STUDIES ON ANTICESTODAL EFFICACY OF *Acacia oxyphylla* (LEGUMINOSAE) AND *Securinega virosa* (EUPHORBIACEAE)

ABSTRACT

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ABSTRACT

The present study involves an investigation on the anthelmintic efficacy of *Acacia oxyphylla* Graham ex Bentham (Family: Leguminosae) and *Securinega virosa* (Roxb. ex Willd.) Baill. (Family: Euphorbiaceae). The aqueous concoction of the plants (stem bark of *A. oxyphylla*, leaves of *S. virosa*) is taken by the Lushai tribe in Mizoram, a state in Northeast India, to cure intestinal worm infections. The *in vitro* activity of the extracts of the two plants and their active principles was tested against the poultry cestode, *Raillietina echinobothrida.* Alterations in the motility of the parasite, structural changes (both morphological and ultrastructural), changes in important enzymes associated with the tegument, free amino acid pool and vital trace elements were taken as parameters for the study. The plants were also tested *in vivo* against the rodent parasite *Hymenolepis diminuta* maintained in adult Wistar rats. Changes in EPG count pre-treatment and post treatment with the plant extracts and the percentage recovery of worms at the end of experimental period were used as parameters for *in vivo* experiment.

1. *In vitro* studies

a) *Motility and mortality studies: In vitro* treatment of the parasite with the plants' extracts, their fractions, and the reference drug praziquantel (PZQ) led to complete immobilization of the parasite followed by death. On exposure to the test solutions, the worms proceeded from a state of vigorous movements to a relaxed condition in which they continued to remain till they attained paralysis. The time taken for paralysis showed a

dose-dependent effect as paralysis occurred at a shorter period of time for higher concentrations of test materials while for lower doses it took a much longer period of incubation. 10 mg/ml dose of crude extract of *A. oxyphylla* took 3.50 ± 0.12 h to cause paralysis in the parasite, which was comparable with the time frame of onset of paralysis in parasites treated with 0.001 mg/ml dose of PZQ. Onset of paralysis within a time span similar with 0.001 mg/ml dose of reference drug occurred at 3.76 ± 0.11 h, with 25 mg/ml dosage of *S. virosa*, demonstrating *A. oxyphylla* to be a faster acting anthelmintic.

b) *Surface topography and ultrastructural studies:* Stereoscan observations on control *R. echinobothrida* revealed normal contour of scolex having four suckers and a rostellum having spines. Trunk of the parasite comprised of a chain proglottides having fine microtriches arranged in a specific direction. On exposure to crude extract of *A. oxyphylla* and its fractions, proglottides became smaller and the surface erupted into blisters all over. Distortion and deformation of scolex was also prominent. A similar kind of distortion and deformation was observed in parasites exposed to *S. virosa* crude extract and its fractions as well. Transmission electron microscopic observations on the control worms revealed the typical cestode morphology with respect to the tegument, subtegument and inner parenchyma. The tegument of *A. oxyphylla* (crude and fractions of crude extract) treated worms was mostly stripped right down to the basal lamina and the remnants of the basal infolds left behind were highly swollen. There was an increase in electron-lucency of the background due to both glycogen loss and vacuole formation and the subtegument showed immense vacuolization and accumulation of debris. There was very little amount of GER remaining with no evidence of Golgi complexes. The parenchymal cytons of the treated parasites showed complete loss of connections with the surrounding parenchyma and the chromatin in the nucleus appeared clumped into large areas of electron-dense heterochromatin. Disintegrated muscle blocks, vacuolated tissue material, distorted subtegumental cytons and loss of cell organelles were some of the major damages seen. When cestodes were treated with the extract of *S. virosa* and its fractions, the distal cytoplasm was perforated and no tegumental discs or mitochondria were visible. The cytoplasm was observed to stream down into the subtegument basal lamina in the form of thin laminar processes. The lamina itself had vacuoles which went down to penetrate and cause disintegration of the subtegumental cytons (making them hardly discernible) between the muscle blocks. Here too the degree of damages was noted to be dose dependent.

c) *Histochemical localization and biochemical analyses of tegumental enzymes*: On treatment with *A. oxyphylla* and *S. virosa* extracts, and PZQ, a decline in the levels of important tegumental enzymes, namely, adenosine triphosphatase, alkaline phosphatase and acid phosphatase was observed histochemically in various structures within the parasite. The same was confirmed with biochemical tests where a decline in the total and specific activities of these enzymes was observed. While control worms showed the highest activity for ATPase followed by AlkPase and AcPase, the treated worms went on to show decrease in the activities of all the three enzymes. AcPase activity declined most (97%) in the parasites, as compared to ATPase (72%) and AlkPase (90%), when treated with *A. oxyphylla*. On treatment with *S. virosa*, AlkPase activity was noticed to decline most (96%), as compared to ATPase (91%) and AcPase (34.5%).

d) *Free amino acid pool*: The free amino acid pool of the control and the treated worms was evaluated using mass spectrometry. Out of the 20 amino acids detected, Gly was found to be at the maximum level, followed by Ala, Ser, Pro, Val, Thr, Cys, Ile/Leu, Asn, Asp, Gln/Lys, Glu, Meth, His and Phe, respectively, in a decreasing order. Parasites treated with plant extracts and reference drug showed a marked decrease in the levels of Meth, Ile/Leu, Phe, Asn Gly, and Tyr. *A. oxyphylla*-treated parasites showed an increase in the level of Thr while *S. virosa* showed an increase of Ala, Pro, Cys, Thr and Trp.

e) *Trace elements studies*: On exposure to the plants' extracts and PZQ, the parasites were seen to lose high amounts of calcium and magnesium ions into the treatment medium as compared to the control groups which retained high levels of the two elements.

2. *In vivo* studies

Each acclimatized adult rat was infected with 5 cysticercoids of *H. diminuta* and later treated with single doses (25 mg and 50 mg/kg body weight for *A. oxyphylla*, and 750 mg and 1500 mg/kg body weight for *S. virosa*) of the two plants' extracts and PZQ, for three days. A significant decline in the post treatment egg count was noticed in all the treatment groups when compared to the pre-treatment egg counts, except for the group treated with 750 mg of *S. virosa* (16.8%-18.2% reduction only). On autopsy, 50 mg *A. oxyphylla* and 1500 mg of *S. virosa* treated groups showed 87% and 83% decline in worm burden, respectively. PZQ reduced the worm recovery rate by 92%. However, 25 mg of *A. oxyphylla* and 750 mg of *S. virosa*/kg dose brought down the worm burden by 80% and 40%, respectively.

3. Active principles testing:

a) *Motility and mortality studies*: The active component of *A. oxyphylla* was isolated from the crude extract of the plant and was identified 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. Virosecurinine, the active component of *S. virosa* was obtained commercially. When *R. echinobothrida* was incubated in the active principles of the two plants, there was a decline in the motility of worms resulting eventually in their death, taking 5.75 ± 0.1h and 8.9 ± 0.01 h for 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 and virosecurinine, respectively.

b) *Surface topography and ultrastructural studies:* Scanning electron microscopy revealed total depletion of microtriches which fused together to form masses. Proglottides were noted to be super-contorted and seemed to be breaking off from the main body at many places due to extreme shrinkage. The suckers in the scolex collapsed and retreated back, pushing the rostellum forward. Transmission electron microscopic observations showed that treatment with active compounds caused the tegument to strip off and the nuclei of subtegumental cytons to swell up severely. Mitochondria were found sparsely in the parasite body and were mostly acristate.

c) *Histochemical localization of tegumental enzymes:* Histochemically, the control worms showed intense activity for all the three tegumental enzymes (ATPase, AlkPase and AcPase) while the treated worms portrayed a diminished or no activity of the enzymes in different structures of the body.

The active principles (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 and virosecurinine) present within the plants seem to bring about all the alterations noticed in the present study by acting transtegumentally and as the tegument of cestodes happens to be the main interface of nutrition, the resulting effect is the instant paralysis and ultimate death of the parasite.

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Thesis

Dedicated To My Family

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DECLARATION

I, Shyamashree Dasgupta, hereby declare that the subject matter of this thesis is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

This is being submitted to the North-Eastern Hill University for the degree of Doctor of Philosophy in Zoology.

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Introduction

Gastrointestinal (GI) parasites are responsible for significant production losses in livestock worldwide (Gill and Lejambre, 1996), particularly under tropical and subtropical climates. A marginal level of nutrition and climate that favours survival of the parasites in most of the year explains why GI infections are more devastating in these regions (Waller, 1997). Among GI parasites, helminthiasis plays a crucial role in small ruminant production leading to enormous economic losses particularly in areas where extensive grazing is practiced (Waller, 2006). It causes loss of production through mortality, weight loss, reduced milk and wool production (Soulsby, 1986).

Control of helminth infections has traditionally been done using anthelmintics (chemotherapy) with best results being obtained when this approach is integrated with proper grazing management (Larsen, 2006; van Wyk *et al.*, 2006, WHO, 2009). In the last 2 - 3 decades there has been over-dependency and even misuse of the chemotherapeutic approach with consequent evolution of anthelmintic resistance (Ngomuo *et al.*, 1990; Prichard, 1994). The broad-spectrum anthelmintics used in the control of different helminths fall into just three classes *viz.* the benzimidazoles, imidothiazoles and macrocyclic lactones (Behnke *et al.*, 2008). Resistance to all the three drug classes now occurs in the major helminth parasites of sheep and goats throughout the world (Waller, 1997).

Moreover, these drugs are unaffordable, inaccessible or inadequately available to the resource poor farmers of the developing countries (Hammond *et al.*, 1997). To add to it, there is a growing concern over drug residues in the food chain and the environment. Alternative approaches to helminth control are needed to circumvent some of the above constraints. Search for novel anthelmintics that are more sustainable and environmental friendly is undoubtedly a sensible approach to the control of parasitic infections. One such alternative could be harnessing of the available ethnoveterinary knowledge (Fajimi and Taiwo, 2005; Roy *et al.*, 2008; 2009; Challam *et al.*, 2010), i.e., the use of medicinal plants with anthelmintic activity. This includes the use of herbal preparations that may offer a cheaper and sustainable alternative to synthetic drugs, provided of course that they have reasonable levels of efficacy.

Evaluation of the activities of medicinal plants claimed for anthelmintic property is getting attention these days (Roy, 2001; Kar *et al.*, 2002; 2004; Pessoa *et al.*, 2002; Alawa *et al.*, 2003; Das *et al.*, 2004 a, b; Githiori, 2004; Iqbal *et al.*, 2004; Roy *et al.*, 2008). Use of plants as a source of medicine has been inherited and is an important component of the health care system in India. The World Health Organisation (WHO, 1999), estimated that 80% of the populations of developing countries rely on traditional medicines, mostly plant drugs, for their primary health care needs. Also, modern pharmacopoeia still contains at least 25% drugs derived from plants and many others which are synthetic analogues built on prototype compounds isolated from plants. Demand for medicinal plants is increasing in both developing and developed countries due to growing recognition of natural products, being non-narcotic, having no undesired effects, easily available at affordable prices and sometime the only source of health care available to the poor (Roy and Tandon, 1996; 1999; Roy, 2003). Medicinal plant sector has traditionally occupied an important position in the socio cultural, spiritual and medicinal arena of rural and tribal lives of India (Roy *et al.*, 2007 a, b; Challam *et al.*, 2010; Dasgupta *et al.*, 2010).

There are about 45,000 plant species in India, with concentrated hotspots in the region of Eastern Himalayas, Western Ghats and Andaman & Nicobar Island (Das *et al.*, 2006). The officially documented plants with medicinal potential are 3000 but traditional practitioners use more than 6000. India is the largest producer of medicinal herbs and is appropriately called the botanical garden of the world. There are currently about 2,50,000 registered medical practitioners of the Ayurvedic system (total for all traditional systems: approximately 2,91,000), as compared to about 7,00,000 of the modern medicine system (Ahmedullah and Nayar, 1999). Millions of rural households use medicinal plants in a self-help mode.

The use of medicinal plants for the prevention and treatment of gastro-intestinal parasitism has its origin in ethnoveterinary medicine. Public, academic and government interest in traditional medicines is growing exponentially due to the increased incidence of the adverse drug reactions and economic burden of the modern system of medicine (Dubey *et al.*, 2004). In the oral traditions, local communities in every climatic condition from the trans-Himalayas down to the coastal plains have discovered the medical uses of thousands of plants found locally in their ecosystem. It is a culture that is of tremendous contemporary relevance because it can on one hand ensure health security to millions of people and on the other hand it can provide new and safe herbal drugs to the entire world.

A number of plants have been used in different parts of the globe to evaluate their anthelmintic efficacy. Leaves, dried flowers and oil from *Chenopodium ambrosioides*, a shrub that originated from Central America and has been distributed around the world, have all been used as anthelmintics since the early 1900s (Guarrera, 1999). *Pavetta owariensis* extract and its proanthocyanin components caused significant reduction in *Schistosoma mansoni* burden in experimentally infected mice (Balde *et al.*, 1986, 1989). *Ruta graveolens*, *Cucurbita maxima*, *Artemesia absinthum* and *Allium sativum* are used traditionally to cure intestinal worm infection (Guarrera, 1999), among which, *C. maxima* is a proven anthelmintic (Srivastava and Singh, 1967). *A. sativum* ingestion inhibits larval development of human nematodes (Bastidas, 1969) and its extract is far more potent than praziquantel against *Taenia saginata* cysticerci (Mohamoud and ElAlfy, 2003). *Lotus pedunculatus*, *L. corniculatus*, *Hedysarum coronarium*, *Onobrychis viciifolia*, *Dorycnium rectum*, *D. pentaphyllum* and *Rumex obtusifolius*, all rich in condensed tannins, induced paralysis in the nematode *Trichostrongylus colubriformis* (Molan *et al.*, 2000). *Dictyocaulus* sp. burden was greatly reduced in red deer calves on treatment with *Medicago sativa*, *L. corniculatus* and *H. coronarium* (Hoskin *et al.*, 2000). *C. ambrosioides* oil is noted to significantly reduce egg per gram count of *Haemonchus contortus* in goats (Kato *et al.*, 2000; Ketzis *et al.*, 2002).

The neem tree (*Azadirachta indica*) is known for its medicinal properties and has been recommended for use against gastro-intestinal nematodes and related problems in many parts of the world (Biswas *et al.*, 2002; Subapriya and Nagini, 2005).

Ethnoveterinary sources from south-east Asia report that cassava forage (*Manihot esculenta*) has been used by traditional healers with success for the control of internal parasitism (Sokerya and Preston, 2003). Consumption of cassava hay resulted in lower faecal egg counts and worm burdens in sheep parasitised with abomasal and intestinal nematodes compared with unsupplemented controls (Sokerya and Preston, 2003; Bunyeth and Preston, 2006). The consumption of leaves of wormwood in the form of powder (*Artemisia brevifolia*), one of the bitterest of plants, has been tested in a controlled study for its anthelmintic activity. Iqbal *et al.*, (2004) demonstrated that the consumption of the whole plant resulted in a 62% reduction of egg counts of the abomasal nematode *H. contortus*. Consumption of fagara leaves (*Zanthoxylum zanthoxyloides*), a native tree from Africa, resulted in reduced egg excretion by the same nematode in sheep, when consumed regularly in small amounts (Hounzangbe-Adote *et al.*, 2005). *Fumaria parviflora* whole plant, *Ananas comosus* stem, *A. indica* seeds, *Caesalpinia crista* seeds showed significant effect on *H. contortus* larvae and adults (Singh *et al.*, 2004; Hordegen *et al.*, 2006) and *F. parviflora* effectively reduced the faecal egg counts of *T. colubriformis* of sheep (Hordegen *et al.*, 2003). Similarly, lespedeza (*Sericea lespedeza*), a grazing perennial legume native of Eastern Asia, showed promising anthelmintic activity when fed fresh or as hay to goats (Min *et al.*, 2004; Shaik *et al.*, 2004; Lange *et al.*, 2006).

Microscopy has a long and distinguished history in the study of helminth parasites and has made a singularly outstanding contribution to understanding how these complex animals organise their lives and relate to their hosts. Increasingly, the microscope has been used as a powerful investigative tool in multidisciplinary approaches to parasitological problems, placing emphasis on functional correlates rather than anatomical detail. In doing so, microscopy has also uncovered a number of attributes of parasites that are of wider significance in the field of biology (Tandon and Roy, 2002). Parasite surfaces have understandably demanded most of the attention of microscopists, largely as a result of the pioneering studies using transmission electron microscopy. Their findings focused the attention of physiologists and immunologists on the tegument and cuticle of helminths and in doing so helped unravel the complex molecular exchanges that are fundamental to understanding host-parasite interactions. Scanning electron microscopy succeeded in augmenting these data by revealing novel micro-topographical features of the host-parasite interface, as well as proving invaluable information in helminth taxonomy and in assessing the efficacy of test substances in drug screens (Roy and Tandon, 1990, 1992, 1993; Roy and Dutta, 2003). Control of helminth parasites has never been more critical: problems of drug resistance demand urgent action to identify exploitable targets for new generation anthelmintics.

Over the years, electron microscopy has helped to reveal the effect of various drugs on parasites. Different anthelmintic drugs have been shown to cause diverse morphological alterations in parasites. Treating mice infected with the Egyptian strain of *S. mansoni*, with garlic resulted in swelling of the tegument accompanied by widespread vacuolization (Riad *et al.*, 2009). Similar results were obtained under the influence of praziquantel (PZQ), as studied by Modha *et al.*, 1990; Staudt *et al.*, 1992; Liang *et al.*, 2002; Mohamed *et al.*, 2006). Comparable drug effects, such as blebbing, swelling of the tegument, loss of spines and distortion of suckers have been observed in *Fasciola gigantica* treated with the natural compound, propolis (Hegazi *et al.*, 2007). Extensive blebbing of *F. hepatica* surface was observed on incubation of the parasite in 0.27 mg/ml of genistein (Toner *et al.*, 2008).

In India, many traditionally used plants have been proven to possess anthelmintic potential. Anthelmintic efficacy of the seeds of *Butea monosperma* syn. *B. frondosa* has been reported against ascarids, stomach worms of sheep and *Ascaridia galli* (Garg and Mehta, 1958; Ramanan, 1960; Satyanarayanrao and Krishnaiah, 1982). Chakraborty *et al.* (1979) studied the anthelmintic properties of *Tribulus terrestris,* a perennial plant common in India. They reported that alcoholic extracts of the dried and ground plants expelled 64 and 79% of experimental infections of *A. galli* in poultry, depending on the concentration used. The alcoholic extract of the stem bark of *Punica granatum* has been evaluated for its proclaimed anthelmintic potential. The extract was found to inhibit transformation of eggs to filariform larvae of *H. contortus* (Prakash *et al.*, 1980). In clinical studies, the plant showed strong anthelmintic efficacy in calves (Pradhan *et al.*, 1992). Various extracts of seeds of *Vernonia anthelmintica* have been proven to function against *Fasciolopsis buski*, *Ascaris lumbricoides and Hymenolepis nana* (Singh *et al.*, 1985).

Jantana, an ayurvedic preparation, was used by Sharma (1993) in a trial with 26 cattle with mild to moderate mixed infections of *Haemonchus* spp., *Strongylus* spp., *Trichostrongylus* spp. and *Nematodirus* spp. Some of the constituents of this herbal preparation are listed as powdered *Artemisia maritima, Brassica nigra, Cassia* *lanceolata, V. anthelmintica, Cuprium sulphas* and *Embelia ribes.* No untoward effects were reported as a result of this treatment. Faecal egg counts were reduced to zero, 7 days after treatment. Ethanol extract of *Centella asiatica* proved to be effective against canine dirofilariasis (Chakraborty *et al.*, 1996). Seeds of cucumber and pumpkin have also been found to have anthelmintic activity against tapeworms (Waller *et al.*, 2001). Similarly, root bark of *Capparis spinosa* and leaves of *Ocimum sanctum* were also found to possess potent anthelmintic activity (Asha *et al.*, 2001; Mali *et al.*, 2005). The crude alcoholic and aqueous exracts of *Cleome viscosa* showed activity against *A. galli* and *Pheritima posthuma* (Mali *et al.*, 2007). Alcohol and aqueous extracts of the roots of *Baliospermum montanum* Muell. Arg were found to exhibit significant anthelmintic activity against *A. galli* (Mali and Wadekar, 2008).

In Northeast India, many traditionally used plants have acquired scientific credence over the period of time. The crude extract of root tubers of *Flemingia vestita* was reported to be effective against *Ascaris suum, in vitro* (Yadav *et al.*, 1992). The extract further proved to be effective and responsible for tegumental alterations in trematodes *Paramphistomum* spp., *F. buski* and cestode *Raillietina echinobothrida* (Roy and Tandon, 1996; Tandon *et al.*, 1997). The crude extract of the leaves of *Cannabis sativa* caused paralysis of *F. buski* followed by death and was found to be more potent than the reference trematocidal drug oxyclozanide (Roy and Tandon, 1997). Aerial shoot extract of *Trifolium repens* and leaf extract of *Strobilanthes discolor*, consumed by Naga tribes, caused significant reductions in faecal egg counts of *Hymenolepis diminuta* (Tangpu *et al.*, 2004; 2006).

Roy *et al.* (2008, 2009) showed that the root peel extract of *Millettia pachycarpa* and shoot extract of *Alpinia nigra* causes severe ultrastructural alterations in the tegument and subtegument of *R. echinobothrida* and *F. buski*, respectively, when treated *in vitro*. Roy and Swargiary, (2009) showed that the shoot extract of *A. nigra* can reduce the activities of several tegumental enzymes in *F. buski*, an intestinal giant fluke of zoonotic importance in Northeast India. Recently, leaf extract of *Lysimachia ramosa*, a plant used traditionally by the natives of Meghalaya, was reported to be efficacious against *F. buski* and *R. echinobothrida* (Challam *et al.*, 2010).

If natural plant products are to be exploited in the future as medicines for human use or for the treatment of livestock, then isolation and characterisation of their active principles become an essential prerequisite to further progress. One of the plants most commonly mentioned in the Nordic literature is male fern (*Dryopteris filix-mas*), a common fern that is widespread throughout the Northern hemisphere. Extracts from powdered rhizomes were first used by the Greeks (circa 350–250 BC) to treat tapeworm infections. This product (oil of aspidium) became an established product in many pharmacopoeia of the Western World and was sold until the end of the 1940s. A number of active compounds have been isolated from this product, and it appears that the anthelmintic constituent is filicic acid (Waller *et al.*, 2001). Members of the family Asteraceae occupy a prominent position in the herbal de-worming literature. The Romans used dried, unexpanded flower heads obtained from several species of the genus *Artemisia* in the first century, for the treatment of *Ascaris, Enterobius* and tapeworm infections (Steinegger and Hansel, 1972). It remained an important member of the European pharmacopoeia until the early 20th century. The active principle was found to be the sesquiterpene lactone, santonin. High fatality of *Panagrellus redivivus* and *Caenorhabditis elegans* resulted on exposure to sedanolide, senkyunolide-N and senkyunolide-J, isolated from *Apium graveolens* seeds (Momin and Nair, 2001). *Melia azedarach* extract proved to be more efficacious than the reference drug piperazine on the cestodes *Taenia solium* (Szewczuk *et al.*, 2003, 2006). More recent pharmacological studies have demonstrated the pharmacological basis of this chemical (Githiori *et al.*, 2003 b; Sukul *et al.*, 2005). Active plant ingredients like palasonin from *Butea monosperma*, santonin from *Artemisia maritima* and benzyl isothiocyanate from *Carica papaya* have demonstrated high anthelmintic efficacy (Tagboto and Townson, 2001; Githiori *et al.*, 2006; Gomez-Galera *et al.*, 2007), but, many plant components have not lived up to their expectations when tested rigorously (Githiori *et al.*, 2003 a), and very few have been found to have curative properties that can compete effectively with synthetic drugs (Hammond *et al.*, 1997). Presently, there are no naturally-derived plant products sold commercially in the markets for the treatment of worm infections (Githiori *et al.*, 2006).

Exceptional natural products, like penicillin is a fungal product and indeed ivermectin itself is a bacterial product, being derived from the soil bacterium *Streptomyces avermitilis* have become widely accepted (Campbell, 1985). Quinine for the treatment of malaria is an obvious example, as is artemisinin or quinghaosu from *Artemisia annula* (Tagboto and Townson, 2001; Taylor and Berridge, 2006). In recent years, the drug artemether, a derivative of artemisinin that is well known for its antimalarial properties (McIntosh and Olliaro, 1999), has gained importance also in schistosomiasis chemotherapy (Xiao *et al.*, 2000 a, b). Scanning electron microscopy showed that artemether caused extensive and severe damage to the tegument of 21-day-old schistosomules, including swelling, erosion, peeling and vesiculization (Xiao *et al.*, 2000 c). Among plant derived molecules that have anti-parasitic activity and have been used as veterinary parasiticides at times there are nicotine, pyrethrum and rotenone. The former two provided templates for the development of synthetic mimics which include the pyrantel group for nematodes and synthetic insecticides, respectively. Rotenone has been known to inhibit NADH-cytochrome *c* oxidoreductase activity of *A. suum* mitochondria, also reported to be a cestocide when tested against *R. echinobothrida* (Takamiya *et al.*. 1984; Lalchhandama, 2008). Some plants, like *Citrullus vulgaris*, which contain condensed tannins, have been demonstrated to be active against cestode parasites of small ruminants (Nguyen *et al.*, 2005).

Acacia spp. are purported to be effective as anthelmintic, anti-bacterial and antifungal agents, and by virtue of their belonging to the Leguminosae family, the most pharmacologically active components of the plants of this genus are known to be the condensed tannins (CTs), a class of phenolic secondary metabolites that are relatively stable in the digestive tracts of animals and rarely have toxic effects. The genus *Acacia* also contains saponins like Acaciaside A and B, which have been proven to have nematocidal and cestocidal activity (Ghosh *et al.,* 1993, 1996).

The natives of Mizoram (Northeast India) use many plants to get rid of intestinal worm infections. The root of *Imperata cylindrica*, fruit and root of *Embelia* *ribes*, stem bark of *Acacia oxyphylla*, leaves of *Securinega* virosa, and root peel of *Millettia pachycarpa* are some of the majorly used anthelmintic plants of the state (Lalfakzuala *et al.*, 2007). Among these plants, two popularly used ones are *A. oxyphylla* and *S. virosa.*

A. oxyphylla is a perennial leguminous plant highly abundant on the mountainous slopes near the rivers and streams in the state of Mizoram and two teaspoons of the aqueous juice of the stem bark of the plant is taken twice a day against gastrointestinal worms in the rural traditional medicine practised among the Lushai tribe. Anthelmintic property of the plant has recently been reported by Roy *et al.* (2007 a). *S. virosa* is a multi-stemmed shrub with creamy green flowers and berry-like white fruits and found abundantly in Mizoram. The natives take three teaspoons of the aqueous concoction of the leaves of the plant twice a day to counter intestinal worm infections. According to Kirtikar and Basu (1975) the bark contains 10 percent of tannic acid, and an alkaloid. In Philippines (Rizal Province), the charcoal of the wood is powdered and used as a cicatrizant of wounds, and a decoction of the leaves is used for cleansing wounds. The juice of the leaves, or the leaves made into paste with tobacco, is used to destroy worms (Kirtikar and Basu, 1975; Caius, 1935). Dalziel (1936) stated that the leaves have laxative properties and are taken in decoction. The root, sometimes with the leaves, is taken for venereal diseases. Dalziel (1936) further says that the bark is sometimes astringent and appears to be poisonous. The West Ashantis use the roots to cure gonorrhea. The Ewe people of Togoland use a decoction of the leaves internally to cure constipation.

Acacia oxyphylla (Leguminosae)

Securinega virosa (Euphorbiaceae)

In India, chickens play an important role in the provision of animal protein for the population, as both poultry meat and eggs are affordable sources. Parasites are common in the tropics where the standard of husbandry is poor and climatic conditions are favourable for the development of parasites (Gedion, 1991). Of the diseases that reduce productivity of rural poultry, parasitic diseases come first. Even though, parasitic diseases are among the major causes that decrease productivity of chickens, they are often neglected (Alemu, 1985; Sonaiya, 1990; Calnek *et al*., 1991). In this respect it is observed that parasitic cestodes in poultry are known to cause retarded growth, enteritis, diarrhea and hemorrhages (Gordon and Jordan, 1982). Heavy infections due to *R. echinobothrida* and other intestinal helminthes may cause death of young birds apart from the loss of egg production in laying chickens (Soulsby, 1986; He *et al.,* 1990; Ojok, 1993).

Although the majority of the evidence on antiparasitic activity of plants has been traditionally based on anecdotal observations, there are currently an increasing number of controlled experimental studies that aim to verify, validate and quantify in a scientific manner such plant activity. There are two approaches that have been employed for this purpose. The first one is through offering plants or plant parts to naturally or experimentally infected animals and quantifying the consequences of their consumption. The second approach is through testing plant extracts and concoctions derived from medicinal plants via *in vitro* systems. Although a number of medicinal plants have been evaluated through these methodologies and have been found to be active against parasites, the purported antiparasitic properties of a large variety of plants have not been reproduced under controlled experimentation.

