as compared to control, and a loss of integrity was noted in their stacking. The basal lamina too showed degradation because of which there was release of internal structures at many places (Fig. 5). Also the parenchyma around the subtegumental cytons was highly oedematous and cell–cell contacts appeared disorganized in many areas. Differentiated cells disappeared, and cellular residues were present in large parts of the tissue. GER and Golgi bodies were nonexistent. Many nuclei exhibited a high degree of chromatin condensation; the cytoplasm in most cells was largely vacuolated (Fig. 6). The mitochondria were found sparsely and wherever present, mostly appeared electron lucent, vesicle-like and acristate (Fig. 7). PZQ treatment too caused the tegument to slough off leaving the basal lamina exposed. The nuclei in the subtegumental cytons were swollen and occupied the major part of it, and the cytoplasm surrounding the



Figs. 1 and 2. Scanning electron micrographs of *R. echinobothrida* treated with 12-amino-7,17-dioxo-2-oxa-8,16-diazatricylo [14.2.2.2 3, 6] tetraicosa-1 (20),3,5,18,21,23-hexaene-12-carboxylic acid. (1) Collapsed and retracted suckers and (2) microtriches fused together to form masses.



Figs. 3 and 4. Scanning electron micrographs of *A. galli* treated with12-amino-7,17-dioxo-2-oxa-8,16-diazatricylo [14.2.2.2 3, 6] tetraicosa-1 (20),3,5,18,21,23-hexaene-12-carboxylic acid. (3) Lips showing irregular wrinkles and shrinkages and (4) cuticle showing aberrations in the regular striations.

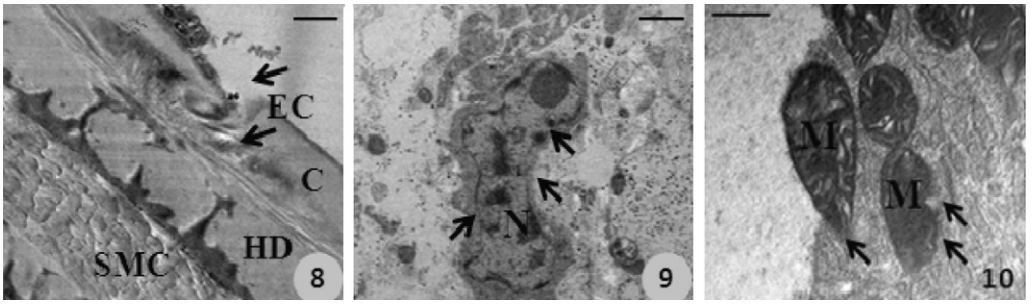


Figs. 5–7. Transmission electron micrographs of *R. echinobothrida* treated with 12-amino-7,17-dioxo-2-oxa-8,16-diazatricylo [14.2.2.2 3, 6] tetraicosa-1 (20),3,5,18,21,23-hexaene-12-carboxylic acid. (5) Stripped off tegument with highly swollen subtegumental cytons (arrows), (6) vacuolated cyton with chromatin clumping in the swollen nucleus and (7) acristate mitochondria.

nuclei was vacuolated. The mitochondria found in the parasite tissue were mostly vesicle-like (Dasgupta et al., 2010).

Ultrastructurally the body wall of control *A. galli* consists of outer epicuticle followed by cuticle, hypodermis and somatic muscle cells. The worms treated with F5-2d revealed a marked change in the epicuticle and cuticle, but a considerable change in the form of disorganization and destruction of hypodermis was noted (Fig. 8). Nucleus showed an irregular shape, accompanying with disruption and disintegration of membrane in many places (Fig. 9). Disappearance of mitochondrial cristae and membrane was observed in the treated worms (Fig. 10).

The present investigation showed that the active compound isolated from *A. oxyphylla* caused extensive damage to the surface of *R. echinobothrida* and *A. galli*, which resulted in a significant reduction in the activity of the parasites leading to paralysis. Most of the effects observed in our study, like loss of microtriches, increased vacuolization, and nonconforming mitochondria, could be accredited to the disruption of intracellular and intercellular transport systems (Ingold et al., 1999). The extensive oedema noted within the parasites internally, may be a result of disturbance of energy-dependent pumps found on the tegument membranes (McKinstry et al., 2008). In the present study, the disorganization seen due to the action of the active compound in the parasite was largely a necrotic volume increase (NVI) within the cells which gave rise to tissue injury and inflammation and lysis (Toner et al., 2008).



Figs. 8–10. Transmission electron micrographs of *A. galli* treated with 12-amino-7,17-dioxo-2-oxa-8,16-diazatricylo [14.2.2.2 3, 6] tetraicosa-1 (20),3,5,18,21,23-hexaene-12-carboxylic acid. (8) Damaged epicuticle along with cuticle, (9) irregular shaped nucleus with membrane disruption and (10) acristate mitochondrial membrane damage.

In nematodes the cuticle is a multi-functional, flexible and durable exoskeleton. It is a highly impervious barrier between the animal and its environment. But there are clear evidences which suggest that transcuticular diffusion is a common means of entry for non-nutrient and non-electrolyte substances in nematodes (Geary et al., 1995). It has also been shown that the transcuticular route is predominant for the uptake of drug levamisole in different nematodes (Alvarez et al., 2007). Structural alterations in the cuticle and cellular components as observed in the present investigation are thus, clear indication of anthelmintic potential of the plant derived active component.

Further investigation is essential to determine whether the plant botanical is also efficacious against cestodes and nematodes *in vivo*, so as to develop an advance treatment for the control of helminthiasis.

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