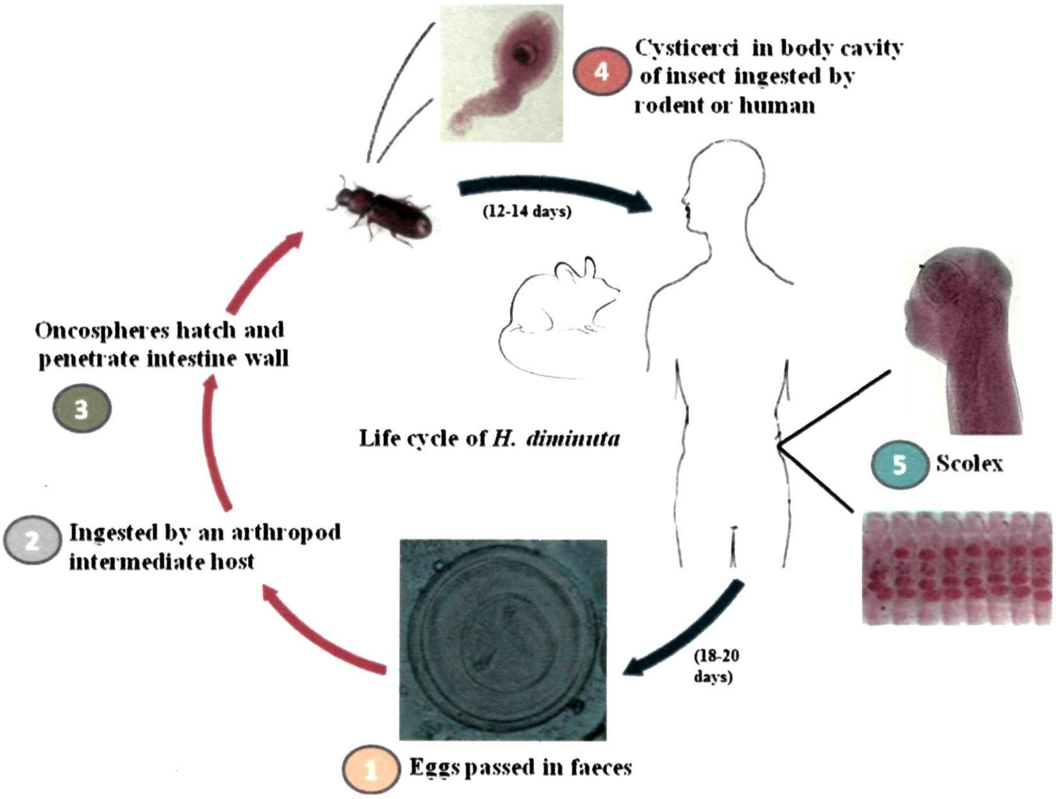
To the best of knowledge, the direct effects of the extracts of the two plants on helminth parasites have not been examined properly. Therefore, in an effort to validate the activities of the plants in a scientific manner and to discover novel anthelmintics from these plants, the present study was designed to investigate the *in vitro* effects of the stem bark of *A. oxyphylla* and the leaves of *S. virosa*, and their active anthelmintic components on *R. echinobothrida*, the cestode of domestic fowl. The same plant parts were also used *in vivo* against *H. diminuta,* the rat intestinal tapeworm which also accidentally infects humans.

The objective of the present investigation is, therefore,

a. to test the *in vitro* and *in vivo* anthelmintic efficacy of traditional medicinal plants, namely, *A. oxyphylla* and *S. virosa*

b. to identify the active principles of the plants responsible for anthelmintic activity

c. to compare the efficacy of these phytochemicals with standard broad spectrum anthelmintics.


4 Cysticerci in body cavity of insect ingested by rodent or human
(12-14 days)
Oncospheres hatch and penetrate intestine wall
3
Life cycle of *H. diminuta*
5 Scolex
2 Ingested by an arthropod intermediate host
(18-20 days)
1 Eggs passed in faeces

Materials and methods

1. Experimental parasites

Mature *Raillietina echinobothrida* (Megnin, 1880) (Class: Cestoda; Subclass: Eucestoda; Order: Cyclophyllidea; Family: Davaineidea), cestode parasite of chicken, and *Hymenolepis diminuta* (Rudolphi, 1819) (Class: Cestoda; Subclass: Eucestoda; Order: Cyclophyllidea; Family: Hymenolepididae), cestode parasite of rats and humans.

2. Plant materials

Acacia oxyphylla Graham ex Bentham (Family: Leguminosae), called 'Khangngo' by the Lushai natives, is a perennial leguminous plant highly abundant on the mountainous slopes near the rivers and streams in the state of Mizoram (Northeast India). *Securinega virosa* (Roxb. ex Willd.) Baill. (Family: Euphorbiaceae), called 'Saisiak' by the natives, is a shrub, about 1m tall with numerous ovoid, white fruits on stems and twigs. It is widely distributed in temperate zone and subtropical zone and was collected from the forests near Aizawl and Lunglei. Both the plants were identified by Dr. H. S. Thapa, Dept. of Botany, Pachhunga University College, Mizoram University, Aizawl. The desired plant parts (stem bark of *A. oxyphylla* and leaves of *S. virosa*) were finally processed for further work.

3. Preparation of plant extracts and fractionation of crude extracts

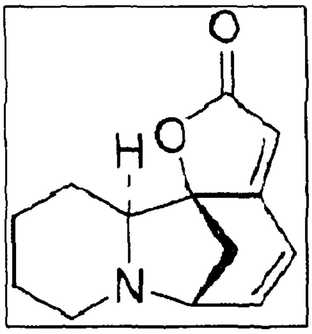
Stem bark of *A. oxyphylla* and fresh leaves of *S. virosa* were collected from Mizoram, India. They were washed gently with distilled water to remove any extraneous materials and dried at 50°C in an oven. After grinding, the material was placed in a reflux flask having rectified spirit (100g/l) for 8 h at 60°C, and the cooled suspension was filtered through Whatman filter paper No.1 to remove the small particulate plant parts. The filtrate is then distilled to remove the solvent. The semisolid crude extracts of the plants thus recovered were dried in an oven at 50°C. In this manner, refluxing was repeated three times. Approximately 2 g and 5 g residue was recovered from 100 g of dried stem bark of *A. oxyphylla*, and dried leaves of *S. virosa*, respectively. Different fractions of the crude alcoholic extract were prepared by dissolving the crude extract with solvents like acetone and methanol in a separating flask, followed by filtration and evaporation of solvent in an oven at 50°C.

The same procedure of fractionation was used with other solvents like, benzene, diethyl ether and ethyl acetate, for both the plant materials, but the crude extracts of the same were not soluble in these solvents.

4. Isolation and identification of active compound

The crude alcoholic extract (2 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 (SUPELCO, 5µm bead size), eluted with a mobile phase composed of methanol-water solution (40 : 60 v/v, also containing 0.1% formic acid) and detected at 220 nm using Agilent 1100 HPLC, that afforded five fractions, Fraction 1 (F1), Fraction 2 (F2), Fraction 3 (F3), Fraction 4 (F4), Fraction 5 (F5). F1: 1400 mg, F2: 178 mg, F3: 27 mg, F4: 50 mg, F5: 310 mg. After testing of these subfractions against the cestode parasite *R. echinobothrida*, two fractions (F1 and F5) were found to be the most active. Among these, F5 proved to have a higher anthelmintic potential and so was further fractionated to yield F5-1d (160 mg) and F5-2d (16 mg). Testing of F5-1d and F5-2d proved that F5-2d was a more potent anthelmintic. The purified F5-2d component was further interpreted through NMR & IR by Dr. P. S. Parameswaran, National Institute of Occeanography, Goa.

Virosecurinine is an isomer of the securinine group of alkaloids and is known to be the active component of *S. virosa* leaves. It was commercially obtained from Pharmeks, Moscow (Code no. PHAR 058226).



Virosecurinine ($C_{13}H_{15}NO_2$)

5. *In vitro* experiments

5.1. Motility and mortality tests

Adult *R. echinobothrida* collected from the intestine of freshly sacrificed domestic fowl were maintained in 0.9% physiological buffered saline (PBS) at 39 ± 1°C and later treated with an incubation media (PBS) containing a particular concentration of crude ethanol extract of the plant, its acetone or methanol fraction, the plants' active principles (F5-2d and virosecurinine), and praziquantel (PZQ; reference cestocidal drug; Distocide) dissolved in 0.1% dimethyl sulphoxide (DMSO). Control worms were simultaneously incubated in the culture medium containing 0.1% DMSO only. A particular concentration was tested with five replicates, each containing a batch of six worms with approximately the same size, weight and maturity. Paralysis was recorded as the state in which there was no visual movement on the part of the worms even on physical stimulation. Death of the worms was ascertained by dipping them in warm PBS, which induced movements in the live worms.

At a concentration of 50 mg and 25 mg crude extracts/ml PBS and their fractions, and 0.01 mg PZQ/ml PBS, the time taken for paralysis and subsequent death of the treated parasites was recorded (Table 1). However, with a view to prolong the time for onset of paralysis and thus allow sufficient time for a significant effect to set in, lower concentrations (5 and 10 mg extract/ ml of PBS, and 0.001 mg PZQ/ ml PBS) of the test materials were also used. On exposure to 10 mg *A. oxyphylla* ethanol extract, 25 mg *S. virosa* ethanol extract and 0.001 mg PZQ, paralysis in parasite tissue was observed to ensue within a comparable time span. Thus, these concentrations were selected to carry on further studies involving other parameters like motility, survivability, ultrastructural, histochemical and biochemical changes, in the treated parasites.

5.2. Surface morphology and ultrastructural studies

Scanning electron microscopy

The paralysed parasite material was fixed in 10% neutral buffered formalin (NBF) at 4°C for 24 h, washed in PBS and dehydrated in ascending grades of acetone to absolute acetone. The specimens were then treated with tetramethyl silane (TMS) for 15 min and air dried at 25°C, following the protocol of Dey *et al.* (1989), modified by Roy and Tandon (1991). The dried material was put on metal stub according to the orientation required and sputter coated with gold in a fine-coat ion sputter, JFC-1100 (JEOL). The gold-coated specimens were viewed using JEOL (JSM 6360) scanning electron microscope at an electron accelerating voltage of 10-15 kV.

Transmission electron microscopy

Soon after paralysis, one specimen (mature proglottid) each from different concentrations of crude extracts, different fractions, reference drug, along with one set of control was fixed in modified Karnovsky's fixative (2% paraformaldehyde, 2.5% gluteraldehyde buffered with 0.1 M sodium cacodylate;

pH 7.4) at 4°C for 4 h followed by secondary fixation in 1% osmium tetra oxide (OsO_4) in the same buffer for 1 h at 4°C. Specimens were dehydrated in graded series of acetone, cleared in propylene oxide and embedded in araldite in beem capsules. Ultra-thin sections (600-900Å) were cut on an RMC ultra microtome, MT-X, with a diamond knife. The ultra-thin sections were then collected on uncoated copper grids, stained with 5% aqueous uranyl-acetate solution for 10 min at 40°C followed by lead citrate (Reynolds, 1963) and examined with a JEM 100 CXII Transmission Electron Microscope (JEOL) at an electron acceleration voltage of 80 kV.

5.3. Histochemical localization of tegumental enzymes

Adenosine triphosphatase (ATPase)

For the demonstration of ATPase activity, calcium method of Maengwyn-Davies *et al.* (1950) (as referenced in Pearse, 1968) was followed.

Tissue preparation:

The frozen sections of 12-14 µm thickness were incubated in a freshly prepared incubation medium (pH 9.9) containing 0.1 M glycine and 0.4 M KCl in saturated sodium acetate, 0.36 M $CaCl_2$, 1.0 M KOH, 0.04 M Na-ATP, distilled water and saturated sodium phosphate, for 0.5-3 h. The sections were then washed in three changes of 1% $CaCl_2$ in 75% ethanol, transferred to 2% cobalt chloride for 3 min. and developed in 1% yellow ammonium sulphide, finally washed in distilled water, dehydrated and mounted in suitable synthetic medium or mounted in glycerine jelly. The activity of ATPase showed as a blackish brown deposit.

Alkaline phosphatase (AlkPase)

A modified coupling azo-dye method described by Pearse (1968) was followed for the determination of AlkPase activity.

Tissue preparation:

The specimens were fixed in 10% NBF at 4°C for 10-16 h, washed thoroughly in running water and processed for frozen sectioning. Sections were cut at 10-15 μm thickness and mounted on clear slides without adhesive and allowed to dry in air for 1-3 h. Frozen sections were incubated in Fast Violet B (2-benzoylamine-4-methoxytoluidine) freshly prepared in 0.1 M stock Tris buffer at pH 10.0 at room temperature (17-20°C) for 15-60 min., washed in running water for 1-3 min., counter stained in Mayer's haemalum (1-2 min), again washed in water for 30-60 min. and mounted in glycerine jelly. The sites of AlkPase activity were coloured brown and the nuclei were coloured dark blue.

Acid phosphatase (AcPase)

The AcPase activity was demonstrated following the modified lead nitrate method of Takeuchi and Tanoue (1951), wherein β-glycerophosphate was used as the substrate.

Tissue preparation:

The live control and treated parasites were fixed in cold formol calcium fluid (prepared with 4% formaldehyde containing 1% $CaCl_2$ at pH 7.0) and kept overnight. The fixed materials were washed extensively in water. Frozen sections were cut at a thickness of 10-15 μm in a cryo microtome (Leica CM 1850) and incubated in a freshly prepared medium containing 2 volumes 2% sodium- β-glycerophosphate, 1 volume 0.1 M acetate buffer (pH 5.0-6.0), 1 volume 2% lead acetate and 0.3 volume 1-5% $MgCl_2$, at 37°C for 2 h. After staining, the sections were rinsed in distilled water and developed in ammoniacal silver nitrate solution (prepared by adding 28% ammonia water drop by drop to 5% aqueous $AgNO_3$ until the precipitate just dissolved) for 30 min. Then the specimens were rinsed in 5% sodium thiosulphate for 5 min, dehydrated, cleared, and mounted in Canada balsam. A brownish precipitate indicated sites of AcPase activity.

5.4. Biochemical analyses

Protein estimation and specific activity

Protein was estimated following the method of Lowry *et al.* (1951), using bovine serum albumin as the standard. Specific activity of the enzymes was expressed as the units of enzyme activity per mg protein.

Tegumental enzymes

Adenosine triphosphatase (ATPase)

The ATPase activity was assayed by estimating the free phosphate released, following the method of Kaplan (1957). Treated parasites were kept at -20°C just after they were paralysed, till they were used for estimation. The estimations were completed within two-three days of sampling during which enzyme activities are supposed to remain unaltered.

Sample Preparation:

The frozen tissues were thawed on ice and 10% homogenate (w/v) was prepared following Kaplan (1957) in glycine buffer at pH 9.1. The homogenate was sonicated for 30 sec using 10% tissue homogenate and Triton-X (0.5% v/v final concentration at 1:1 ratio) before ATPase assay. This treatment was standardized and found to be optimum.

Assay mixture (final volume 1ml) contained the following:

Sodium glycine buffer, pH 9.1	(91.43 μmoles)
$CaCl_2$	(45.71 μmoles)
Na-ATP	(45.71 μmoles)
Tissue extract as enzyme source	0.2 ml

Analysis:

The assay mixture was preincubated for 5 min at 37 ± 1°C before addition of the tissue homogenate to initiate the reaction. The reaction was terminated after 1h by addition of 1 ml of 15% trichloroacetic acid (TCA) to the reaction mixture. The tissue blank was prepared for each assay with the addition of TCA prior to the addition of the tissue extract. The precipitated protein was separated out by centrifugation at 300-400 rpm for 10 min at room temperature.

The phosphate formed was estimated in the supernatant following the method of Fiske and Subbarow (1925). To 0.25 ml of the supernatant was added 0.75 ml water, 0.5 ml 5 N sulphuric acid, 0.5 ml ammonium molybdate (2.5% w/v) and 0.1 ml freshly prepared Fiske and Subbarow reducer (2.5% w/v solution in water of the mixture of 6 part sodium sulphate: 6 part sodium metabisulphite: 1 part 1-amino-3-naphthol-4-sulphonic acid). The reaction mixture was incubated for 10 min and the O.D. taken at room temperature against zero time tissue blank where TCA was added prior to the addition of enzyme. The amount of phosphate present was calculated from a standard graph prepared using different concentrations of a monophosphate (0.1-1 µmole of KH_2PO_4), which was linear.

One unit of ATPase activity was defined as that amount which catalysed the release of 1 µmole of phosphate/h at 37 ± 1°C from ATP. The enzyme activity was expressed as the total activity (units/g wet wt. of tissue) and specific activity (units/mg protein).

Alkaline phosphatase (AlkPase)

The AlkPase activity was estimated following the procedure described by Plummer (1988).

Sample preparation:

A 10% (w/v) tissue homogenate of the parasite was prepared in 125 mM sodium glycine buffer at pH 10.0. The homogenate was centrifuged at 5000 rpm at 0 ± 2°C for 20 min. and the supernatant was used for the enzyme assay.

The reaction mixture (final volume 1ml) of contained the following:

Sodium glycine buffer, pH 10.0	(22.8 µmoles)
p- nitrophenyl phosphate	(114.2 µmoles)
Tissue extract as enzyme source	0.2 ml

Analysis:

The assay mixture minus tissue extract was incubated in a serological water bath at 37 ± 1° C for 5 min. 200 µl of the tissue extract was then added to it and incubated for 20-30 min and the reaction was stopped by adding 5 ml of 0.02 N NaOH solution. For blank, similar procedure was followed using 200 µl of buffer instead of enzyme solution. The increase or decrease in O.D. at 420 nm was recorded and the amount of p- nitrophenol produced was calculated from a linear standard curve prepared using different concentrations (0.01-0.1 mM) of p-nitrophenol.

One unit of AlkPase activity was defined as that amount which catalysed the formation of 1 mM p- nitrophenol/h at 37 ± 1°C. The enzyme activity was expressed as the total activity (units/g wet wt. of tissue) and specific activity (units/mg protein).

Acid phosphatase (AcPase)

The AcPase activity was assayed by estimating the p-nitrophenol product following the method of Plummer (1988) with necessary modification in the concentration of the buffer and substrate.

Sample preparation:

A 10% (w/v) tissue homogenate of the parasite was prepared in buffer containing 125mM sodium acetate and glacial acetic acid at pH 4.5 using a Remi-motor driven Potter-Elvejem type glass homogenizer fitted with a Teflon pestle.

The homogenate was centrifuged at 5000 rpm at 0 ± 2°C for 20 min. and the supernatant was used for the enzyme assay.

The assay mixture (final volume 1ml) contained:

Sodium acetate buffer, pH 4.5	(125 μmoles)
p -nitrophenyl phosphate	(62.5μmoles)
Tissue extract as enzyme source	0.4 ml

Analysis:

The assay mixture minus tissue extract was incubated in a serological water bath fitted with a temperature control unit at 37 ± 1°C for 5 min. 400 μl of the tissue extract was then added to it and incubated for 20-30 min. Subsequently, the reaction was stopped by adding 4 ml of 0.5 N NaOH solution and the O.D. measured at 405 nm. For blank, similar procedure was followed using 400 μl of water instead of enzyme solution. The amount of p-nitrophenol produced was calculated from a linear standard graph prepared using different concentrations (0.01-0.1 mM) of p-nitrophenol.

One unit of AcPase activity was defined as that amount which catalysed the formation of 1 mM p-nitrophenol/h at 37 ± 1°C. The enzyme activity was expressed as the total activity (units/g wet wt. of tissue) and specific activity (units/mg protein).

Free amino acid analysis (FAA)

Sample Preparation:

The experimental samples were dissolved in milli Q water (200 mg/ml). As the sample solutions were very turbid, centrifugation was carried out at 10,000 rpm for 60 min to remove the insoluble material. The supernatant was taken and passed through C18 column (1 c.m. length) to remove the peptides and proteins from the sample. The eluate was collected. Following this, the column was also eluted with 1 ml of 40% acetonitrile and this eluate was combined with the earlier one. The pooled eluate was then lyophilized to a dry powder and stored at -70°C for further experimentation.

Isotopic bar labeling for mass spectrometry:

Before going for isotopic bar labeling the samples as well as the iTRAQ (isobaric Tags for Relative and Absolute Quantification) reagents (Applied Biosystems) were brought to room temperature. The samples were dissolved in 20 µl of dissolution buffer provided in the kit. The samples and standard amino acids were dissolved in equimolar concentrations.

In the first set of experiment Standard amino acid mixture, and the samples A, B and C were labeled with 114, 115, 116 and 117 respectively. In the 2nd set of the experiment keeping the Standard amino acid labeled with 114, the next set of samples i.e. A, C and D were labeled with 115, 116 and 117 respectively.

The sample solutions were centrifuged to bring the solution to the bottom of the tube. In parallel, 70μl of ethanol was added to each iTRAQ reagent vial at room-temperature. The reagent were mixed properly and settled down by a short spin (2,000 rpm for 2 mins). The total contents of the iTRAQ reagents were then transferred to each sample tube as mentioned above. The labeling was carried out for 1 h at room temperature. The reaction mixtures were again vortexed and short spin was applied to them. Each set of sample mixtures were combined by pooling them together. Sample mixtures were aliquoted and dried at 40°C.

Mass spectrometric analysis:

The dried sample was dissolved in 15 μl of 3% acetonitrile and 0.1% formic acid. The mixed samples were introduced to QSTAR- XL MS/MS mass spectrometer using nano ES spray capillaries. The inlet voltage was fixed at 1100 kV. In IDA (Information dependent acquisition) method ions (1+, 2+ and 3+) were scanned from 200-450 m/z. The three most intense ions which exceed at least 10 counts were allowed for MS/MS fragmentation and data was acquired. Ion tolerance was fixed at 50 atomic mass unit (amu). The duration of data acquisition was 10 min with a cycle time of 4.002 sec. From the IDA method the total ions were selected and compared with the standard amino acid mass values from the list. For the estimation of % amino acid composition the peak area for each ion was taken for analysis.

5.5. Trace elements analyses

Fresh worms in control group were directly taken for analyses of their trace metal contents. Cestodes in different treatment groups were harvested immediately after they succumbed to paralytic state, and thoroughly washed with deionised double distilled water. Whole worms were dried in an incubator at 50°C. The dried worms were finely pulverized to powder. The powdered tissue (2 g) was digested in 10 ml of concentrated HNO_3 in an airtight corked conical flask for overnight at 50°C. The fully digested solution was transferred to and kept on a hot plate at 70°C for ~2 h to allow complete evaporation of the acid. After cooling down, 10 ml of deionised water was then added, vigourously mixed with magnetic stirrer, and filtered through Whatman fliter paper (110 mm Φ). The volume was finally made to 100 ml by adding more deionised water to the filtrate. The final solution was directly used for quantitative measurement of the trace elements using a single beam atomic absorption spectrophotometer (Perkin Elmer 3110) at the absorbance wavelengths of 422.6 nm for calcium and 285.2 nm for magnesium.

6. *In vivo* experiments

6.1. Parasites

The tapeworm, *H. diminuta*, was maintained in Wistar rats and flour beetles of the species *Tribolium confusum*. The rats were given food and water *ad libitum*, and all animal procedures were carried out in accordance with the regulations of the Animals (Scientific Procedures) Act, 1986.

6.2. Experimental Conditions

Animals were housed under standard laboratory conditions, air-conditioned with adequate fresh air supply (Air changes 12-15 per hour), room temperature 22 ± 3°C, relative humidity 30-70 %, with 12 h light and 12 h dark cycle. Two animals of same sex and group were housed per cage in standard polypropylene (Size: L 430 x B 270 x H 150 mm) with stainless steel mesh top grill having facilities for holding pelleted food and drinking water in water bottle fitted with stainless steel sipper tube. The animals were provided with food and water *ad libitum* throughout the acclimatization and experimental period.

6.3. Acclimatization and de-worming of rats

The animals were acclimatized five days to laboratory conditions and were observed for clinical signs daily. Veterinary examination of all the animals was performed on the day of receipt and randomization. After five days of acclimatization the rats were de-wormed by administering praziquantel at 200 mg/kg dose for 3 days. All rats were starved for 24 h before cysticercoid inoculation.

6.4. Evaluation of tolerated doses of extracts

The dose that did not produce any sign of toxicity (referred to as maximum tolerated dose; MTD) was determined orally by administering serial doses (50-2000 mg/ kg body weight). With 100 mg/kg dose of *A. oxyphylla,* the treated rats began to show signs of toxicity like convulsions, dullness, restlessness, constipation and diarrhoea within 5 h of inoculation and died in 15 h. On the other hand, onset of toxicity with 2500 mg/kg dose of *S. virosa* was within 8 h of inoculation and death was noted within 24 h for the treated rats. So, dosages of 25 mg and 50 mg/kg body weight were chosen for *A. oxyphylla* testing, and 750 mg and 1500 mg/kg body weight dosages were chosen for *S. virosa* testing.

6.5. Cysticercoid inoculation

Each rat was administered with 5 cysticercoids in 1.5 ml of PBS. Stool examination was performed for the infection from day 18 to 20 post inoculation for the presence of proglottides/eggs to confirm the infection and maintain the infection cycle in beetles.

6.6. Grouping and study design

The infected animals were divided into six groups (of three rats each) on day 20 post inoculation. Animal grouping was done by the method of body weight stratification and randomization. The infected animals were weighed and grouped in to body weight ranges. These body weight stratified rats were distributed to all the study groups in equal numbers if possible such that body weight variation of animals used doesn't exceed ± 20% of the mean body weight.

The selected animals were assigned to different test groups as shown below:

Test Group	Dose (mg/kg)	Daily Dose frequency	No. of rats/group
Untreated control	-	-	3
A. oxyphylla	25	Once	3
	50	Once	3
S. virosa	750	Once	3
	1500	Once	3
Praziquantel	5	Once	3

Anticestodal efficacy was adjudged on the basis of parasite eggs/g (EPG) of faeces count before and after treatment, direct count of surviving worms including scolices (% worm recovery) remaining in small intestines after completion of treatment, and by host clearance of parasite. These were calculated in percentage as follows:

Percentage difference in EPG count = (mean EPG at pre-treatment – mean EPG at post-treatment) X 100/ (mean EPG at pre-treatment)

Percentage worm recovery rate = number of worms recovered at necropsy X 100/number of cysticercoids given

6.7. Route of administration

The test item was administered through oral route.

6.8. Stool examination (pre-treatment)

Approximately 2 g of faeces was collected from each test group from day 18 to 20 post-inoculation and the average eggs/g was counted by modified McMaster's method.

6.9. Frequency of treatment

The animals were dosed once a day except untreated control. The animals were treated for a period of 3 days i.e. from day 21 to 23 post inoculation. All animals were observed twice daily for pre-terminal deaths and morbidity. Dead/moribund animals were autopsied immediately at the earliest.

6.10. Stool examination (post-treatment)

Approximately 2 g of faeces was collected from each test group from day 24 to 26 post-inoculation and the eggs were counted. Another EPG count was made on days 33-35, exactly a week after.

6.11. Necropsy

All the animals were fasted for 24 h on day 35 post inoculation, next day (day 36 post inoculation) the animals were sacrificed using CO_2 asphyxiation. The number of worms in each animal was counted to determine the worm burden.

7. Data Analyses

In vitro

All data were expressed in mean ± S.E.M. for three or five replicates in each group. Comparison of the mean values of the experimental groups against the control groups was made using unpaired Student's t-test, and values of $p < 0.05$ were considered significant in all cases.

In vivo

Statistical analysis was performed using two-way and one-way ANOVA. The significance of difference was accepted at $P < 0.01$.

RESULTS

Efficacy of crude plant extracts and their fractions

In vitro experiments

1. Motility and mortality tests

Raillietina echinobothrida exposed to different concentrations of crude and fractions of crude extract of *A. oxyphylla* and *S. virosa,* and the reference drug PZQ, revealed a decline in the motility of worms resulting eventually in their death (Tables 1, 2; Graphs 1, 2). The control parasites continued to show physical activity for an average timing of 72 h following which they became immobilized. On exposure to the test solutions, the worms proceeded from a state of vigorous movements to a relaxed condition in which they continued to remain till they attained paralysis. The time taken for paralysis showed a dose-dependent effect as paralysis occurred at a shorter period of time for higher concentrations of test materials while for lower doses it took a much longer period of incubation.

On exposure to the various concentrations of *A. oxyphylla* ethanol extract, the tapeworms showed an onset of paralytic state. At 50 mg/ml, spontaneous movements seized in the worms at about 0.6 h while treatment with 25 mg/ml concentration of the crude alcoholic extract induced loss of motility and consequent paralysis in 1.93 h (Table 1). The 5 mg/ml and 10 mg/ml doses took about 4.93 h and 3.5 h, respectively, indicating a dose-dependent effect of the plant material; in comparison, the 0.001 mg/ml dose of the reference drug caused paralysis to set in after 3 h post incubation. The methanol fraction was comparatively less effective than the ethanol extract and death occurred at 3.7 h and 8.06 h for 50 mg and 5 mg, respectively. The acetone fraction proved to be the most potent where death occurred at 1.12 h and 4.19 h for 50 mg and 5 mg, respectively. The observations indicate a significant difference (at $p < 0.05$) between *A. oxyphylla*-treated group and the control group.

The effect of different concentrations of crude ethanol extract of *S. virosa* leaf and its methanol and acetone fractions are depicted in Table 2. Death occurred at 3.42 h and 18.11 h for 50 mg and 5 mg ethanol extract/ ml of PBS, respectively. At 50 mg methanol fraction, spontaneous movements seized in the worms at about 1.05 h while for 5 mg concentration, the worms showed motility for 11.63 h. Death was definite by 2.1 h and 13.7 h, respectively, for the two test concentrations. The ethanol extract was comparatively less effective than the methanol fraction and the acetone fraction proved to be the least effective. Death was recorded at 5.27 h and 21.22 h for 50 mg and 5 mg of acetone fraction, respectively. Here too a significant difference ($p < 0.05$) between *S. virosa*-treated group and control group was observed.

Likewise, 0.01 mg PZQ /ml of PBS brought about a paralytic state at 0.5 h while 0.001 mg/ml dosage took 3.0 h to induce paralysis in the test parasites.

2. Surface morphology and ultrastructural studies

Scanning electron microscopy

Control: The cestode *R. echinobothrida* revealed an elongated body with a long neck leading to a rounded scolex. The scolex had a retractable rostellum surrounded by rows of spines (Figs. 1, 2). Four suckers were arranged sideways around the scolex; each with circlets of hooks that were broad at the base and tapering and bent toward the ends (Fig. 3). The neck gave way to a string of proglottides that were longer than wide (Fig. 4). The tegument revealed a smooth surface with linear striations which when magnified revealed a consortium of layers of minute projections called microtriches, which are similar to microvilli and it sloped gently downwards (Figs. 5, 6).

Treated: As observed with SEM, the strobila of the cestode comprised of a chain of proglottides which, on incubation in various treatment media, became smaller and erupted in blisters all over the surface. Edges of the proglottides became thinner and blunter. Lesions appeared in the surface of the tegument and developed into deep perforations.

Ethanol extract of *A. oxyphylla* caused the parasite to shrivel up with the effect more pronounced in the proglottides than the scolex. The scolex showed an elevated rostellum with majority of the hooks intact and a few cracks beneath the suckers (Figs. 7, 8). The body showed a continuous streaming in the proglottides at many places so that subsequent segments could hardly be differentiated and the microtriches were clumped (Figs. 9, 10). The methanol fraction too showed similar outcome except on a much lower range with very minor effect on the scolex (Figs. 11-14).

The scolex of the parasites treated with the acetone fraction of *A. oxyphylla* revealed severe destruction, so much so that it became a twisted mass where the rostellum and sucker regions were indistinguishable (Fig. 15). The neck region was shrunk into a thin line joining the head with the rest of the body (Fig. 16). The tegument of severely injured worms suffered from focal lesions, peelings and swellings at many places (Fig. 17) and the outer membrane expanded to form deep and tortuous ramifications, leading to the increase in the number of the surface pits (Fig. 18).

On treatment with the ethanol extract and methanol fraction of *S. virosa*, the cestode revealed a contorted body with shriveled scolex. The suckers were no longer elevated and the sunken surface had a porous honey-comb appearance with the hooks around mostly broken and fallen off. Cracks were noted in the scolex, mostly around the sucker area. The ethanol extract brought about a fewer number of lesions on the surface as compared to the methanol fraction which caused widespread focal lesions throughout the body. There were no well defined linear striations but surface infoldings with clumped and unruly microtriches (Figs. 19-26).

Treatment with the acetone fraction of *S. virosa* resulted in damage to the scolex which was wrinkled and showed a decline in the number of hooks on the suckers. The hooks present were mostly clumped and the damage was mostly confined to the general body surface where a lack of demarcation between adjacent proglottides was noticed at many places (Figs. 27-30).

PZQ had a disastrous effect on the scolex which was highly misshapen, to the extent that the suckers could almost just be made out. However, the proglottides did not display much alteration on the whole but the surface was distinctly porous with the microtriches not that prominent (Figs. 31-34).

Transmission electron microscopy

Control: Transmission electron microscopic observations on the control worms revealed the typical cestode morphology with respect to the tegument, subtegument and inner parenchyma. On the very outer surface of the tegument a surface glycocalyx layer is seen to cover the outer plasma membrane (Fig. 35). Below the glycocalyx, the characteristic feature of the cestode tegument, is the presence of numerous microtriches, long spine like processes that are in fact a highly modified form of microvilli. Each microthrix has a hard, pointed, electron dense cap which is separated from the rest of the microthrix by a crescent shaped membranous cap. Below the layer of microtriches, the main syncytial layer of the tegument, the distal cytoplasm is found. This has been seen to contain numerous membrane bound, electron dense rod-like structures, referred to as disc-shaped bodies. Finally numerous mitochondria can also be seen. In the subtegument region, there runs a basal lamina that infolds into the distal cytoplasm at many places, forming the so-called basal infolds. Right underneath the lamina lie blocks of systematically arranged circular and longitudinal muscle bundles. The tegumental nuclei are however not located in this outer layer, but are found within subtegumental cell bodies (cytons), located beneath the circular and longitudinal muscle layers, embedded within the parenchymal tissues and mesenchymal musculature (Fig. 36). Cytoplasmic processes running down from the basal lamina meet these subtegumental cytons with distinct cell bodies having prominent nuclei. These subtegumental cell bodies also contain other cellular elements such as Golgi apparatus and lipid inclusion bodies which are connected to the outer syncytium and areas of glycogen storage by long protoplasmic extensions. The parenchymal tissues fill the spaces between the parasites internal organs (all cestodes and other platyhelminthes being acoelomate organisms). In the parenchymal part, there were numerous well connected parenchymal cytons that were abundant with granular endoplasmic reticulum and other cell organelles including mitochondria with prominent cristae (Fig. 37).

Treated: In worms treated with the crude stem bark extract of *A. oxyphylla* (10 mg/ml PBS), degenerative changes were noticeable in the tegumental ultrastructure; the tegument appeared as stripped right down to the basal lamina and the remnants of the basal infolds left behind were highly swollen (Figs. 38, 39). Even the nuclei of the cytons were devoid of nuclear material due to rupture of the nuclear membrane (Figs. 40). The granular endoplasmic reticulum (GER) near the nuclei was found in very meager numbers and those present had highly dilated cisternae (Fig. 41).

Worms treated with 10 mg methanol fraction/ml PBS showed sloughing off of the tegument with much alteration in the tegumental architecture. There was an increase in electron-lucency of the background due to both glycogen loss and vacuole formation (Fig. 42) and the subtegument showed immense vacuolization and accumulation of debris. There was release of underlying structures to the outside at the basal lamina, which was disrupted at many places. Golgi complexes were scarcely present; the nuclei showed swelling (Fig. 43) and the mitochondria were mostly ruptured (Fig. 44).

The degeneration caused by the acetone fraction of the stem bark was more pronounced than that due to the alcoholic extract. The tegument appeared to be completely sloughed off leaving an exposed basal lamina that was only present in remnants and there was release of underlying structures to the outside at the basal lamina (Fig. 45). The circular muscle layer showed intense degradation and was loosely stacked up with loss in continuity; mitochondria were seen to disappear from the apical zone and those encountered were devoid of cristae (Fig. 46). There was very little amount of GER remaining with no evidence of Golgi complexes. The nuclei encountered in the acetone fraction-treated parasites were electron lucent due to loss of nucleoplasm, and the nucleoli were diffused in appearance (Fig. 47). The parenchymal cytons of the acetone fraction-treated parasite showed complete loss of connections with the surrounding parenchyma and the chromatin in the nucleus appeared clumped into large areas of electron-dense heterochromatin. The mitochondria showed either a loss or a complete lack of cristae altogether and numerous membranous whorls were distinct in the cytoplasm (Fig. 48).

The microthrix layer of the cestode showed a general erosion and clumping effect when treated with the ethanol extract of *S. virosa* which was in tune with the observation made by scanning electron microscopy (Fig. 49). The distal cytoplasm was perforated and no tegumental discs or mitochondria were visible. The cytoplasm also streamed down into the subtegument basal lamina in the form of thin laminar processes (Fig. 49 inset). The lamina itself had vacuoles which went down to penetrate and cause disintegration of the subtegumental cytons (making them hardly discernible) between the muscle blocks. The parenchymal cytons too were vacuolated with mitochondria that were ruptured and acristate and prominent distorted nuclei which mostly had a round nucleolus (Figs. 50, 51).

When treated with the methanol fraction of *S. virosa* (25 mg/ml), the cestode tegument was majorly stripped off, peeling out a significant layer of the basal lamina as well (Fig. 52). The muscle layers were slightly disorganized and the subtegument region was immensely vacuolated. Mitochondria were scarce and ruptured, and the cristae were mostly broken and scattered in the mitochondrial lumen (Fig. 53). The GER was hardly evident and if present, as in the few instances were dilated. Apart from this, an increase in the nuclear heterochromatin content and swelling of the perinuclear space were a few other pronounced effects observed. Almost all the subtegumental cytons were compressed and elongated with long misshapen nuclei which too were vacuolated (Fig. 54).

The acetone fraction brought about a general disorganization in the tissue material along with disintegration of the parenchymal cytons (Figs. 55-57).

PZQ's effect on the parasite was mostly restricted to the tegument which had sloughed off exposing the basal lamina (Fig. 58). Muscle blocks were a bit disrupted with slight tear evident at some places. Autophagosomes could be seen interspersed between these muscle blocks in the subtegument. The subtegumental and parenchymal cytons showed considerable damage and had round electron dense mitochondria with well-formed cristae (Figs. 59, 60). An overall increase in electron lucency of the background and vacuole formation was observed in all treated worms.

3. Histochemical localization of tegumental enzymes

Intense ATPase, AlkPase and AcPase activities were observed in the tegument and subtegument areas of control *R. echinobothrida.* ATPase showed maximum staining intensity in the tegument, subtegument, somatic musculature and parenchyma followed by AlkPase and AcPase activity (Table 3).

Control worms showed high ATPase intensity throughout the parasite tissue, particularly, the tegument, subtegument and parenchyma (Fig. 61). No ATPase activity was observed in the parenchyma and only mild activity was noted in the tegument, subtegument and muscle blocks of *A. oxyphylla* treated parasite (Fig. 62). Treatment with *S. virosa* caused reduction in the general ATPase staining intensity, particularly in the tegument and subtegument regions, which showed a marked decrease. Only region to retain intense stain was the parenchyma. Mild staining was observed in the tegument and the subtegument (Fig. 63). PZQ caused the parasite to lose tegumental enzymes on a large-scale and ATPase activity was located very mildly only in the subtegument (Fig. 64).

AlkPase activity was mostly concentrated in the tegument and subtegument of control worms with moderate activity in the parenchyma (Fig. 65). In case of *A. oxyphylla* treated parasites, AlkPase activity was present in the subtegument, gradually reducing towards the muscle layers (Fig. 66). The activity was overall diminished in parasites treated with *S. virosa* (Fig. 67). PZQ caused the AlkPase activity to concentrate in the subtegument and muscle with very little or no activity in the parenchyma (Fig. 68).

Control worms which showed intense AcPase activity in the tegument (Fig. 69). *A. oxyphylla* treatment led to almost total inactivation of the enzyme activity in the parasite tissue (Fig. 70). The somatic musculature was the only part of the tissue to show mild AcPase stain in *A. oxyphylla* treated *R. echinobothrida*. *S. virosa* treatment resulted in tapeworm tegument damage which is manifested by reduced histochemical staining throughout the tissue (Fig. 71). However, PZQ resulted in extensive diminishing of the enzyme throughout the tissue (Fig. 72).

4. Biochemical analyses

Tegumental enzymes

Table 4 shows the effect of the crude stem bark extract of *A. oxyphylla*, leaf extract of *S. virosa* and PZQ on the tissue activities of the tegumental enzymes in *R. echinobothrida*. At the biochemical level, the activity of ATPase in the parasite was found to be highest, averaging about 155.28 units/g wet weight, followed by AlkPase (31.14 units/g wet wt) and AcPase (6.06 units/g wet wt) (Graphs 3, 4). The activity of ATPase in the parasite decreased significantly by 72%, 91% and 96%, respectively; in treatments with crude stem bark extract of *A. oxyphylla*, leaf extract of *S. virosa* and PZQ. A significant decrease in the activity of AlkPase (of about 96% and 98%, respectively) was observed when the parasite was treated with *S. virosa* leaf extract and PZQ, while a relatively less decrease in activity (of about 90%) was observed in treatments with *A. oxyphylla* (Graph 5). The tissue activity of AcPase decreased significantly in parasites treated with both *A. oxyphylla* and PZQ. The percentage decrease of AcPase activity was recorded as 97%, 34.5% and 96% in parasites treated with crude extract of *A. oxyphylla*, *S. virosa* and PZQ, respectively, compared to the controls. Thus, the effectiveness of the different treatment groups in reducing tissue ATPase, AlkPase and AcPase is in the order PZQ > *S. virosa* > *A. oxyphylla*, PZQ > *S. virosa* > *A. oxyphylla* and *A. oxyphylla* > PZQ > *S. virosa*, respectively.

Free amino acids

As depicted in Table 5, the FAA pool of the control worm consists of amino acids: isoleucine (Ile), leucine (Leu), asparagine (Asn), serine (Ser), proline (Pro), valine (Val), methionine (Met), histidine (His), threonine (Thr), cysteine (Cys), aspartate (Asp), glutamine (Gln), lysine (Lys), glutamic acid (Glu), tyrosine (Tyr), tryptophan (Trp), phenylanaline (Phe), arginine (Arg), glycine (Gly) and alanine (Ala). The quantitative analysis data has also been depicted in Table 5. Out of the amino acids detected, Gly was found to be at the maximum level, followed by Ala, Ser, Pro, Val, Thr, Cys, Ile/Leu, Asn, Asp, Gln/Lys, Glu, Meth, His and Phe, respectively, in a decreasing order. Other amino acids detected, namely, Arg, Tyr and Trp were at comparatively lower levels. Quantitatively, the level of the various FAAs in the parasite was significantly affected by the treatment with the two test materials, and also with the reference drug PZQ (Table 5, Graph 6). Parasites treated with plant extracts and reference drug showed a marked decrease in the levels of Meth, Ile/Leu, Phe, Gly, Tyr and Asn, though an increase in the levels of Thr (except PZQ treated parasite which remained unchanged in Thr level). *A. oxyphylla*-treated parasites showed no increase in the levels of any other amino acids and *S. virosa* showed an increase of 40% (Ala), 6.3% (Pro), 100% (Cys) and 50% (Trp). Further, in contrast to a 150% increase in PZQ treated parasites, Ser did not show any change in level following treatment with *S. virosa and A. oxyphylla. A.*

oxyphylla treatment failed to cause any alterations in Pro and Val while PZQ treated parasite showed a decrease in the same. PZQ treatment resulted in the increase of Ser, Gln/Lys and Trp levels within the parasite and consistently portrayed a decrease or no change (Ala, Thr, Cys, Glu, Arg, His) in the levels of all other amino acids. While the Gln/Lys levels showed a decrease in *A. oxyphylla*-treated parasites and a 25% increase in PZQ-treated parasites, its level was observed to remain unchanged with *S. virosa*-treatment.

5. Trace elements analyses

The quantitative observations on two vital trace elements, namely, Ca and Mg, in control *R. echinobothrida*, and *R. echinobothrida* treated with the crude extracts of *A. oxyphylla, S. virosa*, their different fractions, and PZQ are presented in Tables 6 to 8 and in Graphs 7 to 9. The concentrations of Ca and Mg in the control worms maintained in 0.9 % PBS was about 45.2 and 6.2 ppm. Cestodes exposed to 10 mg/ml of ethanol extract and acetone fraction of *A. oxyphylla* showed a significant reduction in the levels of the two elements (9.7 ± 0.13 and 3.6 ± 0.4 for ethanol extract; 9.8 ± 0.61 and 4.4 ± 0.17 for acetone fraction), and a pronounced increase in their levels in the incubation media (33.1 ± 0.87 and 5.2 ± 0.05 for ethanol extract; 18.7 ± 1.45 and 5.6 ± 0.07 for acetone fraction). The untested plant media in both the cases showed a much less concentration of the two metals than in the effluent. Methanol fraction of the plant did not bring about any significant reduction in the concentration of the elements and the effluent showed a much less Ca concentration than the untested plant media.

Treatment with the ethanol extract of *S. virosa* caused the concentration of Ca and Mg to decrease in the cestode tissue. The level of Ca got raised from 18.6 ± 0.02 in the untested plant media to 35.9 ± 0.67 in the incubation media. The concentrations of Ca and Mg in the cestodes treated with the methanol fraction of *S. virosa* were reduced to 9.2 ± 0.47 and 4.4 ± 0.3, and the concentration of Ca rose from 18.5 ± 0.01 in untested plant media to 36.3 ± 0.87 in the effluent. Acetone fraction too caused reduction in the levels of Ca and Mg in the parasite tissue and the concentration of Ca in the treated media was lower than that of the untreated plant media.

PZQ brought about a decrease in the level of Ca and Mg in the parasite tissue (10.1 ± 0.8 and 5.7 ± 0.37), and the effluent showed Ca and Mg levels of 11.4 ± 0.61 and 5.8 ± 0.46, respectively, as compared to their levels of 10.7 ± 0.02 and 2.6 ± 0.01 in the untested PZQ media.

Table 1: ***In vitro*** **efficacy of ethanol extract of** ***A. oxyphylla*** **stem bark, its fractions and reference drug praziquantel on** ***R. echinobothrida*** **(Number of worms in each test medium, n=6)**

Control/treated cestode with	*Concentration (mg /ml)*	*Paralysis (h)*	*Death (h)*
Control	–	–	72.0 ± 0.06
Ethanol extract	5	4.93 ± 0.12	7.70 ± 0.07
	10	3.50 ± 0.12	5.53 ± 0.1
	25	1.93 ± 0.27	3.60 ± 0.26
	50	0.6 ± 0.31	2.40 ± 0.2
Acetone fraction	5	2.25 ± 0.08	4.19 ± 0.09
	10	1.70 ± 0.25	2.71 ± 0.12
	25	1.30 ± 0.13	1.87 ± 0.12
	50	0.39 ± 0.23	1.12 ± 0.08
Methanol fraction	5	6.12 ± 0.07	8.06 ± 0.06
	10	3.92 ± 0.09	6.03 ± 0.06
	25	2.93 ± 0.03	4.59 ± 0.02
	50	2.04 ± 0.25	3.70 ± 0.3
PZQ	0.001	3.0 ± 0.14	9.8 ± 0.21
	0.01	0.5 ± 0.01	7.3 ± 0.15

Values given as mean ± SE from 5 replicate assays

$p < 0.05$ *vs*. control value. student's t- test

Graph 1: ***In vitro*** **efficacy of ethanol extract of** ***A. oxyphylla*** **stem bark, its fractions and reference drug praziquantel on** ***R. echinobothrida*** **(Number of worms in each test medium, n=6)**

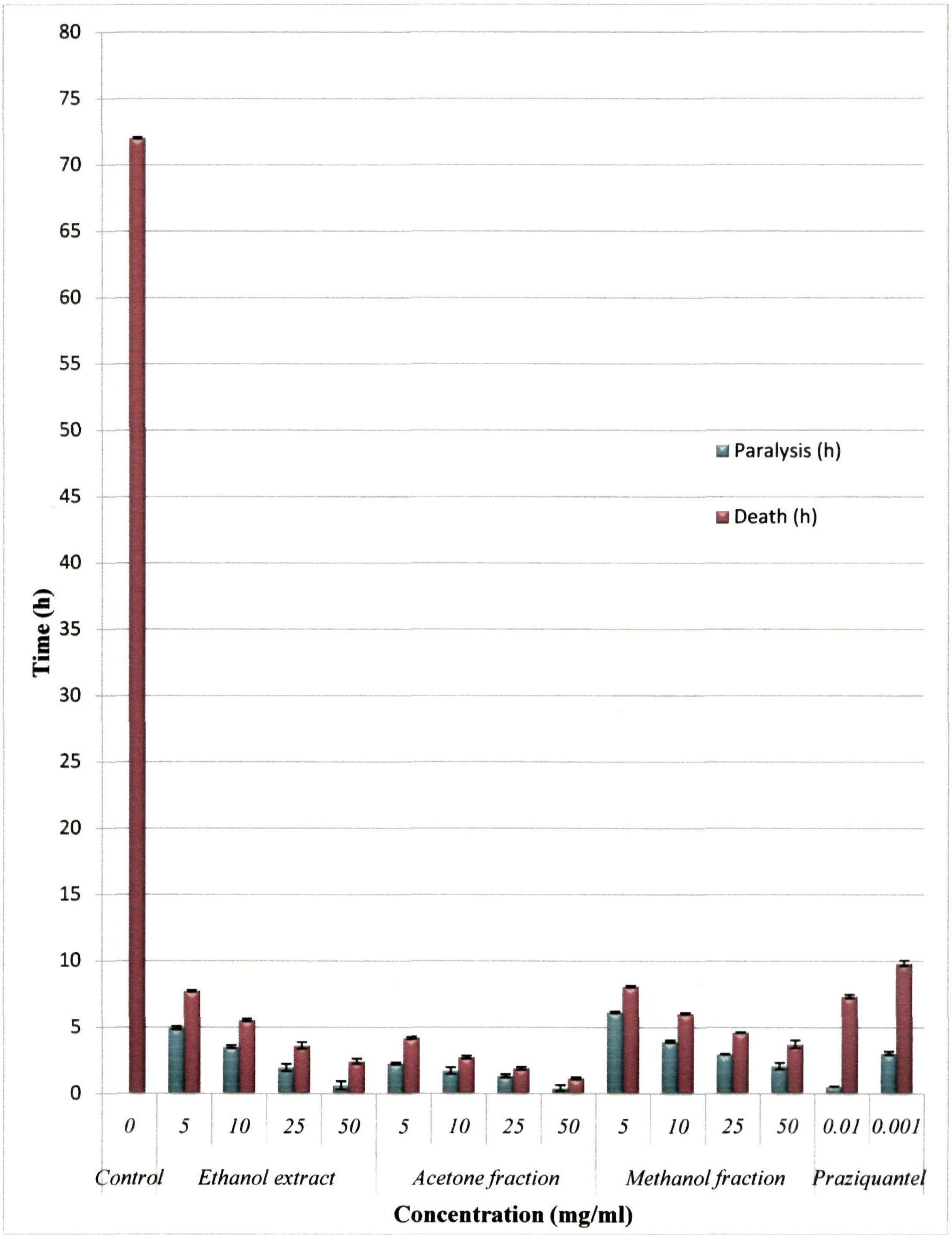


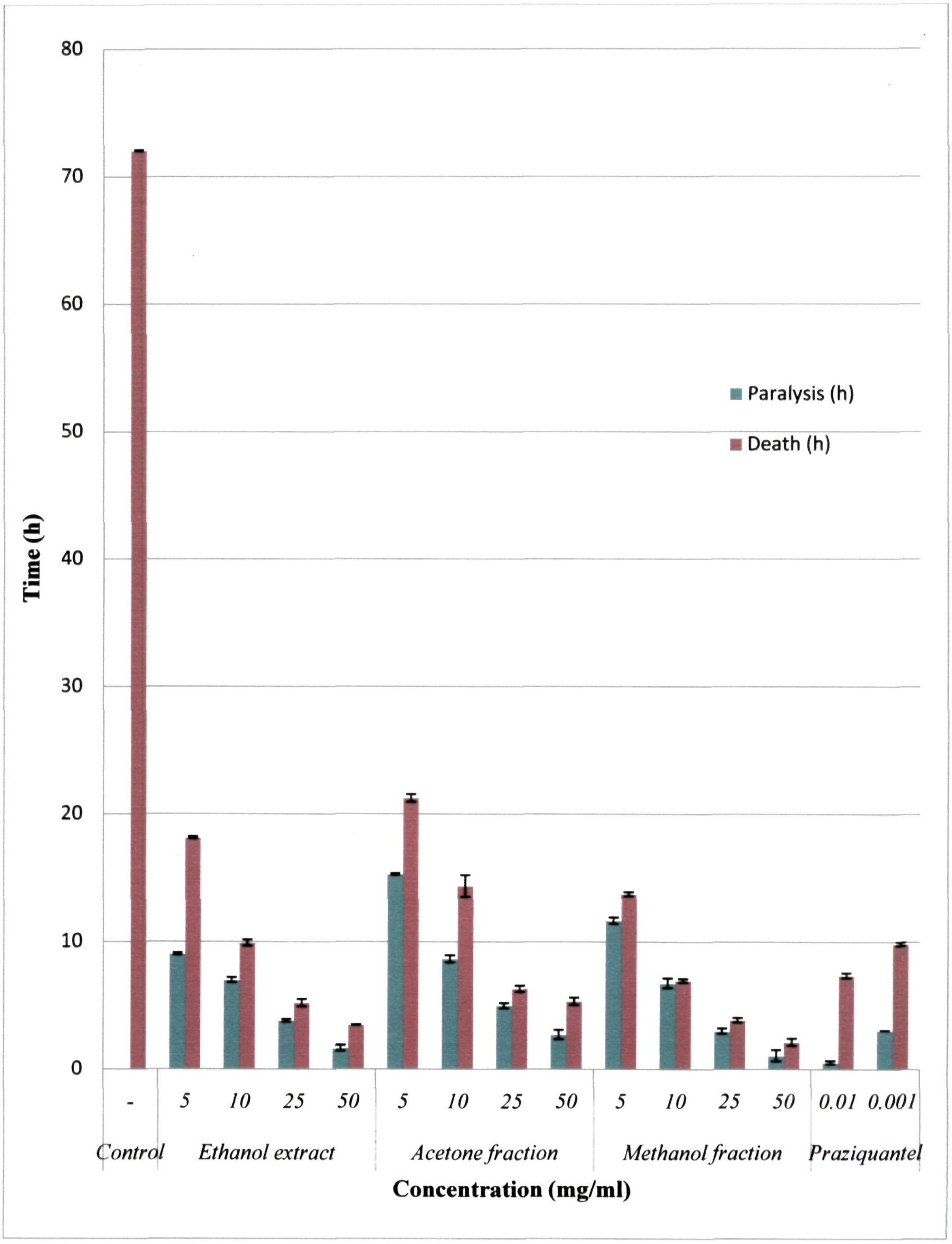
Table 2: ***In vitro*** **efficacy of ethanol extract of *S. virosa* leaf, its fractions and reference drug praziquantel on *R. echinobothrida* (Number of worms in each test medium, n=6)**

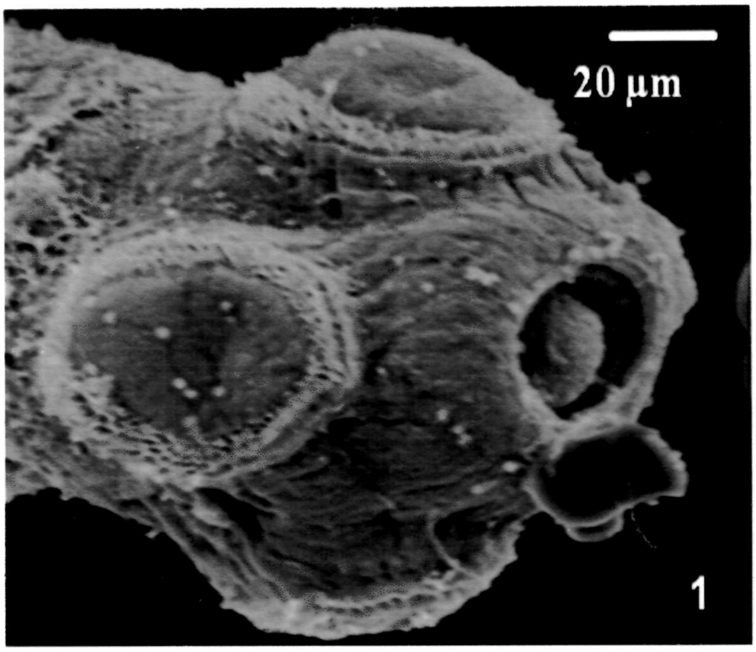
Control/ treated cestode with	*Concentration (mg /ml)*	*Paralysis (h)*	*Death (h)*
Control	-	-	72.0 ± 0.06
Ethanol extract	5	9.01 ± 0.09	18.11 ± 0.09
	10	6.95 ± 0.21	9.88 ± 0.25
	25	3.76 ± 0.11	5.15 ± 0.3
	50	1.62 ± 0.24	3.42 ± 0.01
Acetone fraction	5	15.25 ± 0.08	21.22 ± 0.31
	10	8.62 ± 0.29	14.33 ± 0.87
	25	4.94 ± 0.22	6.27 ± 0.26
	50	2.67 ± 0.37	5.27 ± 0.29
Methanol fraction	5	11.63 ± 0.25	13.70 ± 0.18
	10	6.7 ± 0.39	6.9 ± 0.16
	25	2.95 ± 0.22	3.8 ± 0.2
	50	1.05 ± 0.45	2.1 ± 0.29
PZQ	0.001	3.00 ± 0.14	9.8 ± 0.21
	0.01	0.50 ± 0.01	7.3 ± 0.15

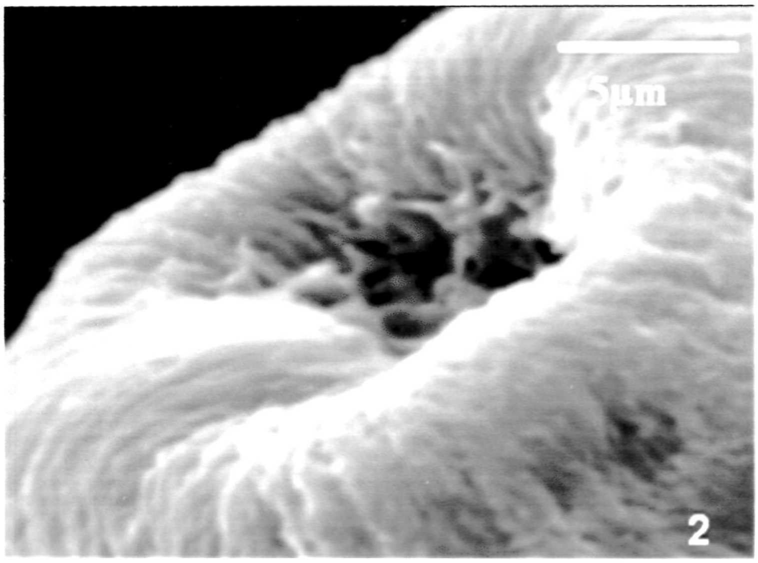
Values given as mean ± SE from 5 replicate assays

$p < 0.05$ *vs*. control value, student's t- test

Graph 2: ***In vitro* efficacy of ethanol extract of *S. virosa* leaf, its fractions and reference drug praziquantel on *R. echinobothrida* (Number of worms in each test medium, n=6)**



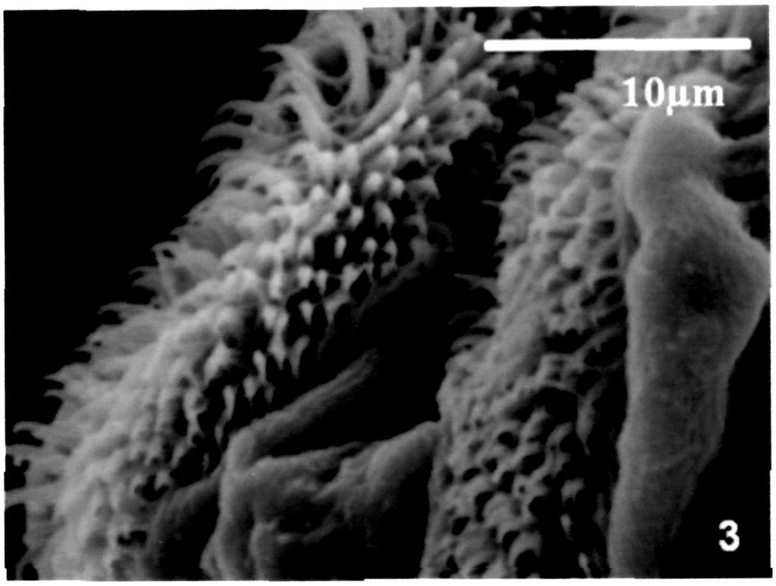


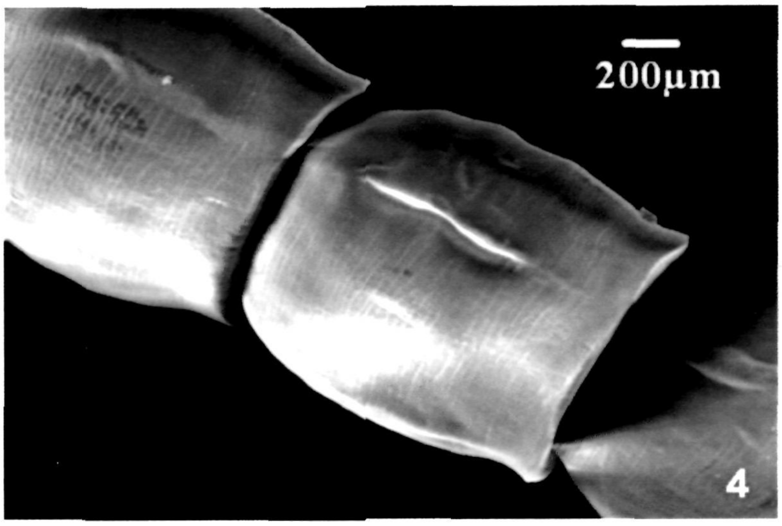


Figs. 1, 2: Scanning electron micrographs of control *R. echinobothrida*

Fig. 1. *R. echinobothrida* scolex with a retractable rostellum, and four suckers.

Fig. 2. Retractable rostellum with rows of spines inside.

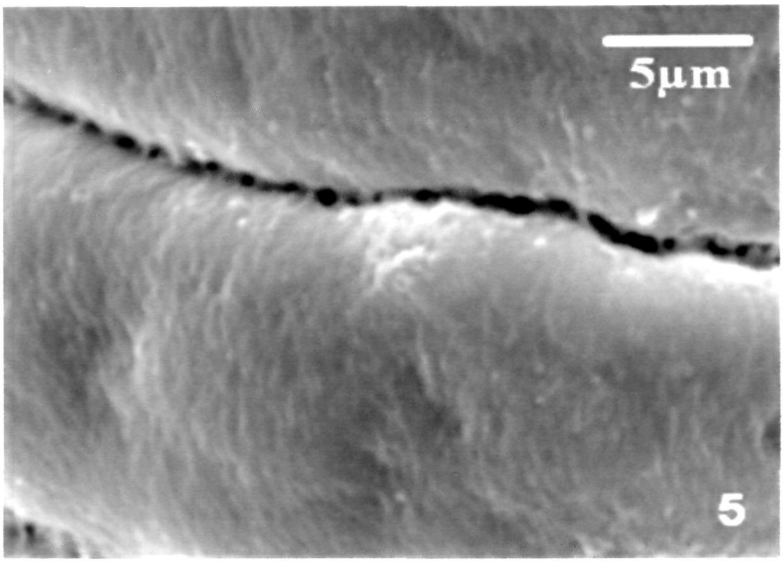


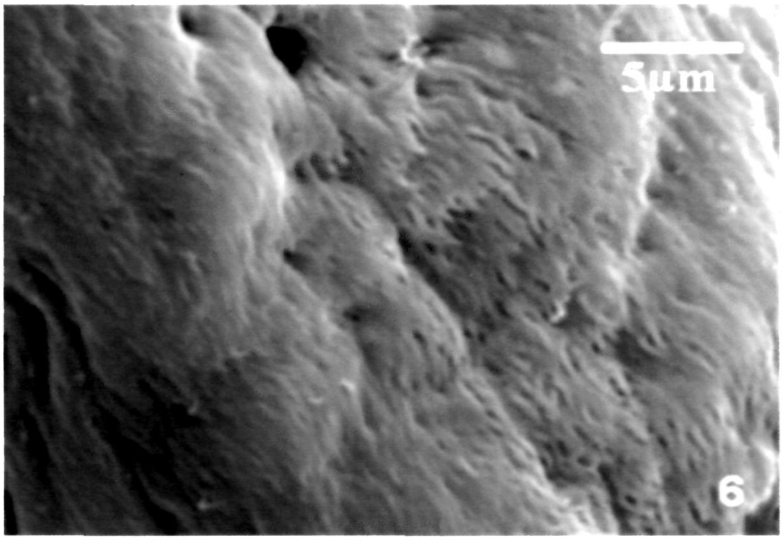


Figs. 3, 4: Scanning electron micrographs of control *R. echinobothrida*

Fig. 3. *R. echinobothrida* sucker showing circlets of hooks that are broad at the base and tapering and bent towards the ends.

Fig. 4. Tapeworm segments or proglottides which are longer than wide and covered with striated tegument.

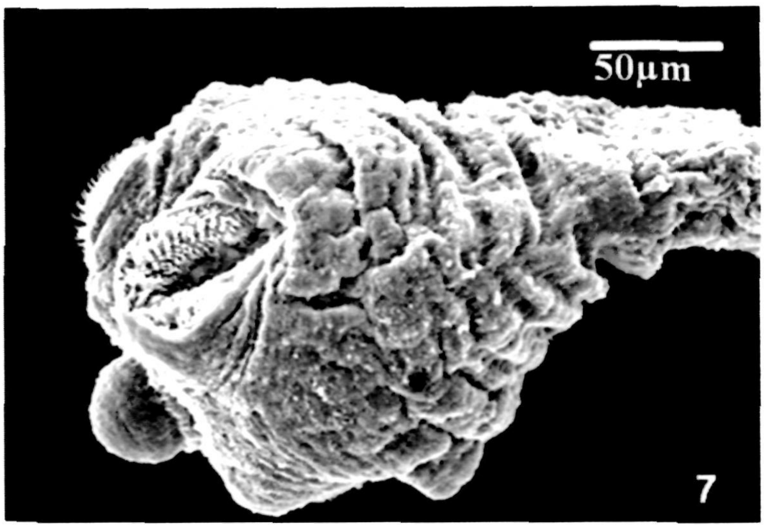


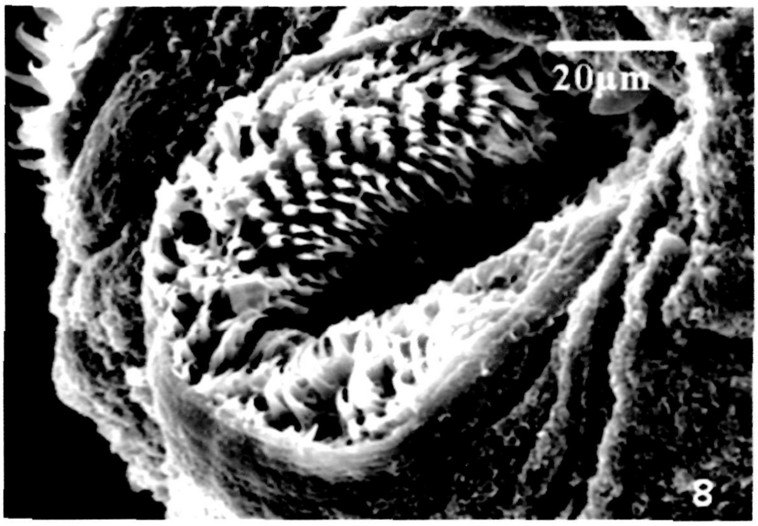


Figs. 5, 6: Scanning electron micrographs of control *R. echinobothrida*

Fig. 5. Closer view of a *R. echinobothrida* proglottid showing linear striations having a smooth tegument.

Fig. 6. A single striation from a proglottid when magnified shows layers of microtriches gently sloping downwards.

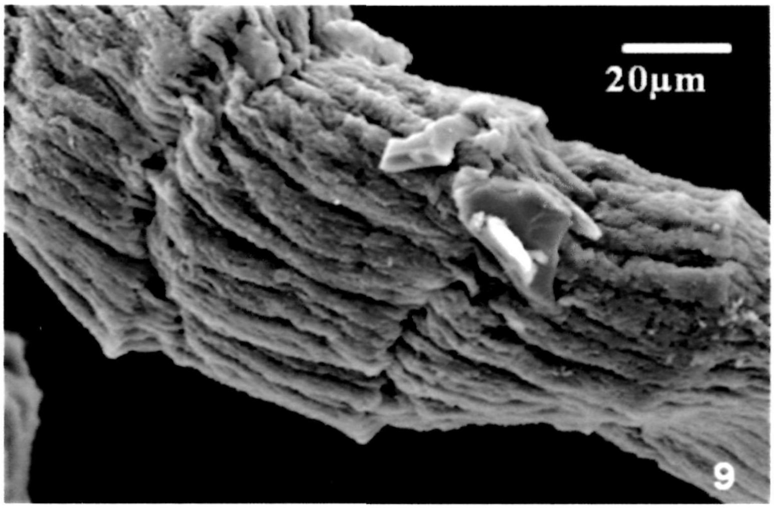


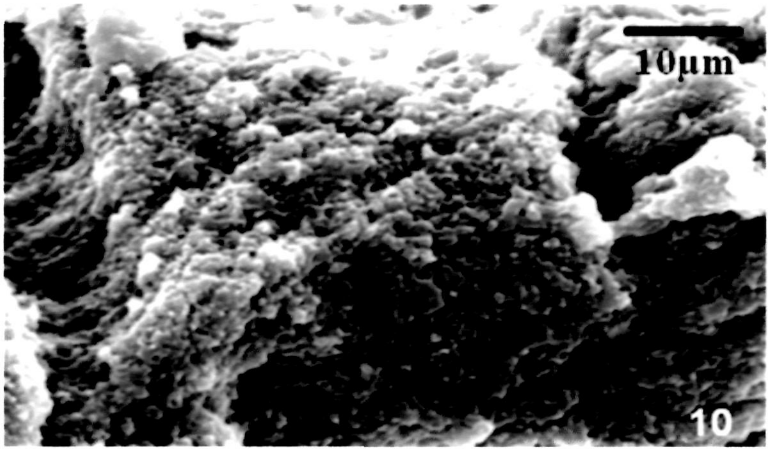


Figs. 7, 8: Scanning electron micrographs of *R. echinobothrida* treated with ethanol extract of *A. oxyphylla* (10 mg)

Fig. 7. Deformed scolex showing elevated rostellum accompanied by cracks at the base of the suckers.

Fig. 8. A closer view of the suckers showing typical hooks with abnormal folding and shrinkage.

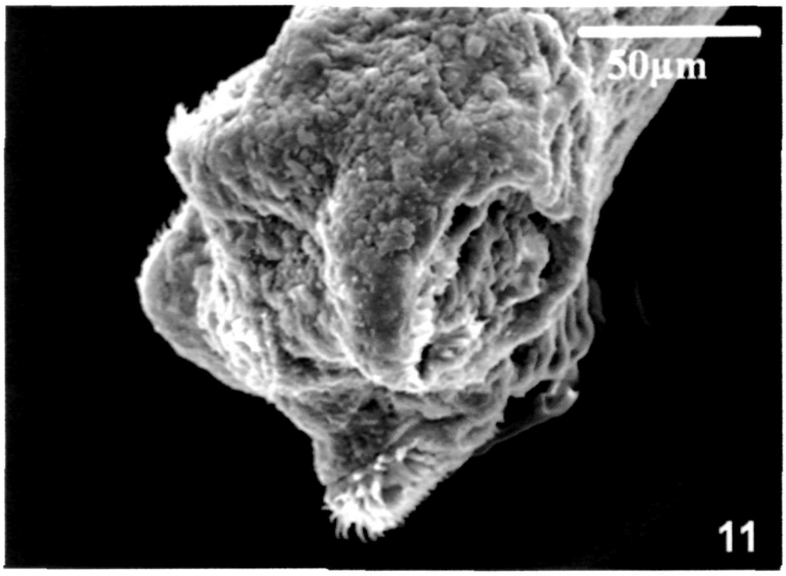


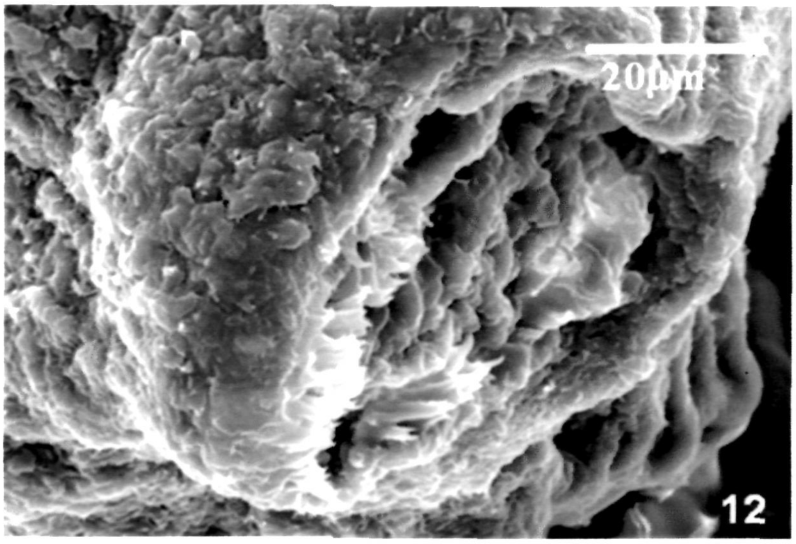


Figs. 9, 10: Scanning electron micrographs of *R. echinobothrida* treated with ethanol extract of *A. oxyphylla* (10 mg)

Fig. 9. Body shrinkage with little differentiation between adjacent proglottides.

Fig. 10. Minor infoldings of tegument with pit formations and no microtriches.

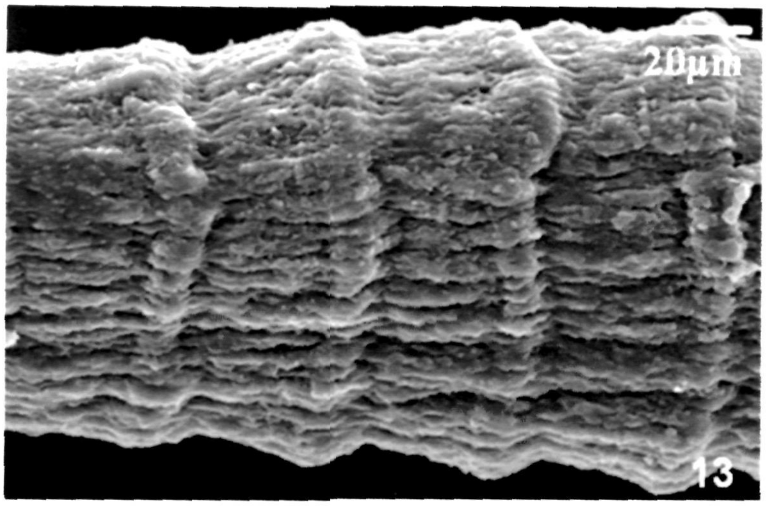


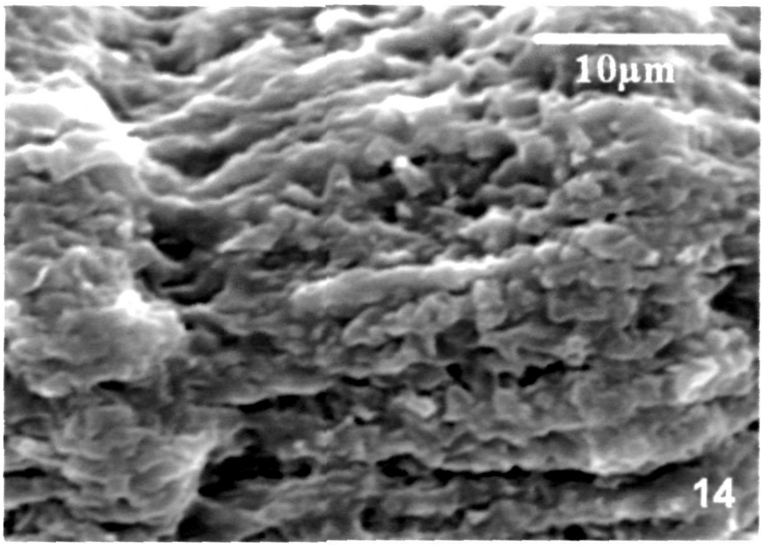


Figs. 11, 12: Scanning electron micrographs of *R. echinobothrida* treated with methanol fraction of *A. oxyphylla* crude extract (10 mg)

Fig. 11. Deformed scolex with shrunken suckers.

Fig. 12. Magnified view of a sucker showing sloughing off of hooks.

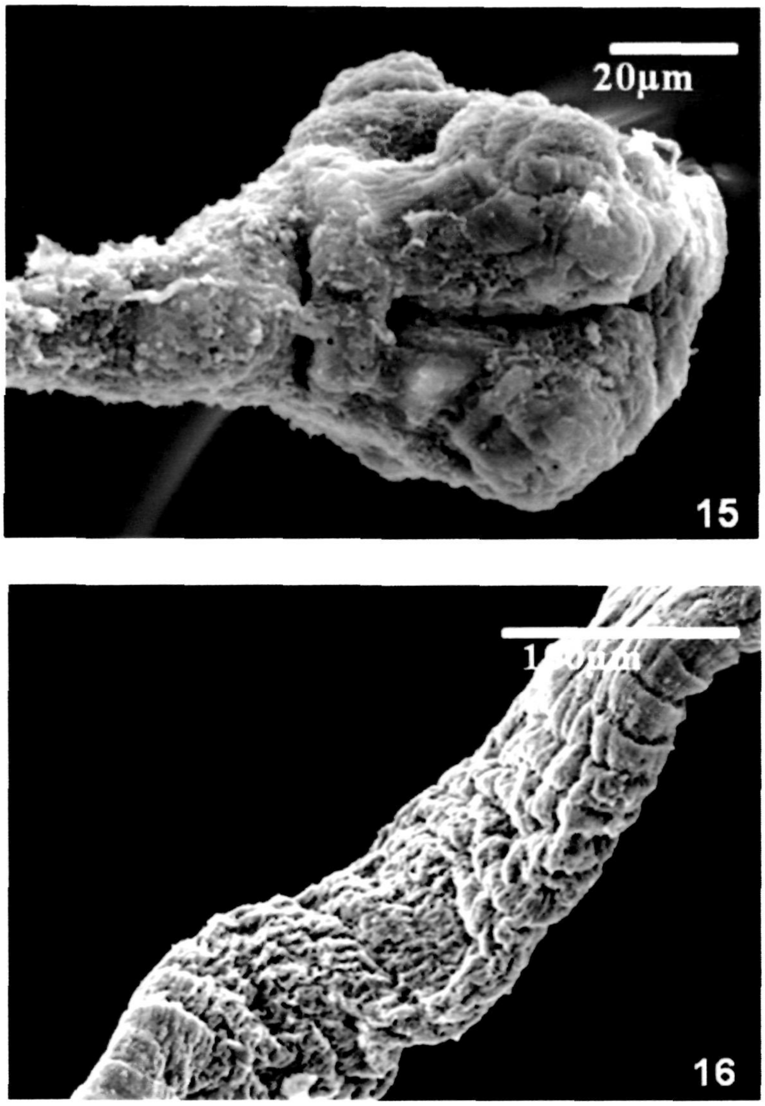




Figs. 13, 14: Scanning electron micrographs of *R. echinobothrida* treated with methanol fraction of *A. oxyphylla* crude extract (10 mg)

Fig. 13. Deformed proglottides having lack of demarcation between subsequent segments.

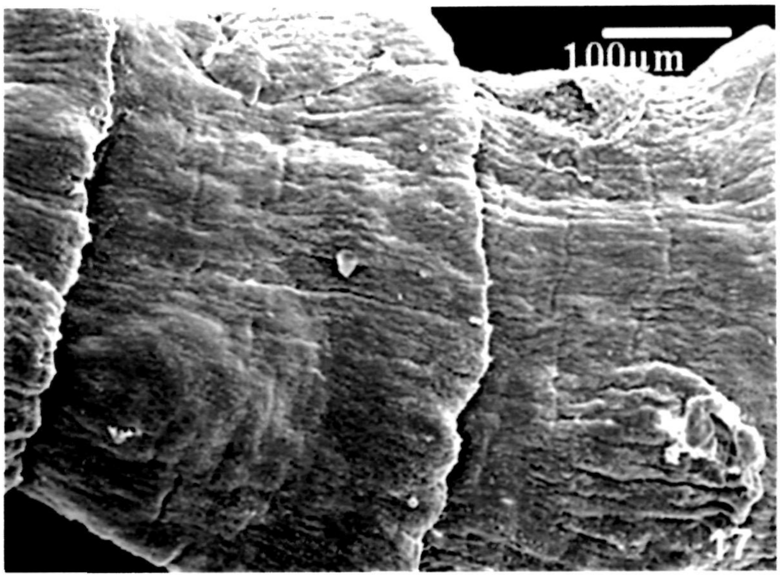
Fig. 14. Enlarged view of a proglottid showing shrinkage and pit formation.

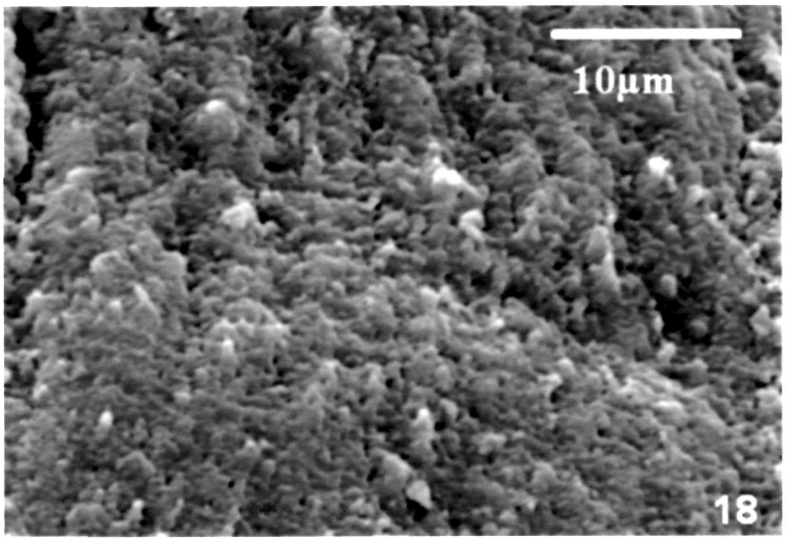


Figs. 15, 16: Scanning electron micrographs of *R. echinobothrida* treated with acetone fraction of *A. oxyphylla* crude extract (10 mg)

Fig. 15. Twisted and strained scolex with no discernible sucker.

Fig. 16. Highly contorted parasite body with major tegumental erosion.

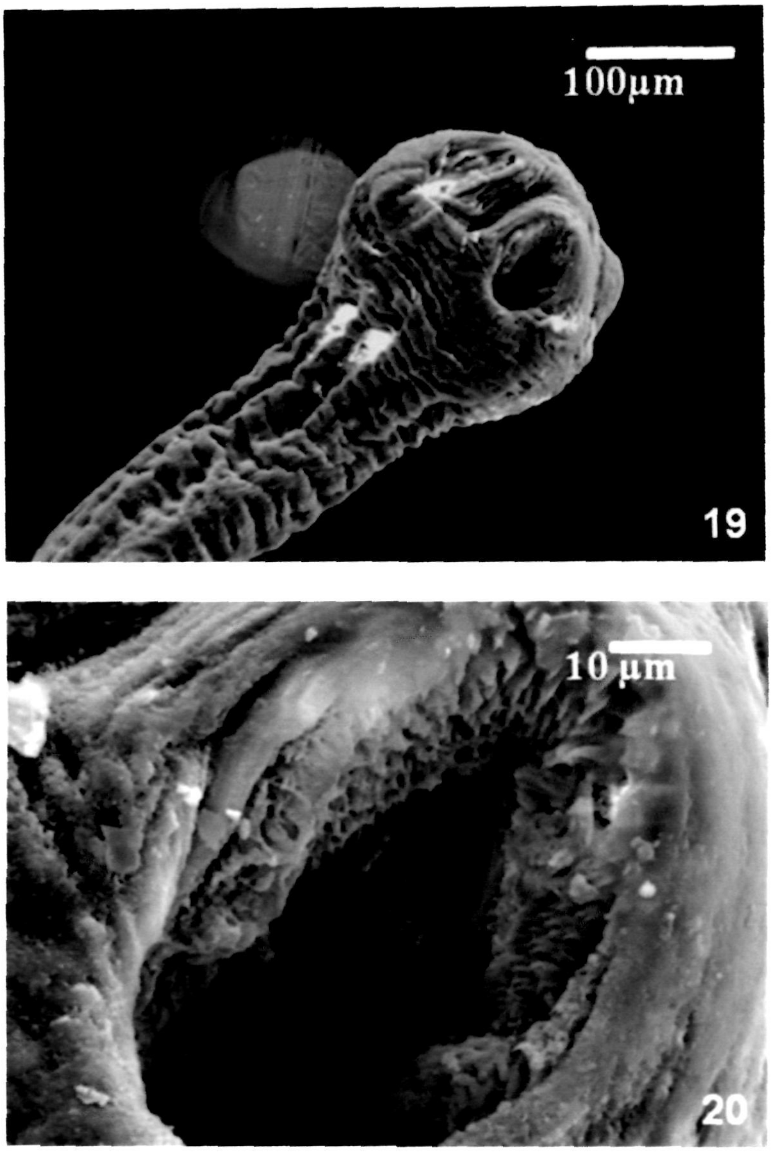




Figs. 17, 18: Scanning electron micrographs of *R. echinobothrida* treated with acetone fraction of *A. oxyphylla* crude extract (10 mg)

Fig. 17. Scarring, swelling and focal peeling of tegumental tissue.

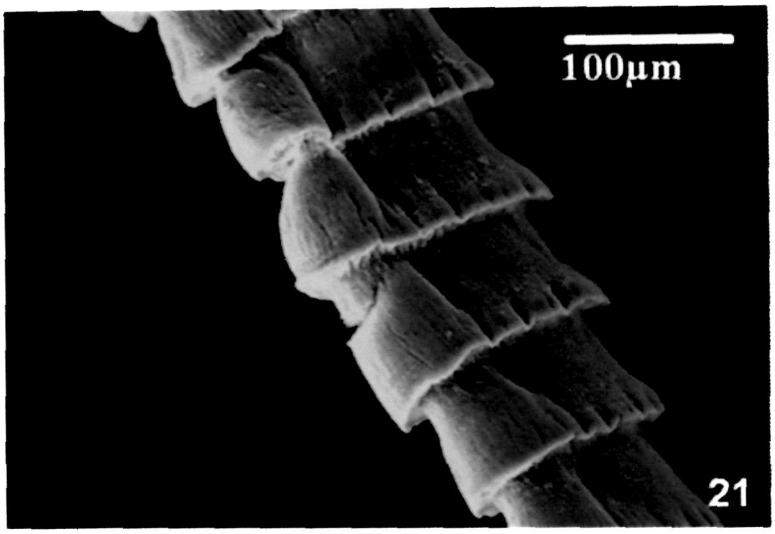
Fig. 18. Enlarged view of a proglottid showing deformed and clumped microtriches.

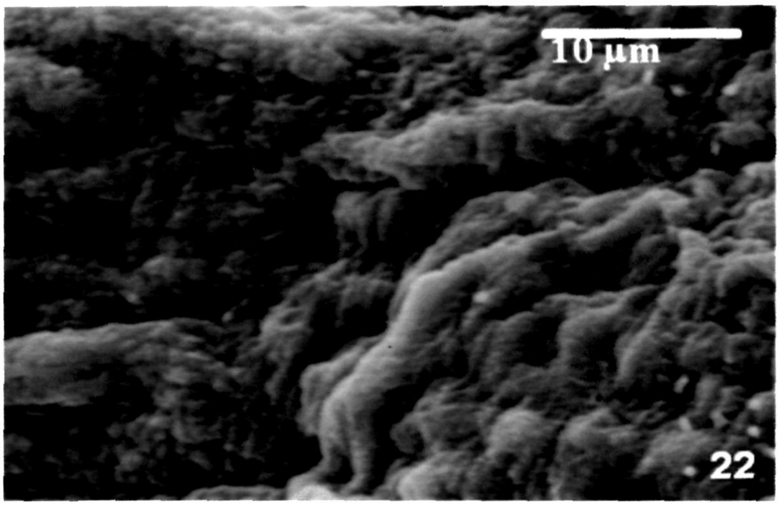


Figs. 19, 20: Scanning electron micrographs of *R. echinobothrida* treated with ethanol extract of *S. virosa* (25 mg)

Fig. 19. Contorted cestode body with shriveled scolex.

Fig. 20. Shrunken sucker with a porous appearance. A slight loss of hooks is also seen.

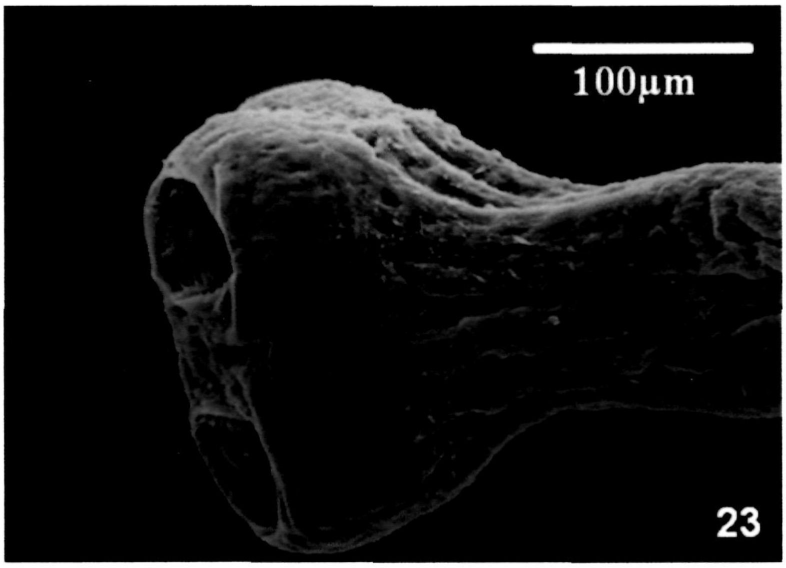


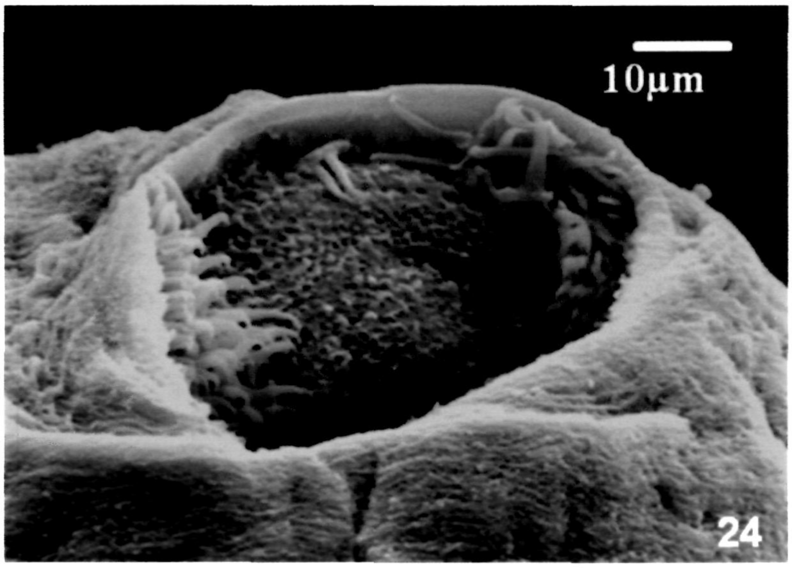


Figs. 21, 22: Scanning electron micrographs of *R. echinobothrida* treated with ethanol extract of *S. virosa* (25 mg)

Fig. 21. Wrinkled body with some proglottides breaking off at places.

Fig. 22. Infolding of the parasite surface leading to the disappearance of tegumental smoothness.

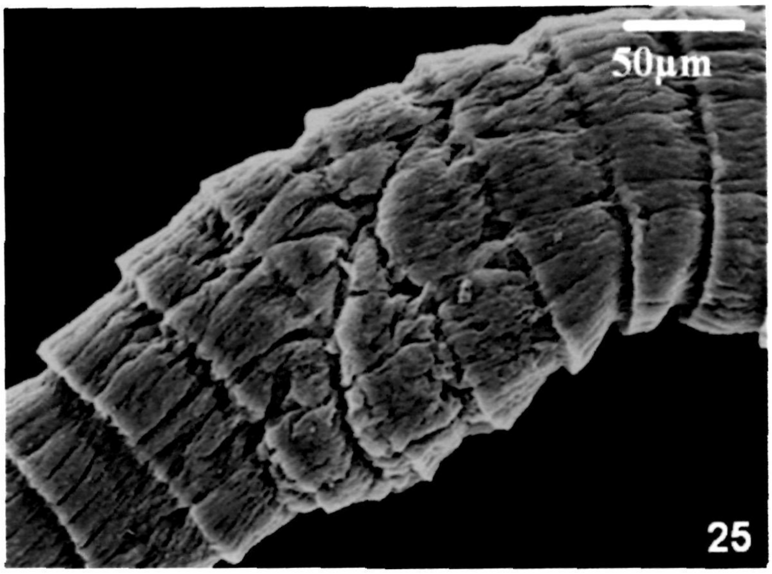


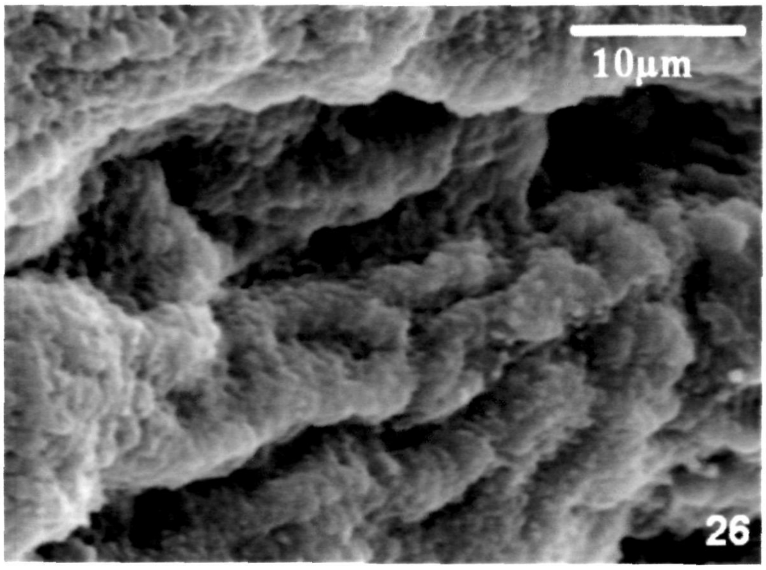


Figs. 23, 24: Scanning electron micrographs of *R. echinobothrida* treated with methanol fraction of *S. virosa* crude extract (25 mg)

Fig. 23. Contorted cestode body with shriveled scolex.

Fig. 24. Sunken sucker with a porous appearance. Many hooks can be seen to be breaking off. Cracks appeared around the sucker.

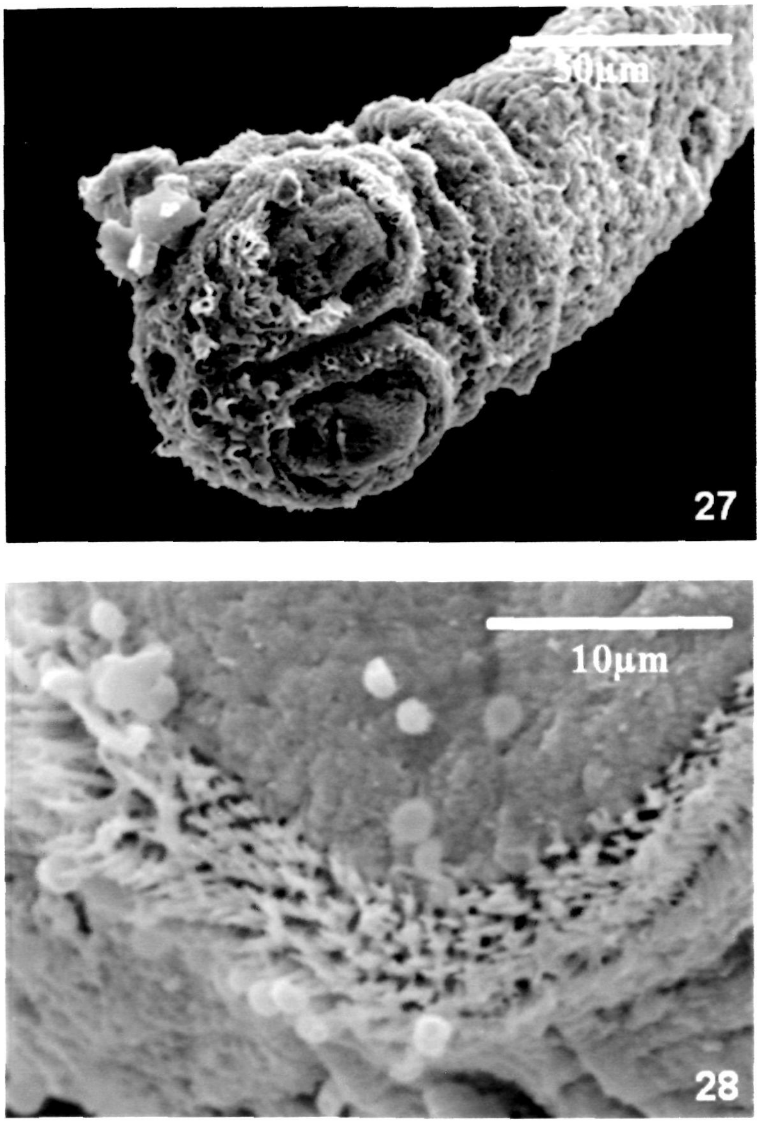




Figs. 25, 26: Scanning electron micrographs of *R. echinobothrida* treated with methanol fraction of *S. virosa* crude extract (25 mg)

Fig. 25. Widespread lesions can be seen at points where few proglottides have swollen in contrast to the general shrinking noticed.

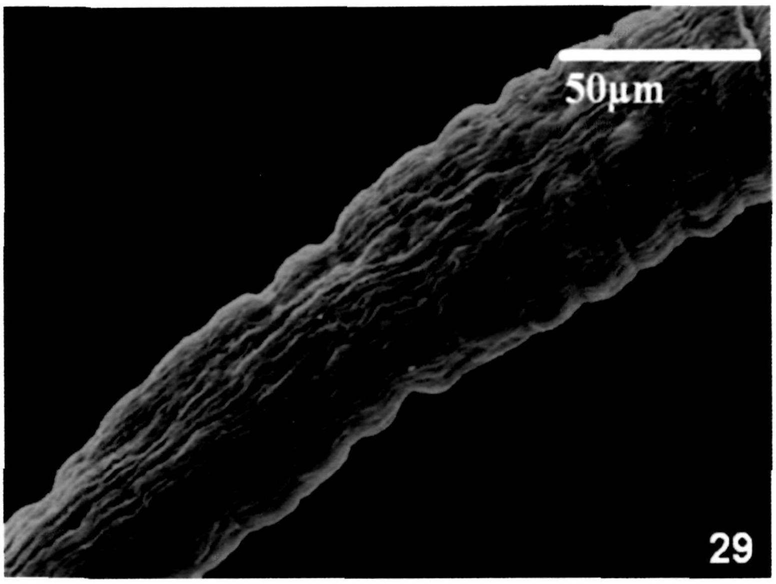
Fig. 26. *Deep infoldings of the tegument with severe clumping of microtriches.*

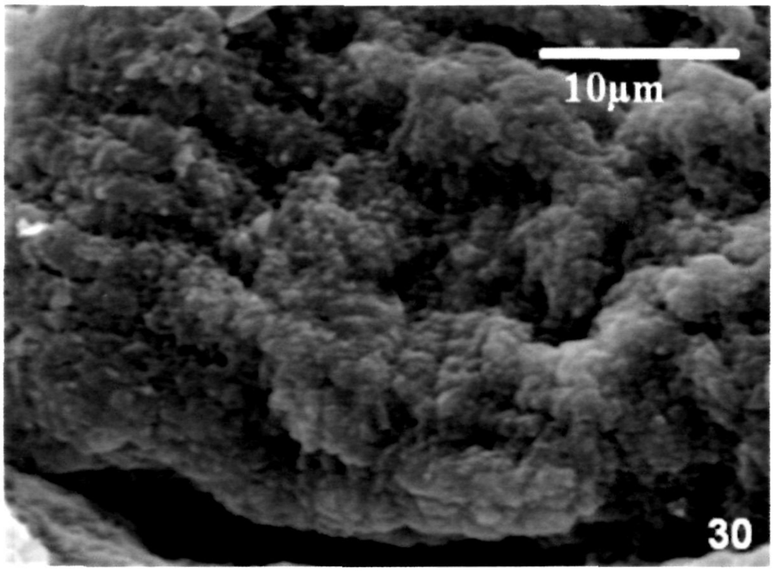


Figs. 27, 28: Scanning electron micrographs of *R. echinobothrida* treated with acetone fraction of *S. virosa* crude extract (25 mg)

Fig. 27. Deformation of general tegument and scolex.

Fig. 28. Sucker rim with clumped hooks.

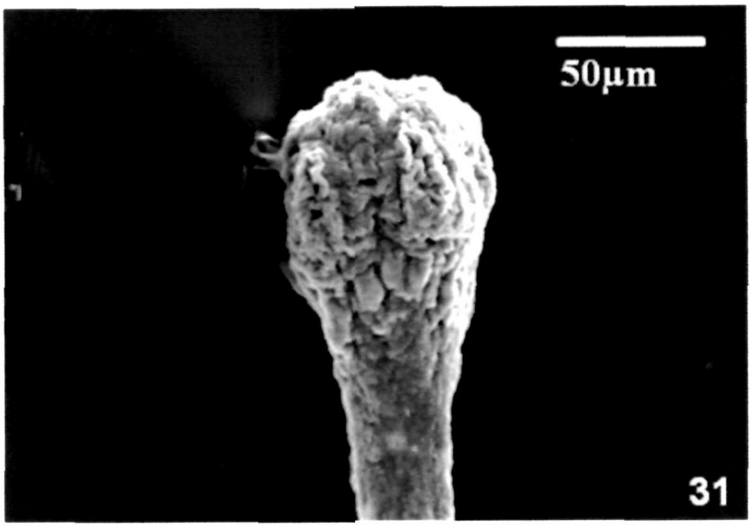


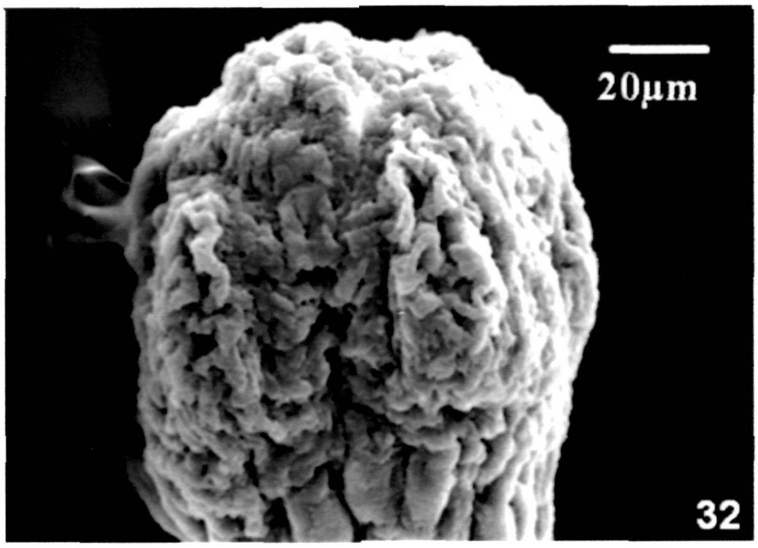


Figs. 29, 30: Scanning electron micrographs of *R. echinobothrida* treated with acetone fraction of *S. virosa* crude extract (25 mg)

Fig. 29. Shrunk and deformed neck region.

Fig. 30. Enlarged view of clumped microtriches.

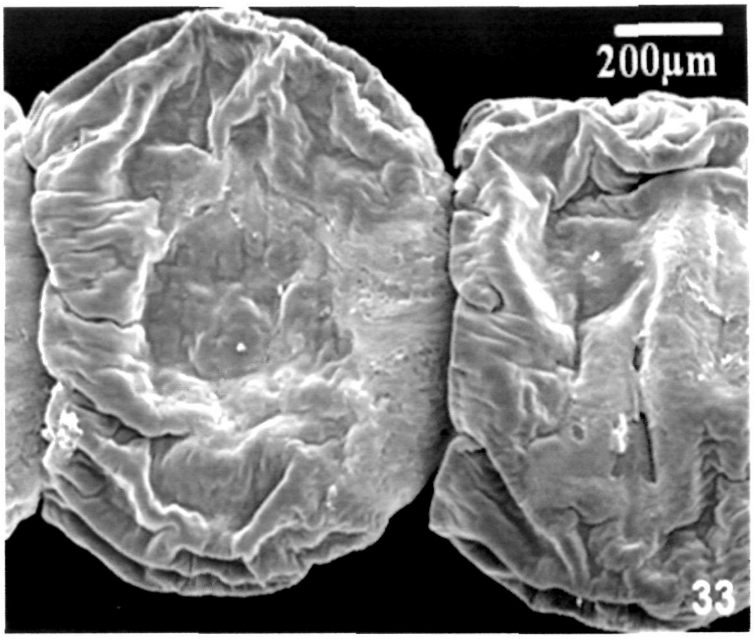


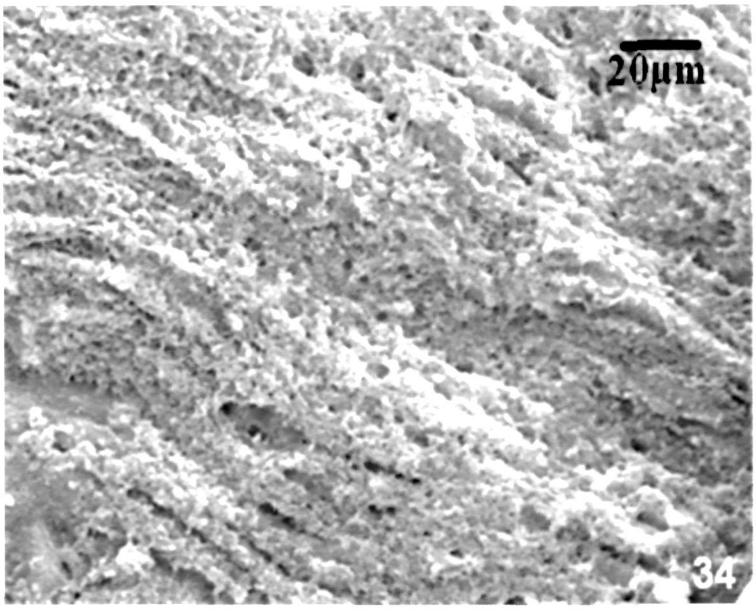


Figs. 31, 32: Scanning electron micrographs of *R. echinobothrida* treated with praziquantel (0.001 mg)

Fig. 31. Shriveled scolex where the suckers are just slightly discernible.

Fig. 32. A magnified view of the scolex showing the suckers.

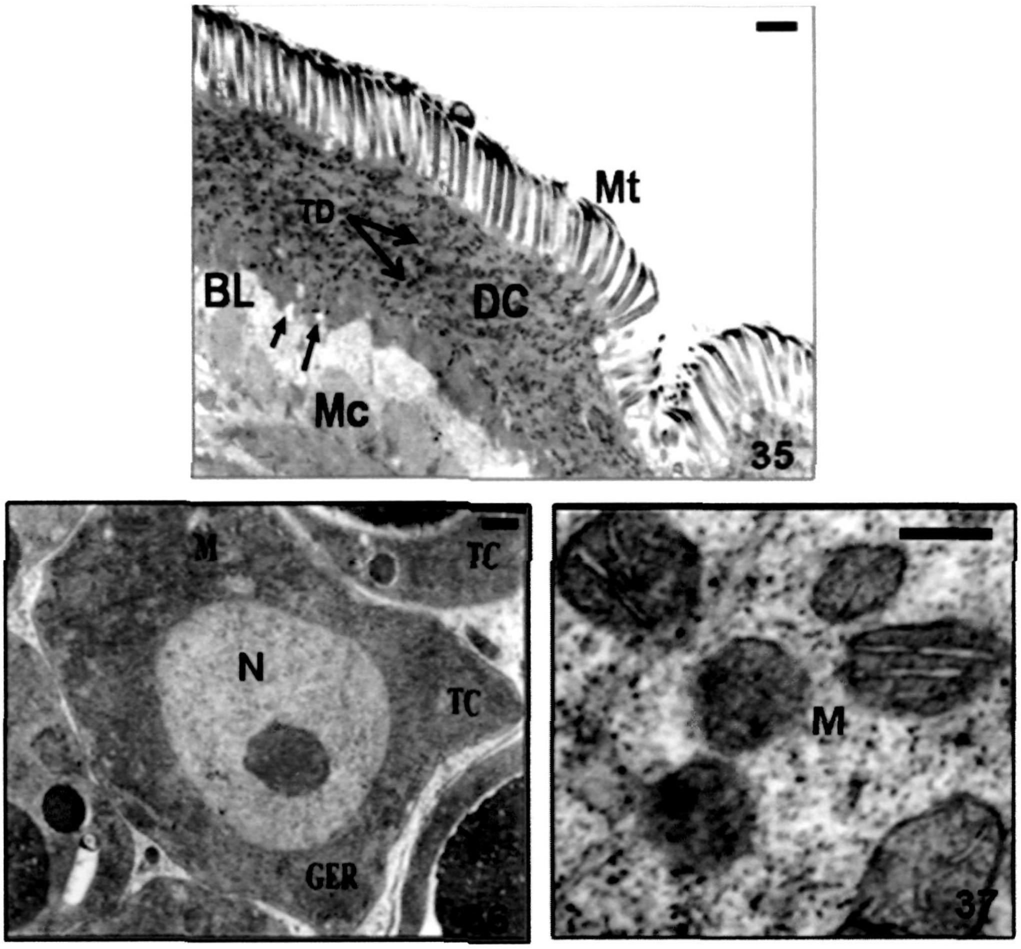




Figs. 33, 34: Scanning electron micrographs of *R. echinobothrida* treated with praziquantel (0.001 mg)

Fig. 33. Slight wrinkles were present on the general body surface of the parasite.

Fig. 34. On magnification, the wrinkled surface showed pit formation with the microtriches not that prominent.



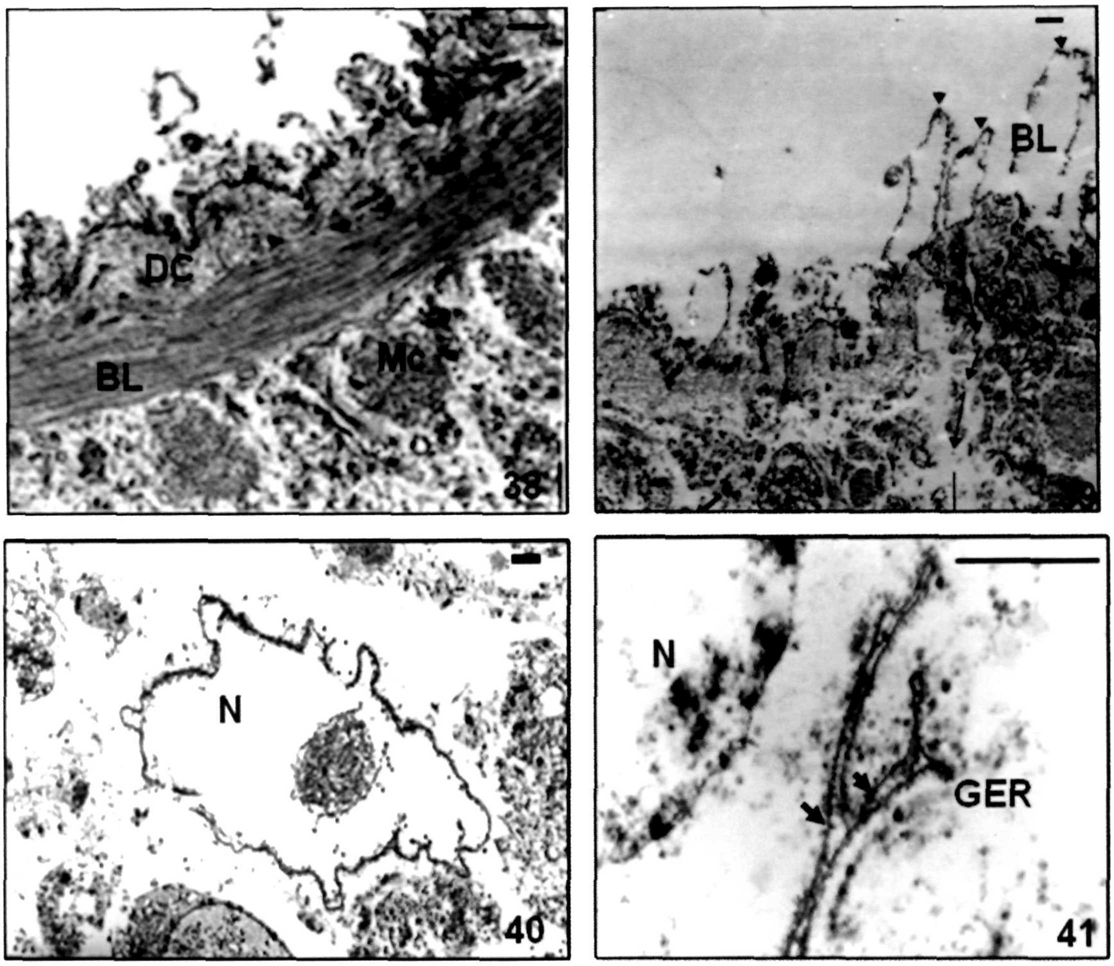
Figs. 35-37: Transmission electron micrographs of control *R. echinobothrida*

Fig. 35. Control tegument with intact microthrix layer (Mt); distal cytoplasm (DC) electron-dense with tegumental discs (TD); non-disrupted basal lamina (BL) with basal infolds (arrows) and well organized subtegumental muscle blocks (Mc).

Fig. 36. Tegumental cytons (TC) retaining normal connections with each other and having abundant granular endoplasmic reticulum (GER) and mitochondria (M) and other cell inclusions; nucleus (N) with no clumping of chromatin.

Fig. 37. Electron-dense mitochondrial matrix with prominent cristae. The mitochondria (M) here are seen to lie in granular parenchyma suggesting the presence of abundant glycogen.

All bars 0.5 µm



Figs. 38-41: Transmission electron micrographs of *R. echinobothrida* treated with ethanol extract of *A. oxyphylla* (10 mg)

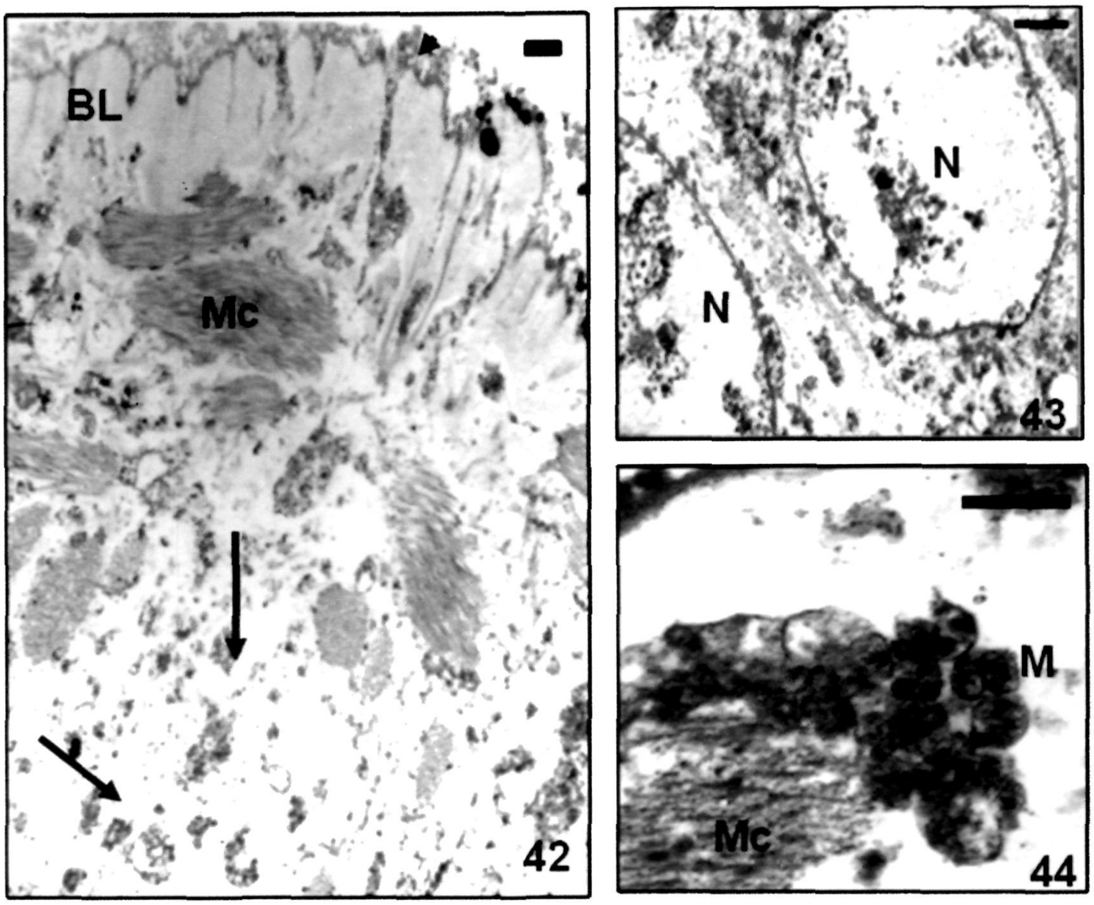
Fig. 38. Tegument sloughed leaving remnants of distal cytoplasm.

Fig. 39. Disrupted basal lamina with swollen basal infolds. Arrows show the streaming of glycogen loss due to release of materials to the exterior.

Fig. 40. Electron-lucent nuclei due to release of nuclear material to the surrounding parenchyma. The fibrous nature of the nucleolus is to be noted.

Fig. 41. Subtegumental cyton with dilated GER cisternae (arrows).

All bars 0.5 μm



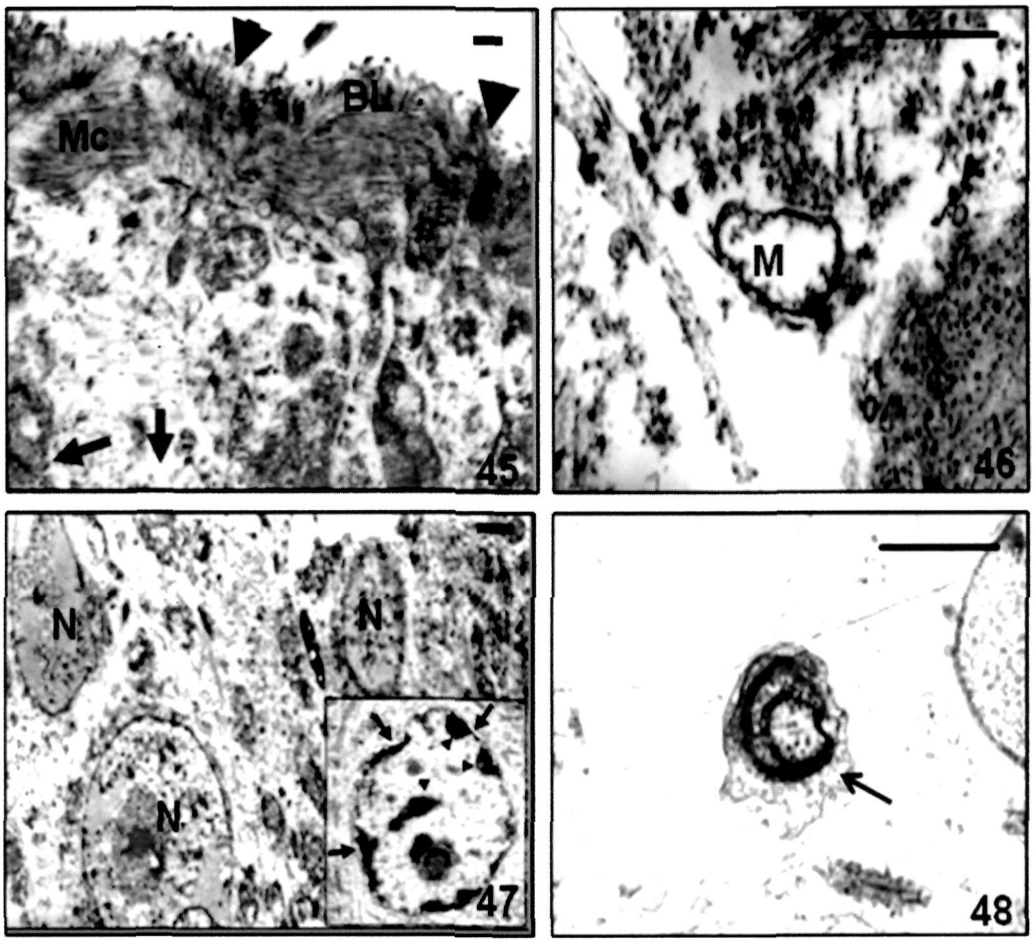
Figs. 42-44: Transmission electron micrographs of *R. echinobothrida* treated with methanol fraction of *A. oxyphylla* crude extract (10 mg)

Fig. 42. Sloughed off tegument with increased electron lucency in the subtegument.

Fig. 43. Well connected cytons but with swollen nuclei.

Fig. 44. Abundant mitochondria interspersed between muscle cells.

All bars 0.5 µm



Figs. 45-48: Transmission electron micrographs of *R. echinobothrida* treated with acetone fraction of *A. oxyphylla* crude extract (10 mg)

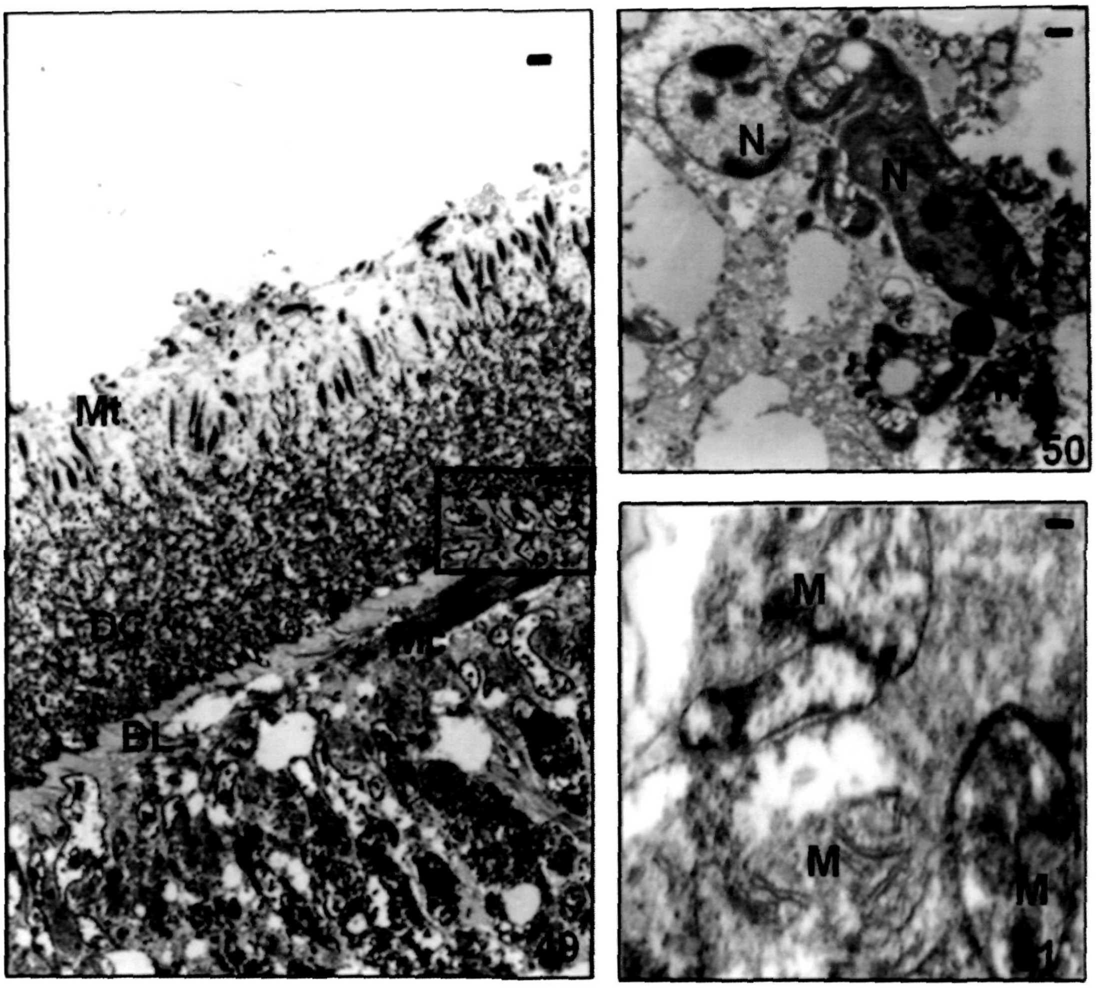
Fig. 45. Stripped-up tegument (arrowheads) of parasite treated with acetone fraction showing intense degradation of longitudinal and circular muscle layers; breaking up of tegumental cells (arrows).

Fig. 46. Mitochondria devoid of cristae.

Fig. 47. Tegumental cells showing distorted nuclei lying in an electron-lucent back-ground. [Inset: Clumped chromatin of nucleus (arrowheads); swellings of the perinuclear space (arrows)].

Fig. 48. Membranous whorls seen throughout the parenchyma of the parasite (arrow).

All bars 0.5 μm



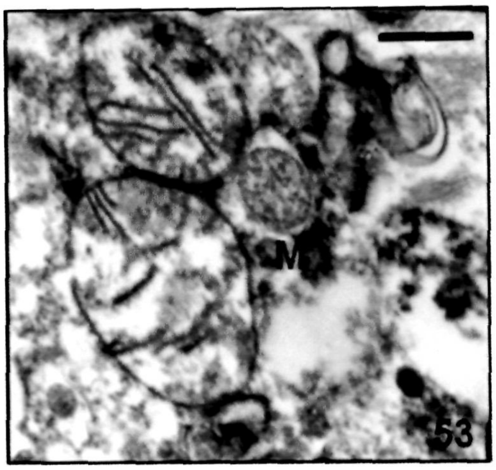
Figs. 49-51: Transmission electron micrographs of *R. echinobothrida* treated with ethanol extract of *S. virosa* (25 mg)

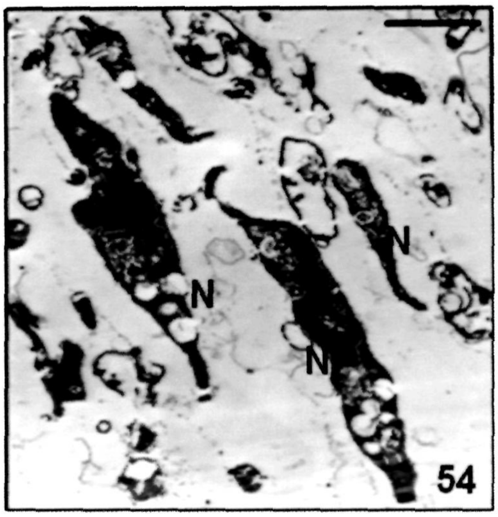
Fig. 49. Erosion of microtriches, perforation of distal cytoplasm, vacuolated basal lamina and subtegumental region. [Inset: streaming of cytoplasm into basal lamina].

Fig. 50. Subtegumental cytons with distorted nuclei.

Fig. 51. Ruptured mitochondria and cristae released into the cytosol.

All bars 0.5 μm





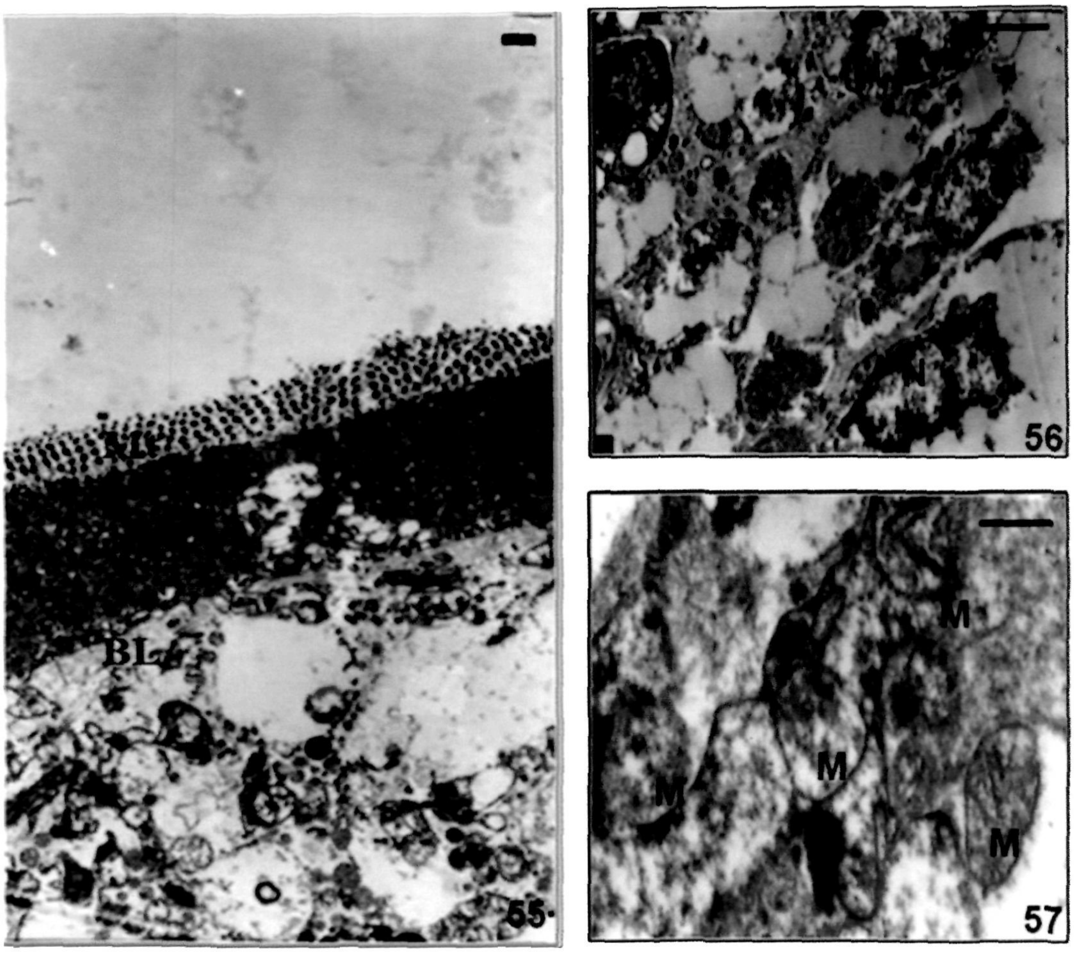
Figs. 52-54: Transmission electron micrographs of *R. echinobothrida* treated with methanol fraction of *S. virosa* crude extract (25 mg)

Fig. 52. Exposed basal lamina of the parasite; subtegumental muscle layers and cytons.

Fig. 53. Broken and loose cristae inside ruptured mitochondria.

Fig. 54. Subtegumental cytons with compressed nuclei; no cytoplasm can be seen around the respective nuclei.

All bars 0.5 μm



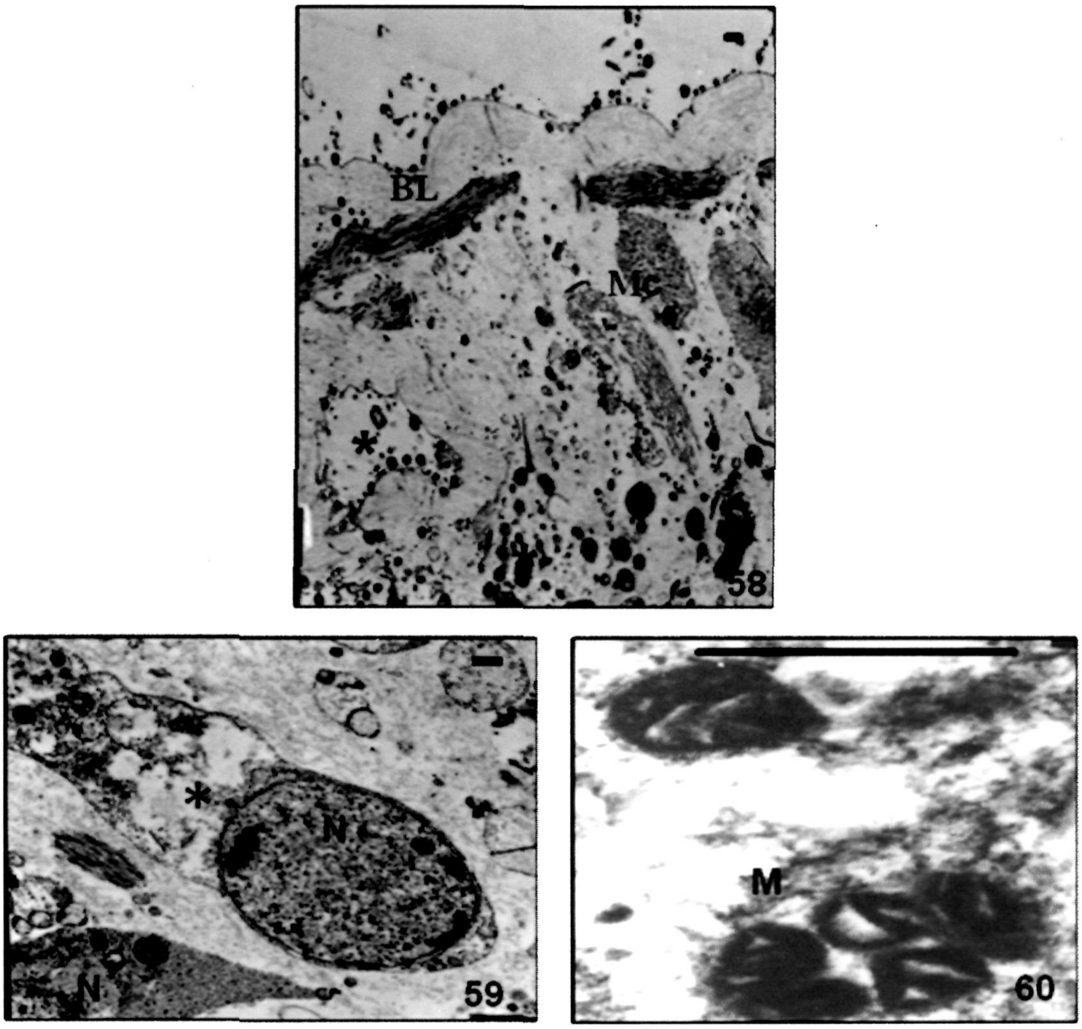
Figs. 55-57: Transmission electron micrographs of *R. echinobothrida* treated with acetone fraction of *S. virosa* crude extract (25 mg)

Fig. 55. Intact microthrix layer with slight vacuolisation in the distal cytoplasm, disrupted basal lamina, no discernible muscle bundles and vacuolated subtegument.

Fig. 56. Deformed nuclei of subtegumental cytons.

Fig. 57. Slight distortion in mitochondria with lack of cristae in some.

All bars 0.5 µm



Figs. 58-60: Transmission electron micrographs of *R. echinobothrida* treated with praziquantel (0.001 mg)

Fig. 58. Stripped tegument with exposed basal lamina, slightly disorganised muscle layers, and autophagosomes (*).

Fig. 59. Vacuolated cyton with intact nucleus.

Fig. 60. Abundant mitochondria sighted throughout the parasite tissue.

All bars 0.5 μm

Table 3: Histochemical localization of ATPase, AlkPase and AcPase in various structures of *Raillietina echinobothrida* treated with ethanol extracts of *A. oxyphylla* and *S. virosa*, and reference drug praziquantel

Control/Treated cestode with	*ATPase*				*AlkPase*				*AcPase*			
	T	**ST**	**SM**	**P**	**T**	**ST**	**SM**	**P**	**T**	**ST**	**SM**	**P**
Control	++++	++++	+++	+++	++++	+++	+++	++	++++	++	++	++
A. oxyphylla **(10 mg/ml)**	+	+	+	-	NP	+	-	-	-	-	+	-
S. virosa **(25 mg/ml)**	++	++	++	+++	+	+	+	-	+	+	+	+
PZQ (0.001 mg/ml)	NP	+	-	-	-	+	-	-	+	+	-	-

T-Tegument; ST-Subtegument; SM- Somatic musculature; P- Parenchyma; NP- Structure not present
++++ Very intense activity, +++ Intense activity, ++ Moderate activity, + Mild activity, - No activity

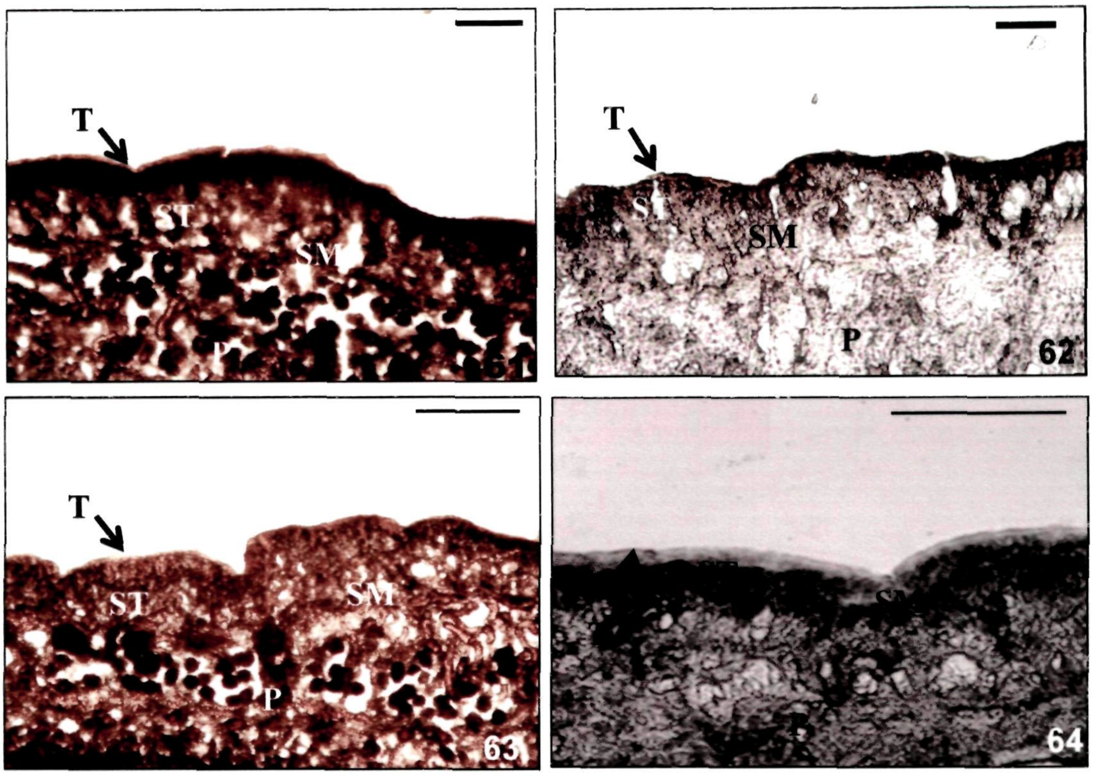


Fig. 61-64: Adenosine triphosphatase (ATPase) activity in *R. echinobothrida*, frozen sections

Fig. 61. Cross section of control worm showing very intense ATPase activity in the tegument (T), subtegument (ST), somatic musculature (SM) and parenchyma (P)

Fig. 62. Stem bark extract of *A. oxyphylla* treated section showing reduced enzyme activity

Fig. 63. Leaf extract of *S. virosa* treated section showing reduced enzyme activity in tegument and subtegument

Fig. 64. Praziquantel treated section showing only mild enzyme activity in the subtegument

All bars 50 µm

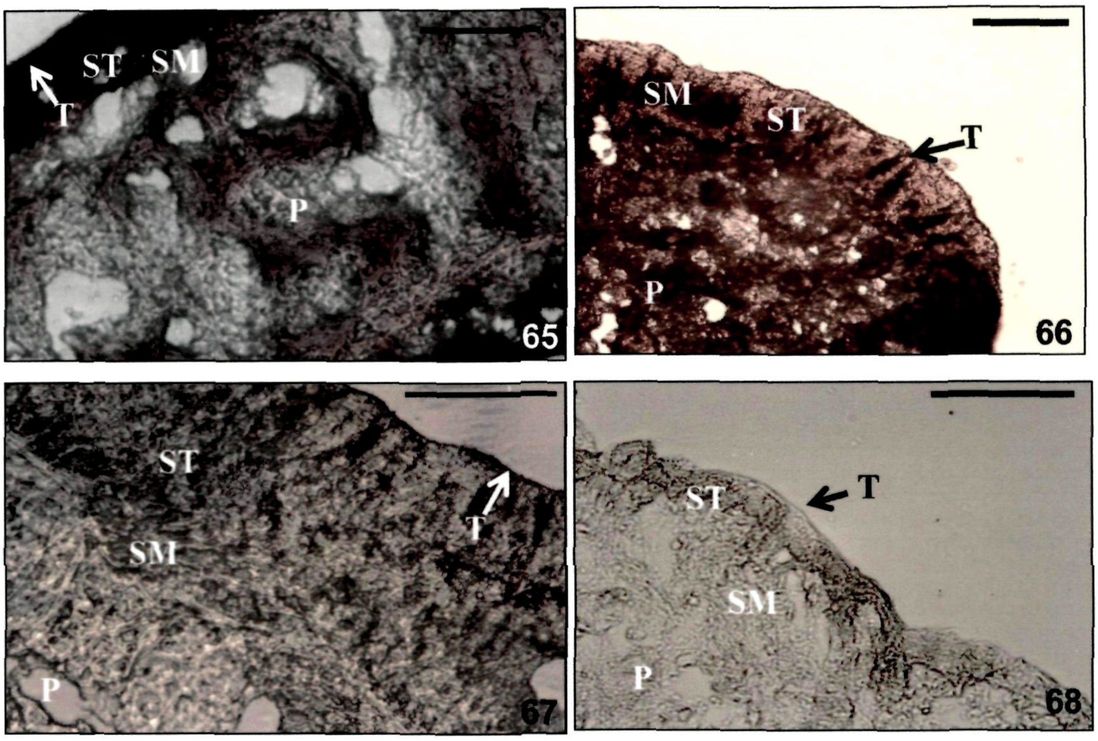


Fig. 65-68: Alkaline phosphatase (AlkPase) activity in *R. echinobothrida*, frozen sections

Fig. 65. Cross section of control worm showing very intense AlkPase activity in the tegument, subtegument, somatic musculature and parenchyma

Fig. 66. Stem bark extract of *A. oxyphylla* treated section showing reduced enzyme staining

Fig. 67. Leaf extract of *S. virosa* treated section showing overall diminished enzyme activity

Fig. 68. Praziquantel treated section showing almost total decline in enzyme activity

All bars 50 µm

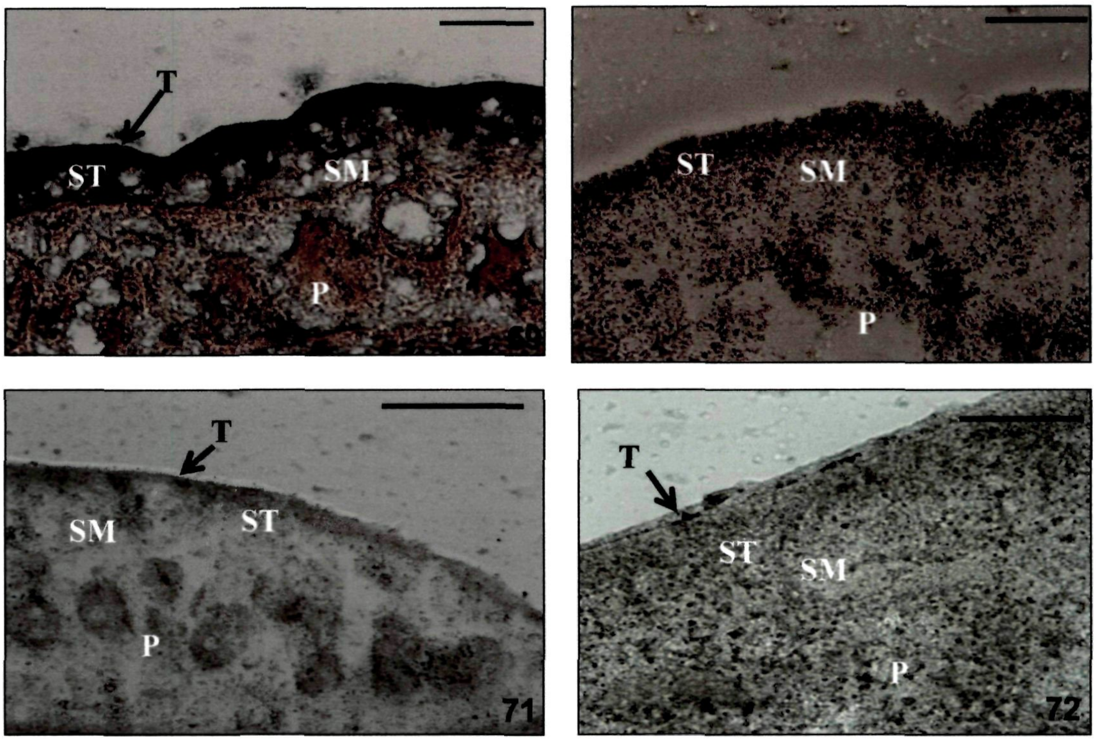


Fig. 69-72: Acid phosphatase (AcPase) activity in *R. echinobothrida*, frozen sections

Fig. 69. Cross section of control worm showing intense AcPase activity

Fig. 70. Stem bark extract of *A. oxyphylla* treated section showing mild enzyme activity in the subtegument

Fig. 71. Leaf extract of *S. virosa* treated section showing mild enzyme activity in the parenchyma

Fig. 72. Praziquantel treated section showing no enzyme activity in the somatic musculature and parenchyma

All bars 50 μm

Table 4: Effect of ethanol extracts of *A. oxyphylla* and *S. virosa*, and reference drug praziquantel on the activity of different tegumental enzymes of *R. echinobothrida*

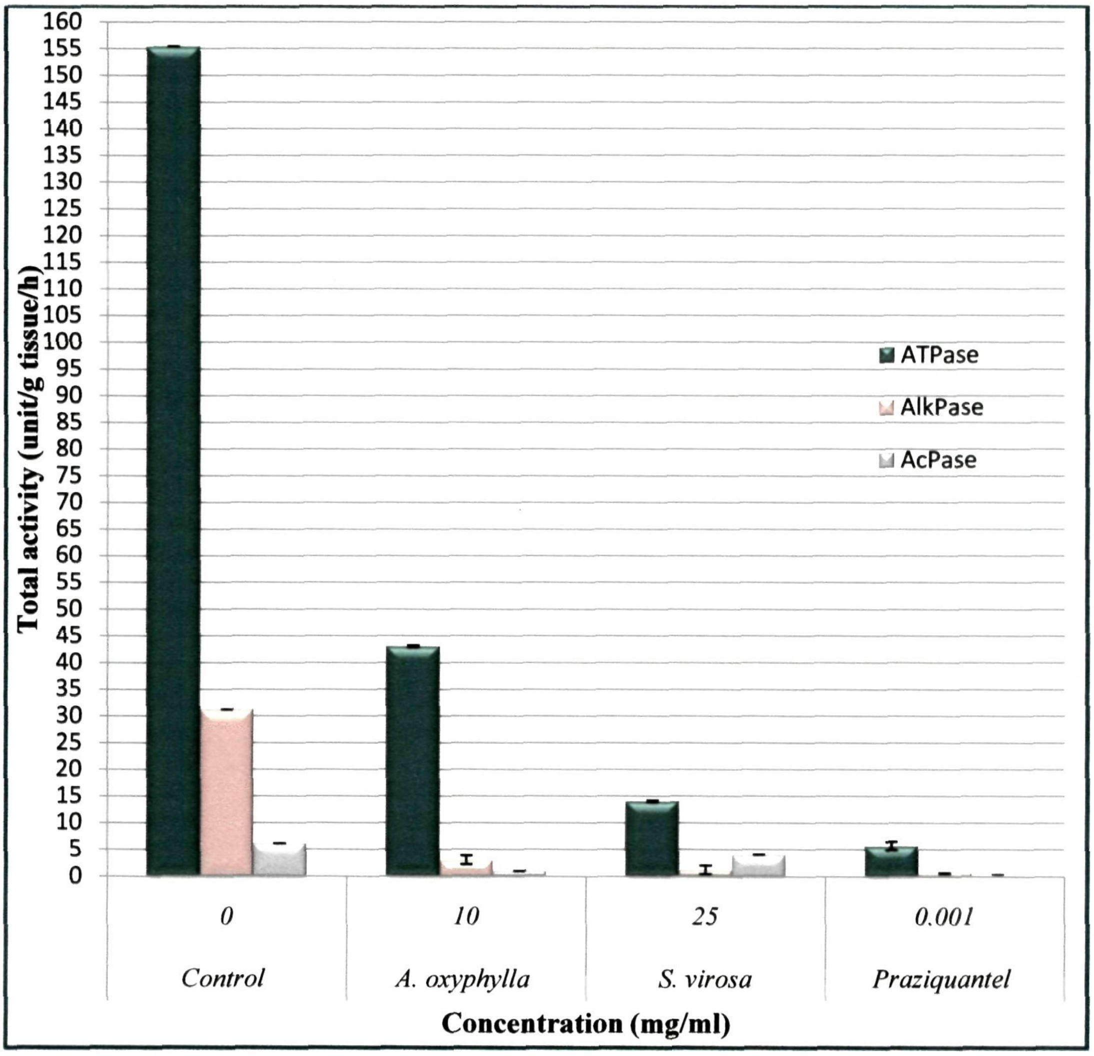
Control/Treated cestode with	*Enzyme activity (total / specific activity)*			*% decline after treatment*		
	ATPase	AlkPase	AcPase	ATPase	AlkPase	AcPase
Control	155.28 ± 0.003/	31.14 ± 0.001/	6.06 ± 0.01/	–	–	–
	3.19 ± 0.0	0.68 ± 0.11	0.43 ± 0.17			
***A. oxyphylla* (10 mg/ml)**	42.98 ± 0.17/	2.98 ± 0.8/	0.99 ± 0.01/	72	90	97
	1.36 ± 0.01	0.12 ± 0.01	0.42 ± 0.19			
***S. virosa* (25 mg/ml)**	13.98 ± 0.17/	1.19 ± 0.8/	3.97 ± 0.01/	91	96	34.5
	0.76 ± 0.01	0.18 ± 0.01	0.84 ± 0.19			
PZQ (0.001 mg/ml)	5.62 ± 0.8/	0.55 ± 0.05/	0.25 ± 0.01/	96	98	96
	2.37 ± 1.3	0.36 ± 0.4	0.007 ± 0.1			

Values given as mean ± SE from 5 replicate assays

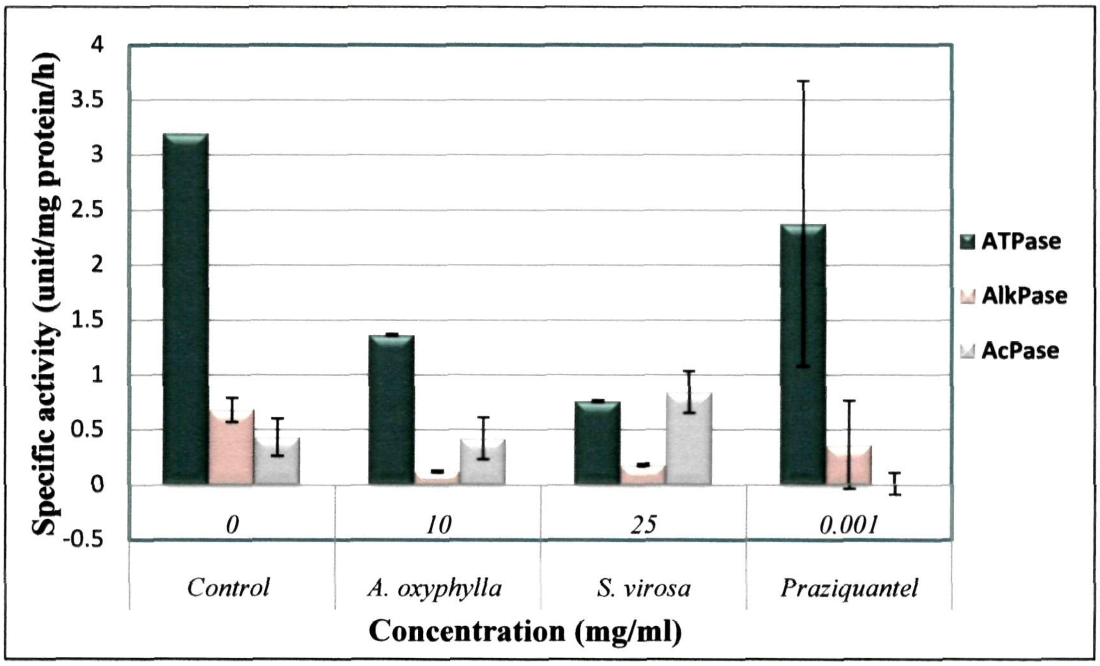
[a]Enzyme activity as specific unit which consumes 1.0 mmol substrate/ g wet wt. tissue /h

[b]Specific activity as unit/ mg protein/ h

Graph 3: Alterations in total enzyme activities of *R. echinobothrida* treated with extracts of *A. oxyphylla* and *S. virosa*, and reference drug praziquantel



Graph 4: Alterations in specific enzyme activities of *R. echinobothrida* treated with extracts of *A. oxyphylla* and *S. virosa*, and reference drug praziquantel



Graph 5: Percentage reduction in enzyme activity of *R. echinobothrida* treated with extracts of *A. oxyphylla* and *S. virosa*, and reference drug praziquantel

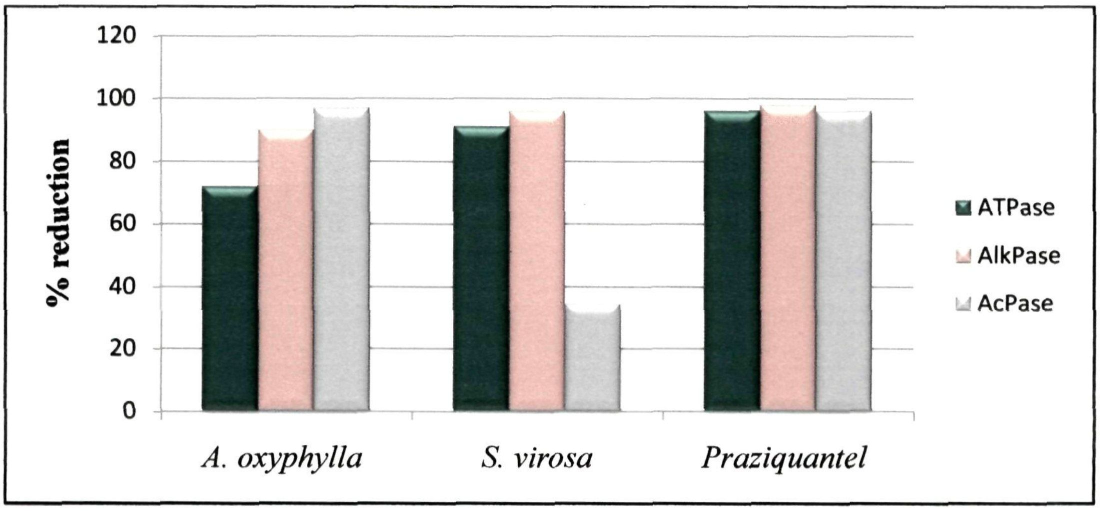


Table 5: Effect of crude extracts of *A. oxyphylla* and *S. virosa*, and reference drug praziquantel on free amino acid pool (nmol/g wet weight) of *R. echinobothrida*

Control/Treated cestode with ⇨ Amino acids ⇩	Control	*A. oxyphylla*	*S. virosa*	PZQ
Ileu / Leu	76.24 ± 1.02	11.9 ± 0.06 (-84.4)	16.68 ± 0.03 (-78.12)	7.15 ± 0.32 (-90.6)
Asn	75.70 ± 1.26	28.0 ± 2.04 (-63)	67.47 ± 1.94 (-10.9)	52.6 ± 1.42 (-30.5)
Ser	95.15 ± 0.1	95.15 ± 0.05 (0)	95.15 ± 0.05 (0)	237.87 ± 0.05 (+150)
Pro	86.86 ± 0.86	86.86 ± 0.96 (0)	92.40 ± 1.02 (+6.3)	77.61 ± 1.06 (-10.6)
Val	85.36 ± 0.08	85.36 ± 0.98 (0)	39.73 ± 0.18 (-53.4)	49.80 ± 0.12 (- 41.6)
Meth	67.01 ± 0.16	26.8 ± 0.01 (-60)	0.00 ± 0.4 (-100)	0.00 ± 0.01 (-100)
His	64.45 ± 0.09	32.23 ± 0.03 (-50)	64.45 ± 0.08 (0)	64.45 ± 0.06 (0)
Thr	83.89 ± 0.06	139.80 ± 0.03 (+66.6)	111.2 ± 0.06 (+32.6)	83.89 ± 0.85 (0)
Cys	82.54 ± 0.02	0.00 ± 0.21 (-100)	165.1 ± 0.03 (+100)	82.54 ± 0.37 (0)
Asp	75.13 ± 1.31	36.5 ± 1.4 (-51.4)	70.83 ± 2.1 (-5.7)	68.69 ± 0.99 (-8.57)
Gln/Lys	68.43 ± 0.18	34.21 ± 0.18 (-50)	68.43 ± 0.26 (0)	85.54 ± 0.24 (+25)
Glu	68.00 ± 0.26	18.55 ± 0.22 (-72.7)	55.64 ± 0.19 (-18.2)	68.00 ± 0.01 (0)
Tyr	55.21 ± 0.06	16.56 ± 0.03 (-70)	36.8 ± 0.08 (-33.34)	46.0 ± 0.04 (-16.7)
Trp	48.96 ± 0.02	24.48 ± 0.07 (-50)	73.44 ± 0.11 (+50)	97.92 ± 0.08 (+100)
Phe	60.53 ± 0.06	15.13 ± 0.02 (-75)	15.13 ± 0.04 (-75)	15.13 ± 0.03 (-75)
Arg	57.40 ± 0.02	28.7 ± 0.02 (-50)	57.4 ± 0.02 (0)	57.4 ± 0.02(0)
Gly	133.21 ± 0.08	0.00 ± 0.04 (-100)	66.7 ± 0.06 (-50)	99.1 ± 0.1 (-25.6)
Ala	110.00 ± 0.1	66.00 ± 0.12 (-40)	154.00 ± 0.07 (+40)	110.0 ± 0.09(0)
Total FAA	1394.07	746.23 (- 46.5)	1250.55 (- 10.3)	1303.69 (- 6.5)

Values given as mean ± SE from 5 replicate assays

Percentage increase (+) or decrease (-) of the FAA levels is given in parentheses

Graph 6: **Effect of crude extracts of *A. oxyphylla* and *S. virosa,* and reference drug praziquantel on free amino acid pool (nmol/g wet weight) of *R. echinobothrida***

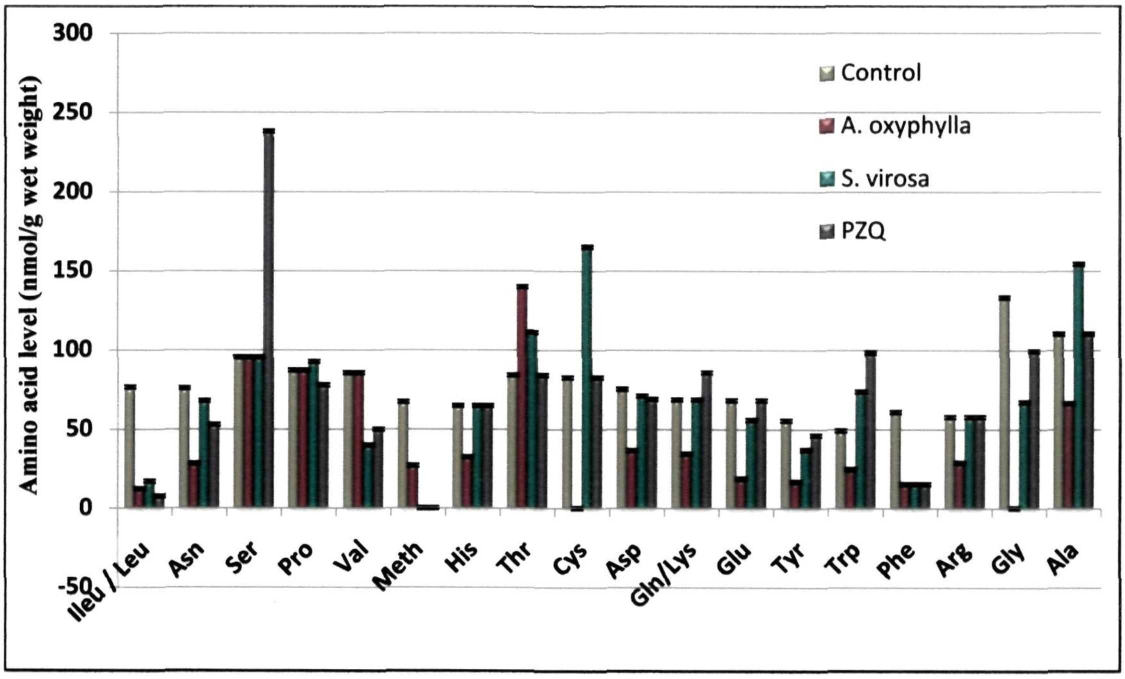


Table 6: Effect of ethanol extracts of *A. oxyphylla* and *S. virosa*, and reference drug praziquantel on the levels of trace elements (ppm) in *R. echinobothrida*, effluent and untested plant media

Control/Treated cestode with	**Test parasite/media**	**Ca**	**Mg**
Control	Parasite tissue	45.2 ± 1.22	6.2 ± 0.07
***A. oxyphylla* (10 mg/ml)**	Parasite tissue	9.7 ± 0.13	3.6 ± 0.4
	Effluent	33.1 ± 0.87	5.2 ± 0.05
	Untested plant media	9.5 ± 0.01	1.84 ± 0.02
***S. virosa* (25 mg/ml)**	Parasite tissue	10.2 ± 0.46	4.6 ± 0.11
	Effluent	35.9 ± 0.67	6.0 ± 0.37
	Untested plant media	18.6 ± 0.02	2.79 ± 0.01
PZQ (0.001 mg/ml)	Parasite tissue	10.1 ± 0.8	5.7 ± 0.37
	Effluent	11.4 ± 0.61	5.8 ± 0.46
	Untested PZQ media	10.7 ± 0.02	2.6 ± 0.01

Values given as mean ± SE from 5 replicate assays

$p < 0.05$ *vs.* control value, student's t- test

Graph 7: Effect of ethanol extracts of *A. oxyphylla* and *S. virosa,* and reference drug praziquantel on the levels of trace elements (ppm) in *R. echinobothrida*, effluent and untested plant media

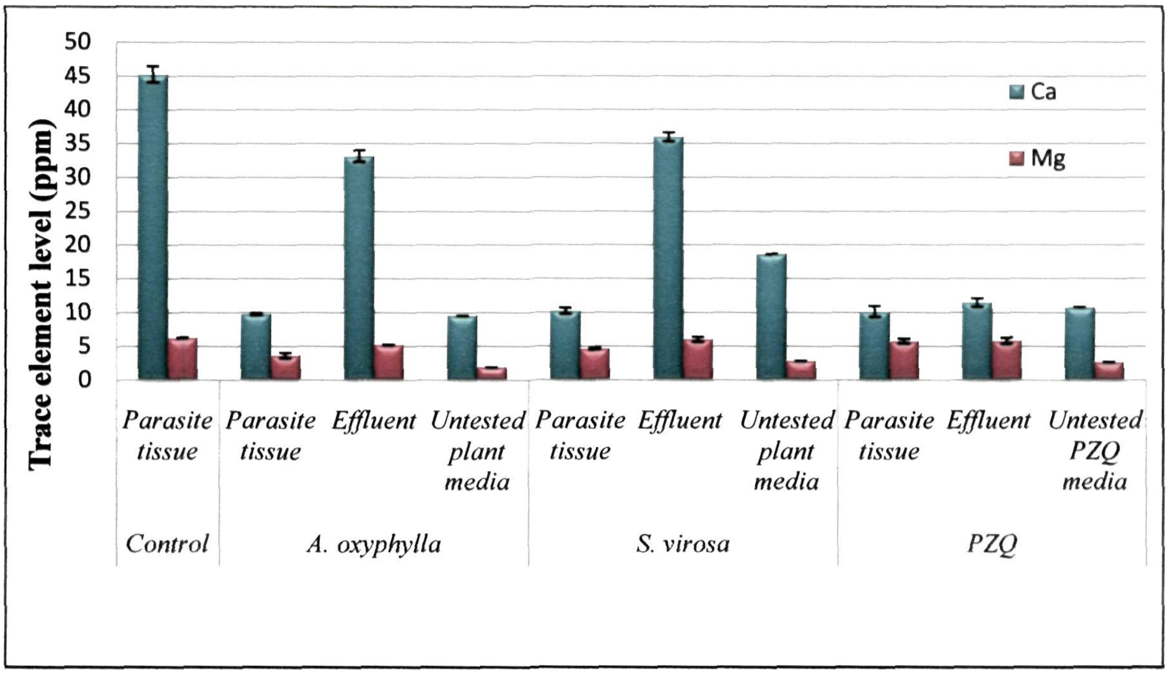


Table 7: Effect of methanol fractions of *A. oxyphylla* and *S. virosa*, and reference drug praziquantel on the levels of trace elements (ppm) in *R. echinobothrida*, effluent and untested plant media

Control/Treated cestode with	Test parasite/media	Ca	Mg
Control	Parasite tissue	45.2 ± 1.22	6.2 ± 0.07
A. oxyphylla **(10 mg/ml)**	Parasite tissue	33.9 ± 1.37	5.1 ± 0.07
	Effluent	5.4 ± 0.64	5.7 ± 0.11
	Untested plant media	11.0 ± 0.01	2.6 ± 0.02
S. virosa **(25 mg/ml)**	Parasite tissue	9.2 ± 0.47	4.4 ± 0.3
	Effluent	36.3 ± 0.87	5.9 ± 0.11
	Untested plant media	18.5 ± 0.01	0.15 ± 0.02
PZQ (0.001 mg/ml)	Parasite tissue	10.1 ± 0.8	5.7 ± 0.37
	Effluent	11.4 ± 0.61	5.8 ± 0.46
	Untested PZQ media	10.7 ± 0.02	2.6 ± 0.01

Values given as mean ± SE from 5 replicate assays

$p < 0.05$ *vs*. control value, student's t- test

Graph 8: Effect of methanol fractions of *A. oxyphylla* and *S. virosa,* and reference drug praziquantel on the levels of trace elements (ppm) in *R. echinobothrida,* effluent and untested plant media

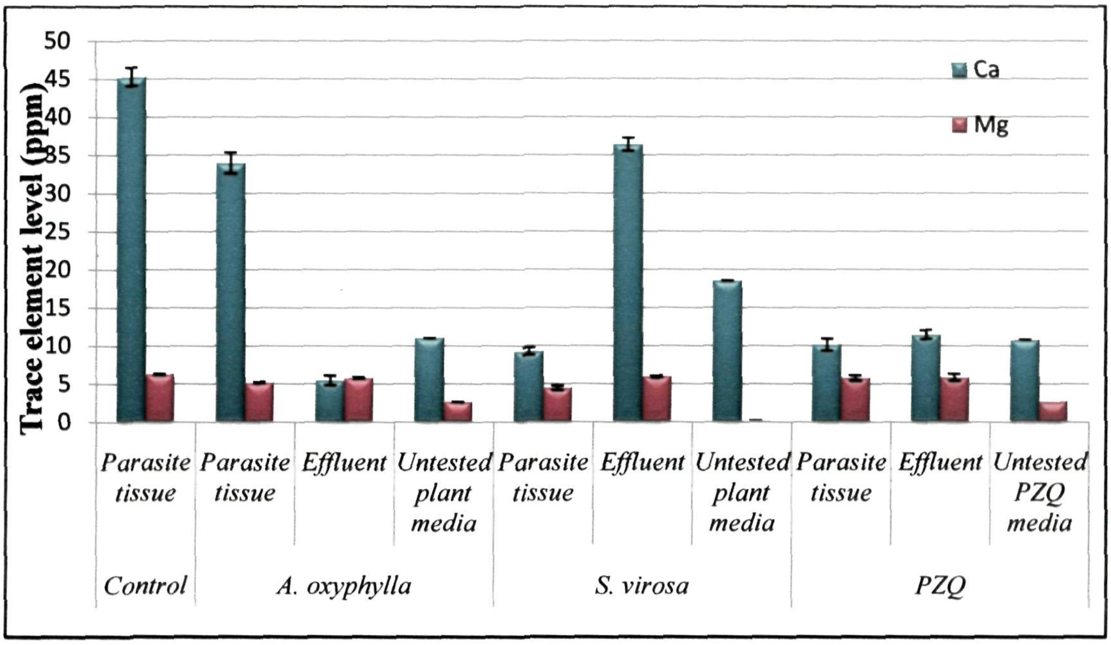


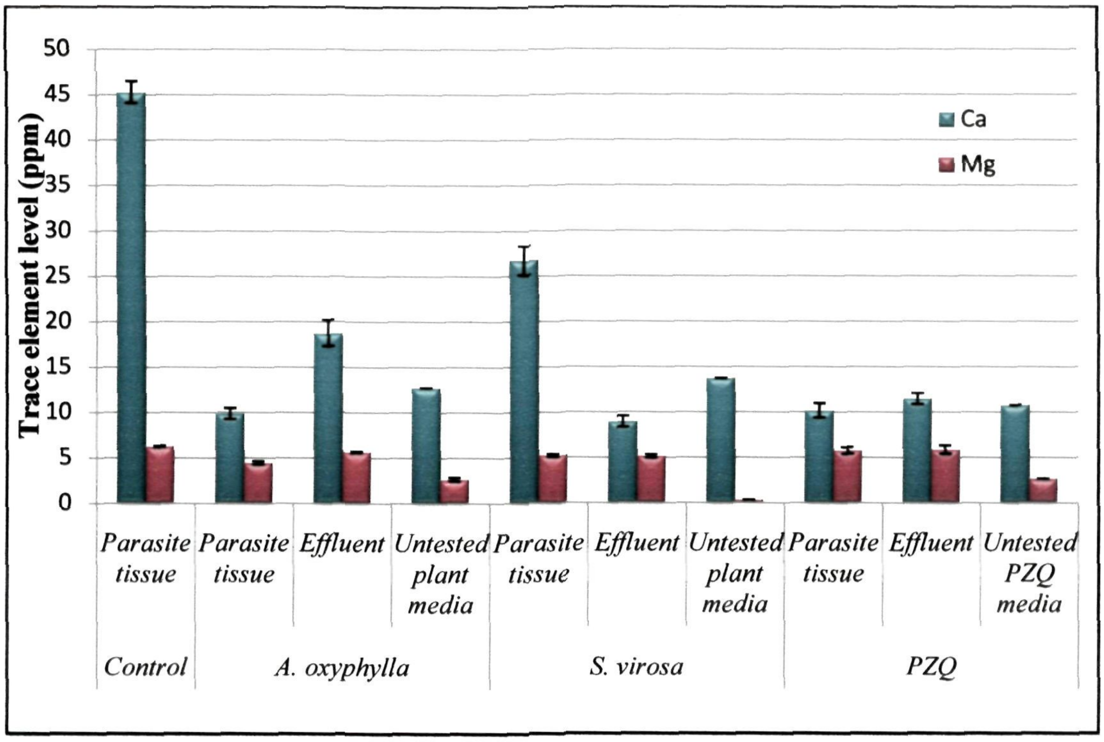
Table 8: Effect of acetone fractions of *A. oxyphylla* and *S. virosa*, and reference drug praziquantel on the levels of trace elements (ppm) in *R. echinobothrida*, effluent and untested plant media

Control/Treated cestode with	**Test parasite/media**	**Ca**	**Mg**
Control	Parasite tissue	45.2 ± 1.22	6.2 ± 0.07
A. oxyphylla **(10 mg/ml)**	Parasite tissue	9.8 ± 0.61	4.4 ± 0.17
	Effluent	18.7 ± 1.45	5.6 ± 0.07
	Untested plant media	12.68 ± 0.01	2.6 ± 0.2
S. virosa **(25 mg/ml)**	Parasite tissue	26.7 ± 1.61	5.2 ± 0.14
	Effluent	8.9 ± 0.62	5.1 ± 0.18
	Untested plant media	13.7 ± 0.02	0.27 ± 0.01
PZQ (0.001 mg/ml)	Parasite tissue	10.1 ± 0.8	5.7 ± 0.37
	Effluent	11.4 ± 0.61	5.8 ± 0.46
	Untested PZQ media	10.7 ± 0.02	2.6 ± 0.01

Values given as mean ± SE from 5 replicate assays

$p < 0.05$ *vs.* control value, student's t- test

Graph 9: Effect of acetone fractions of *A. oxyphylla* and *S. virosa*, and reference drug praziquantel on the levels of trace elements (ppm) in *R. echinobothrida*, effluent and untested plant media



In vivo experiments

1. Treatment

The results are presented in Tables 9 and 10 (Graphs 10 and 11). It is obvious from Table 9 that pre-treatment EPG count of untreated infected group of rats did not show any significant change on days 24-26. Administration of 25 mg of *A. oxyphylla* extract/kg body weight reduced the post treatment EPG counts by about 62%. However, 50 mg extract/kg body weight reduced EPG count from an average of 20,350 to an average of 4,350 i.e., 78% reduction (significant at $p<0.01$).

The administration of 1500 mg of *S. virosa* extract/kg body weight induced significant reduction ($p<0.01$) of EPG. The values decreased from 19250 ± 984.9 to 6850 ± 427.2 and 6100 ± 180.3 post treatment (days 24-26 and days 33-35, respectively) i.e. about 64 and 68% reductions, respectively. The extract equivalent to 750 mg of *S. virosa* extract/kg body weight did not significantly reduce post treatment EPG count. Single oral administration of PZQ (5 mg/kg) also significantly reduced the EPG counts at all time intervals. In these rats, pre-treatment EPG count was about 20, 200 which reduced to about 1600 and 350 on days 24-26 and days 33-35, respectively, after the drug administration, inducing 92.1 and 98.3 percent reductions, respectively.

Treatment with 750 mg/kg dose of *S. virosa* extract resulted into comparatively low worm recovery rate; PZQ reduced the worm recovery rate by 92%. The 25 and 50 mg of *A. oxyphylla* extract/kg body weight and 1500 mg of *S.*

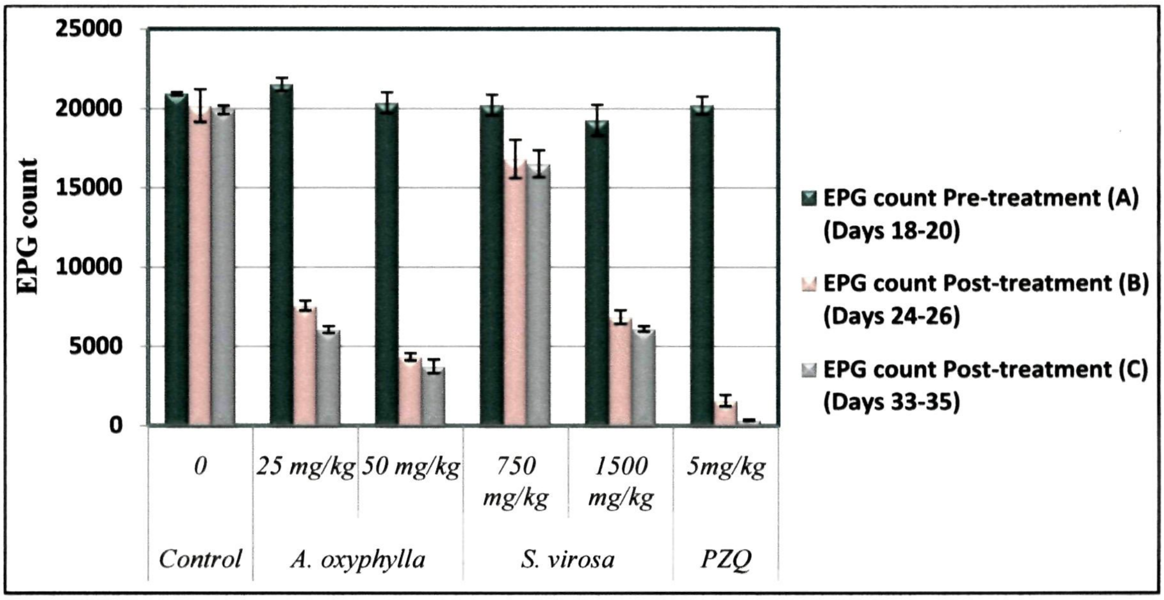
virosa extract/kg body weight showed a 80%, 87% and 83% decline in worm burden. However, 750 mg/kg dose brought down the worm burden by only 40%.

Table 9: ***In vivo*** **anticestodal efficacy of ethanol extracts of *A. oxyphylla* and *S. virosa*, and reference drug praziquantel against *H. diminuta* infections in albino rats by egg per gram of faeces (EPG) count**

Control/Treated	**EPG count[a]**			**Difference in EPG count[a]**		**% reduction in EPG[a]**	
cestode with	**Pre-treatment (A) (Days 18-20)**	**Post-treatment (B) (Days 24-26)**	**Post-treatment (C) (Days 33-35)**	**(A and B)**	**(A and C)**	**(A and B)**	**(A and C)**
Control	20900 ± 100	20150 ± 1033.2	19900 ± 264.6	1150 ± 705.3	1000 ± 264.6	5.5 ± 3.4	4.8 ± 1.2
A. oxyphylla							
25 mg/kg	21500 ± 409.3	7550 ± 304.1	6050 ± 217.9	13950 ± 708.9	15450 ± 396.9	62.1 ± 1.7	71.8 ± 0.9
50 mg/kg	20350 ± 650	4350 ± 229.1	3750 ± 433	16000 ± 638.3	16600 ± 934.1	78.6 ± 1.1	81.5 ± 2.4
S. virosa							
750 mg/kg	20200 ± 655.7	16800 ± 1221.7	16500 ± 866	3400 ± 1231.9	3700 ± 1014.9	16.8 ± 5.8	18.2 ± 4.7
1500 mg/kg	19250 ± 984.9	6850 ± 427.2	6100 ± 180.3	12400 ± 1011.2	13150 ± 1089.7	64.2 ± 2.8	68.1 ± 2.3
PZQ							
5mg/kg	20200 ± 556.8	1600 ± 360.6	350 ± 50	18600 ± 458.3	19850 ± 507.4	92.1 ± 1.7	98.3 ± 0.2

[a] Values are mean ± SEM. n = 3 animals in each group. P < 0 01 (two-way ANOVA)

Graph 10: ***In vivo*** **effect of ethanol extracts of *A. oxyphylla* and *S. virosa*, and reference drug praziquantel (PZQ) on *H. diminuta* EPG count**



Graph 11: Percentage reduction in EPG count after treatment with extracts of *A. oxyphylla* and *S. virosa*, and reference drug praziquantel (PZQ)

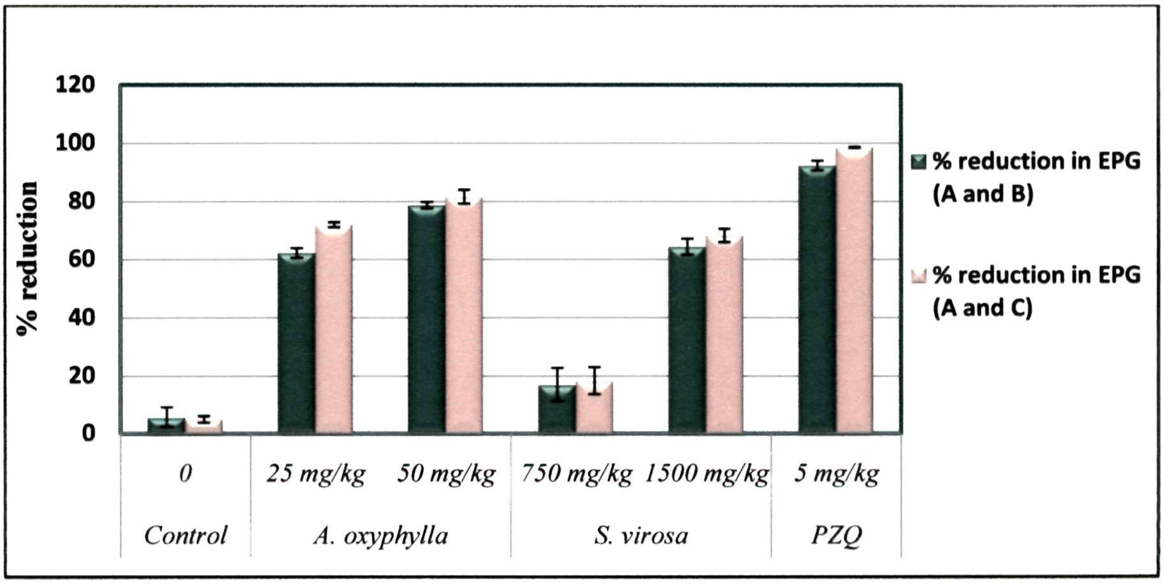


Table 10: *In vivo* anticestodal efficacy (worm recovery rate) of ethanol extracts of *A. oxyphylla* and *S. virosa,* and reference drug praziquantel on *H. diminuta*

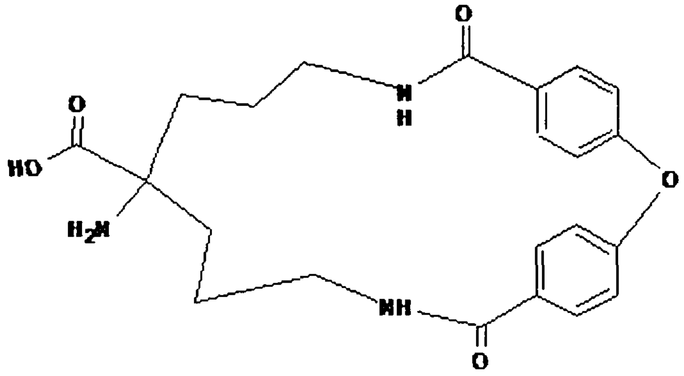
Control/Treated cestode with	No. of cysticercoids/rat	No. of worms/group[a]	Worm recovery rate (%)[a]
Control	5	4.5 ± 0.5	90 ±10
A. oxyphylla			
25 mg	5	1 ± 0.5	20 ± 10
50 mg	5	0.65 ± 0.35	13 ±7
S. virosa			
750 mg	5	3 ± 0	60 ±1
1500 mg	5	0.85 ± 0.15	17 ± 3
PZQ			
5 mg	5	0.4 ± 0.1	8 ± 2

[a] Values are mean ± SEM. n = 3 animals in each group. $P < 0.01$ (one-way ANOVA)

Efficacy of active principles

1. Purified active component of *A. oxyphylla*

The active anthelmintic component isolated from *A. oxyphylla* was identified 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 ($C_{22}H_{25}N_3O_5$) having the structure provided below:



($C_{22}H_{25}N_3O_5$)

2. Motility and mortality studies

When *R. echinobothrida* was incubated in F5-1d, 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, virosecurinine and PZQ, there was a decline in the motility of worms resulting eventually in their death (Table 11, Graph 12). The control parasites continued to show physical activity for an average timing of 72 h following which they became immobilized.

At 1 mg F5-1d and 1 mg 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/ml of PBS, spontaneous movements seized in the worms at about

0.5 h while treatment with virosecurinine induced loss of motility and consequent paralysis in 1.25 h. Death occurred at an average timing of 9.0 h, 5.75 h and 8.9 h for F5-1d, 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 and virosecurinine, respectively, and PZQ (0.001 mg/ml) caused the worms to die within 9.8 h. The observations indicate a significant difference (at $p < 0.05$) between the active principles-treated groups and the control group.

2. Surface morphology and ultrastructural studies

Scanning electron microscopy

Fraction 5-1d of *A. oxyphylla* caused the worms to contort and shrivel up. The surface alterations usually appeared as pronounced shrinking and a general smoothening of tegument because of a major diminution of microtriches. Formation of a large number of small pits gave the surface a total porous façade (Figs. 73-76).

Treatment 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 of *A. oxyphylla* led to total depletion of microtriches which fused together to form masses. Proglottides were noted to be super-contorted and seemed to be breaking off from the main body at many places due to extreme shrinkage. The suckers in the scolex collapsed and retreated back, pushing the rostellum forward. However, the spines around the rostellum were retained and arranged flawlessly (Figs. 77-80).

When treated with virosecurinine, the cestodes seemed to be affected more as the suckers had retracted back giving the rostellum a markedly elevated appearance (Fig. 81). The suckers were porous with fewer hooks that were all clumped together wherever present (Fig. 82). The effect on the proglottides too was more prominent as they were not distinctly separate and each proglottid seemed to be gradually melting into the other. The surface was much clumped once again but with lesser infoldings (Figs. 83, 84).

Transmission electron microscopy

Parasites treated with Fraction 5-1d had no tegument as such apart from threads of basal lamina at some places (Fig. 85). The muscle layers were slightly fuzzy, particularly the longitudinal muscles and increased electron lucency was observed. Nuclei of the parenchymal cytons were immensely distended and showed a decrease in the nucleoplasm content while retaining their nucleolus at the same time (Fig. 86). Mitochondria observed were found to be normal throughout the parasite tissue (Fig. 87).

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 treatment caused the tegument to strip off and the nuclei of subtegumental cytons to swell up severely (Fig. 88). Chromatin was condensed into heterochromatin in the nucleus and the cytoplasm of the cytons was highly vacuolated (Fig. 89). Mitochondria were found sparsely in the parasite body and were mostly acristate (Fig. 90).

Virosecurinine treatment caused the microthrix layer and part of distal cytoplasm to dissolve leaving the basal lamina and the muscle layers disrupted (Fig. 91). Unlike the methanol fraction of *S. virosa*, the subtegumental cytons did not have swollen and vacuolated nuclei. It was only the parenchyma that had bloated and deformed nuclei with no distinct nuclear material or distinct cytoplasm around it to mark the cytons (Fig. 92). Mitochondria were found in large numbers but they were mostly distorted and lacked the cristae (Fig. 93).

3. Histochemical localization of tegumental enzymes

The results for the activity of the tegumental enzymes in different parts of the parasite tissue are depicted in Table 12. Control worms showed intense ATPase activity throughout the parasite tissue, particularly, the tegument, subtegument and parenchyma (Fig. 94). ATPase activity was present mildly only in the subtegument and somatic musculature of the 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 treated parasites (Fig. 95). Moderate enzyme activity was noted in the muscles and parenchyma of virosecurinine treated worms (Fig. 96). PZQ caused the parasite to lose tegumental and subtegumental enzymes on a large-scale and ATPase activity was located very mildly only in the subtegument (Fig. 97).

AlkPase activity was mostly concentrated in the tegument and subtegument of control worms with moderate activity in the parenchyma (Fig. 98). In case of 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 treated parasites, AlkPase activity was present very mildly in the subtegument and muscle blocks (Fig. 99). The activity was present only in the muscles in parasites treated with virosecurinine (Fig. 100). PZQ caused the AlkPase activity to concentrate in the subtegument and muscle with very little activity in the parenchyma (Fig. 101).

Control worms showed intense AcPase activity in the tegument (Fig. 102). 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 treatment led to almost total inactivation of AcPase activity in the parasite tissue (Fig. 103). Virosecurinine treatment resulted in reduced enzyme staining throughout, with mild activity in the subtegument (Fig. 104). However, PZQ resulted in extensive diminishing of the enzyme throughout the tissue (Fig. 105).

Thus, when treated with the active principles of the two plants, AlkPase activity was reduced majorly with virosecurinine, and AcPase activity was reduced most 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.

Table 11: ***In vitro*** **efficacy of fraction F5-1d of** ***A. oxyphylla,*** **active principles F5-2d and virosecurinine, and reference drug praziquantel on** ***R. echinobothrida*** **(Number of worms in each test medium, n=6)**

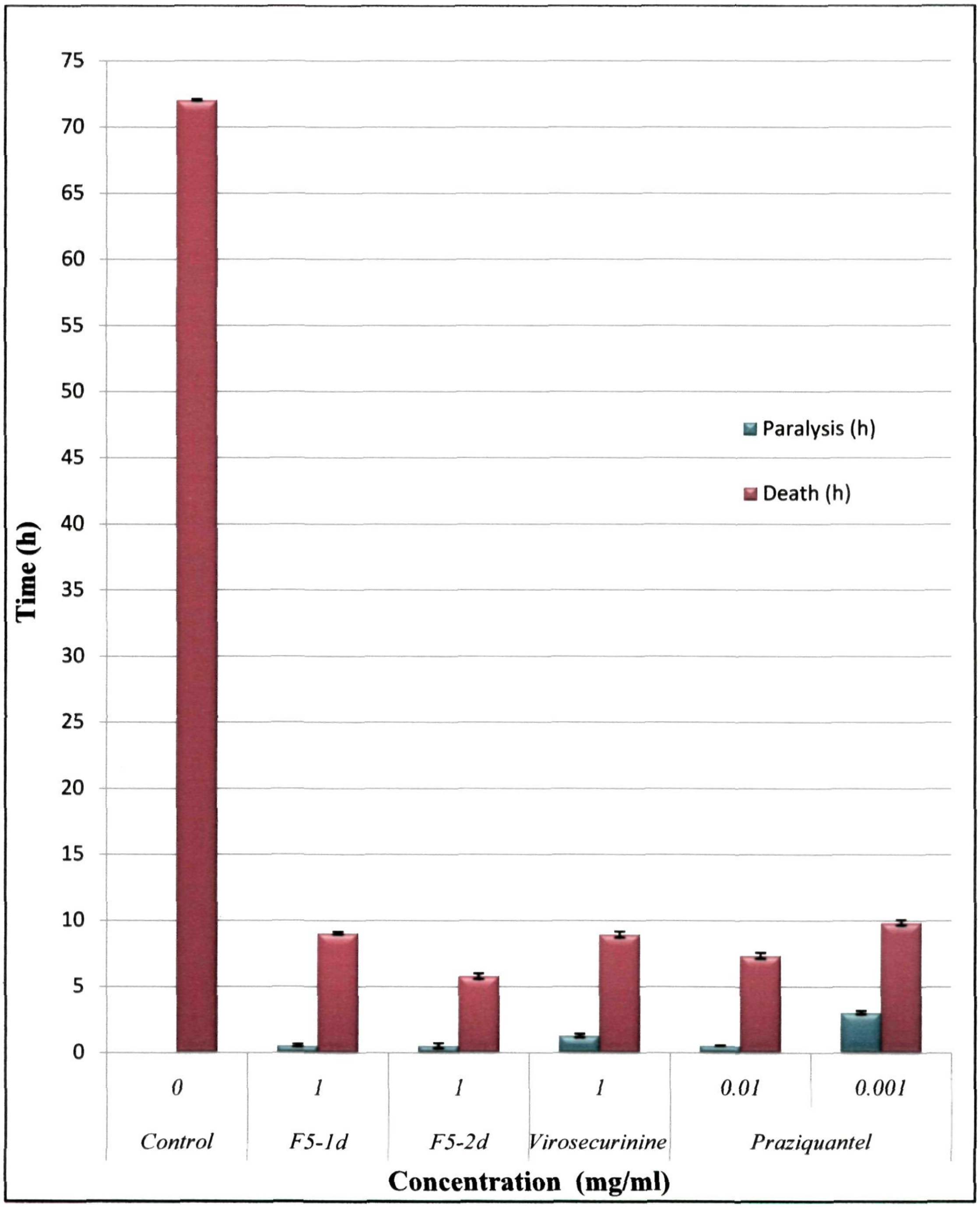
Control/treated cestode with	*Concentration (mg /ml)*	*Paralysis (h)*	*Death (h)*
Control	_	_	72.0 ± 0.06
F5-1d	1	0.5 ± 0.1	9.0 ± 0.01
F5-2d	1	0.5 ± 0.2	5.75 ± 0.1
Virosecurinine	1	1.25 ± 0.15	8.9 ± 0.01
PZQ	0.001	3.0 ± 0.14	9.8 ± 0.21
	0.01	0.5 ± 0.01	7.3 ± 0.15

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

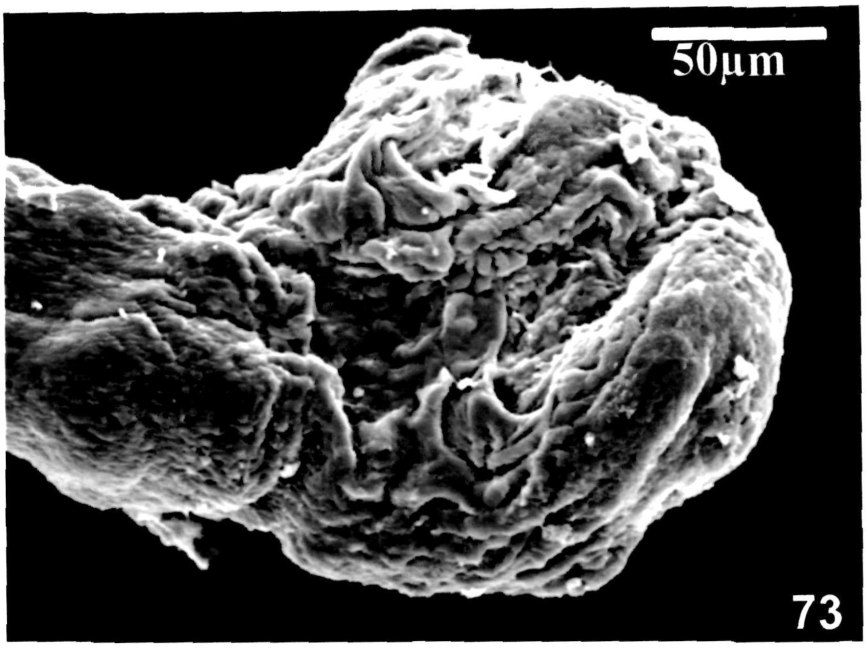
Values given as mean ± SE from 5 replicate assays

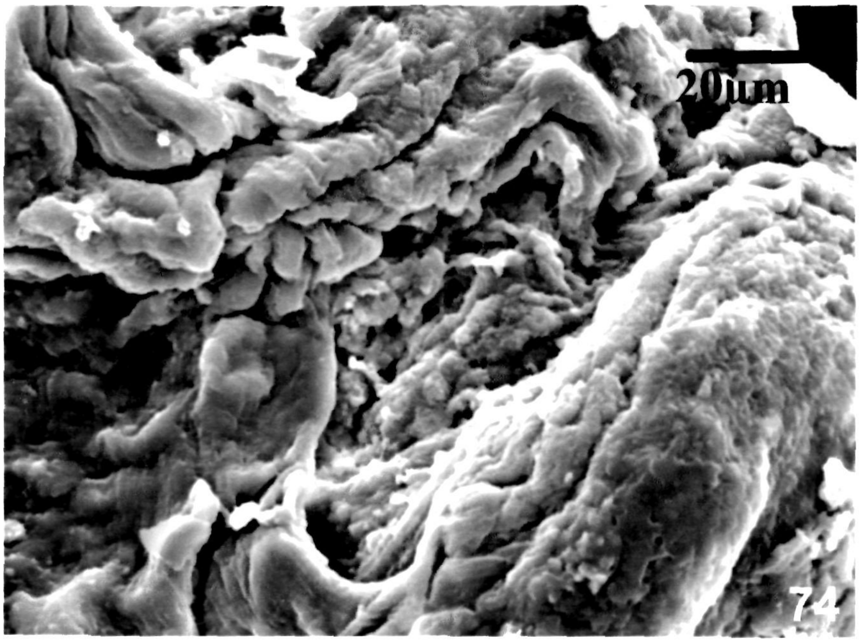
$p < 0.05$ *vs*. control value, student's t- test

Graph 12: ***In vitro*** **efficacy of fraction F5-1d of** ***A. oxyphylla,*** **active principles F5-2d and virosecurinine, and reference drug praziquantel on** ***R. echinobothrida*** **(Number of worms in each test medium, n=6)**



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

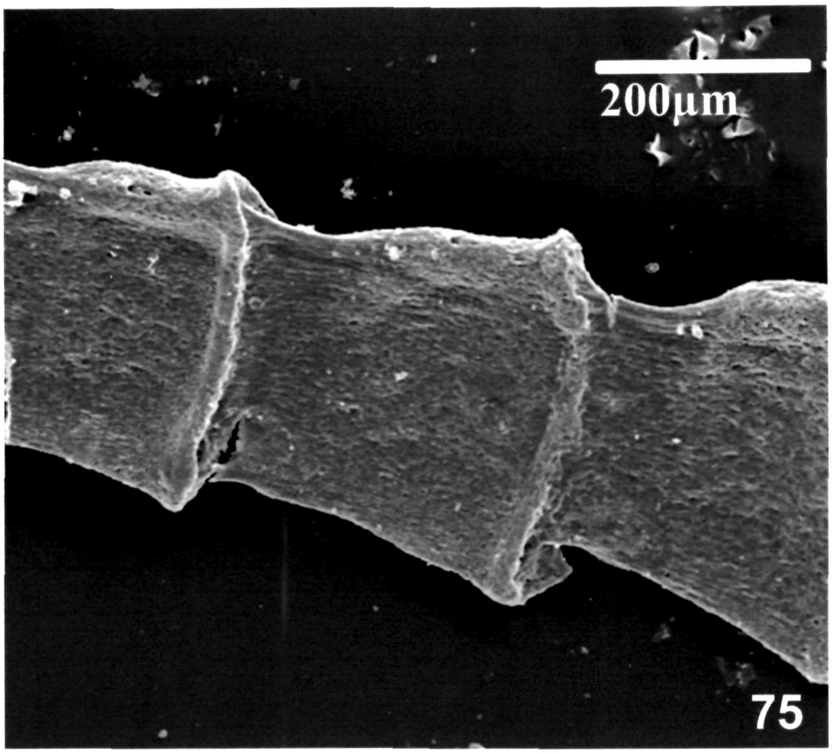


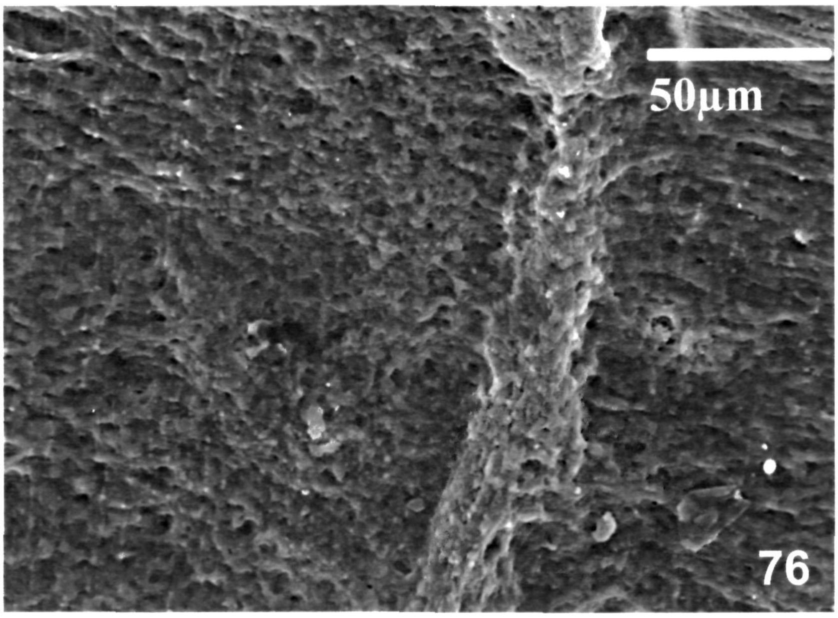


Figs. 73, 74: Scanning electron micrographs of *R. echinobothrida* treated with F5-1d fraction of *A. oxyphylla* (1 mg)

Fig. 73. Twisted scolex with distorted suckers.

Fig. 74. Magnified view of a distorted sucker.

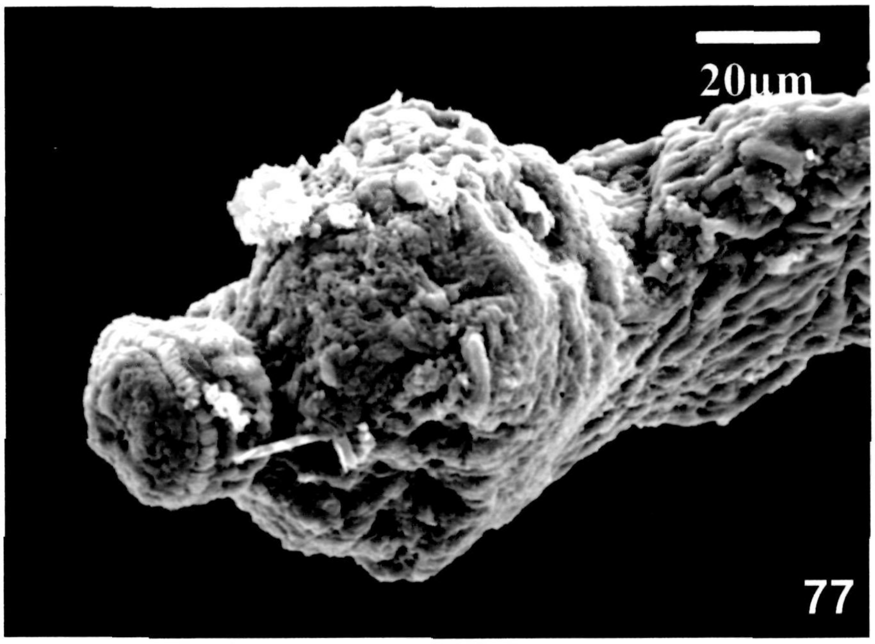


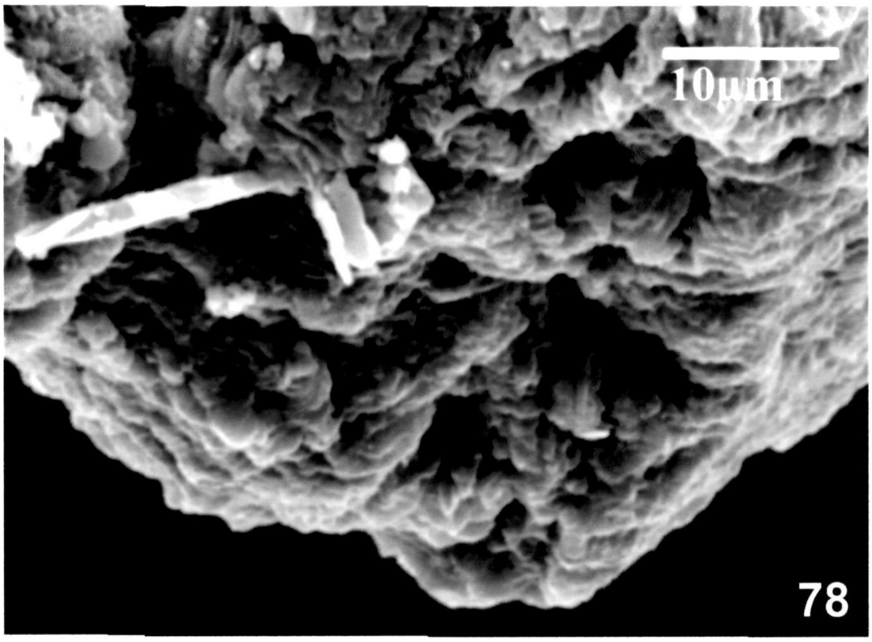


Figs. 75, 76: Scanning electron micrographs of *R. echinobothrida* treated with F5-1d fraction of *A. oxyphylla* (1 mg)

Fig. 75. String of shrunk proglottides.

Fig. 76. A magnified view showing pit formations in the tegument.

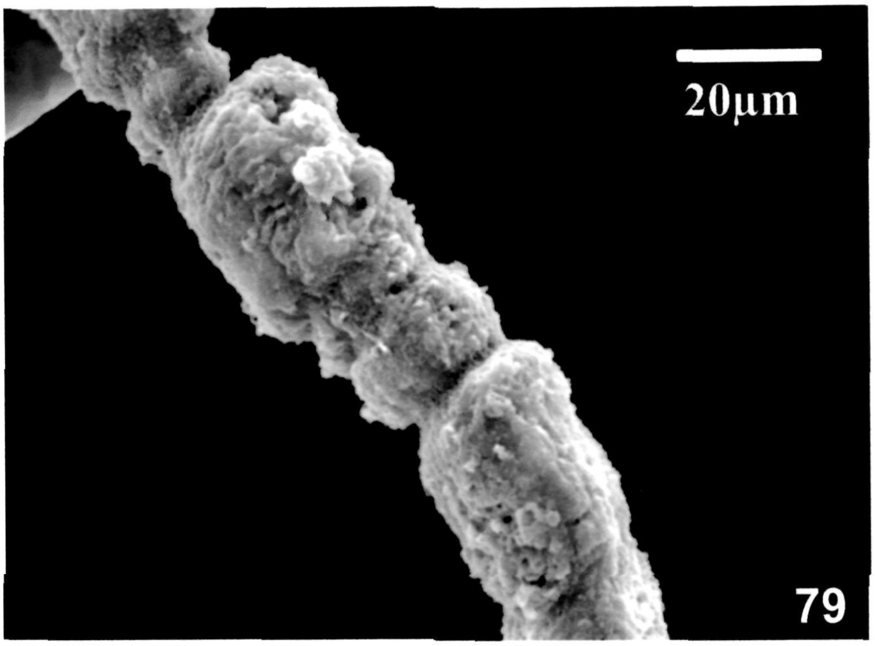


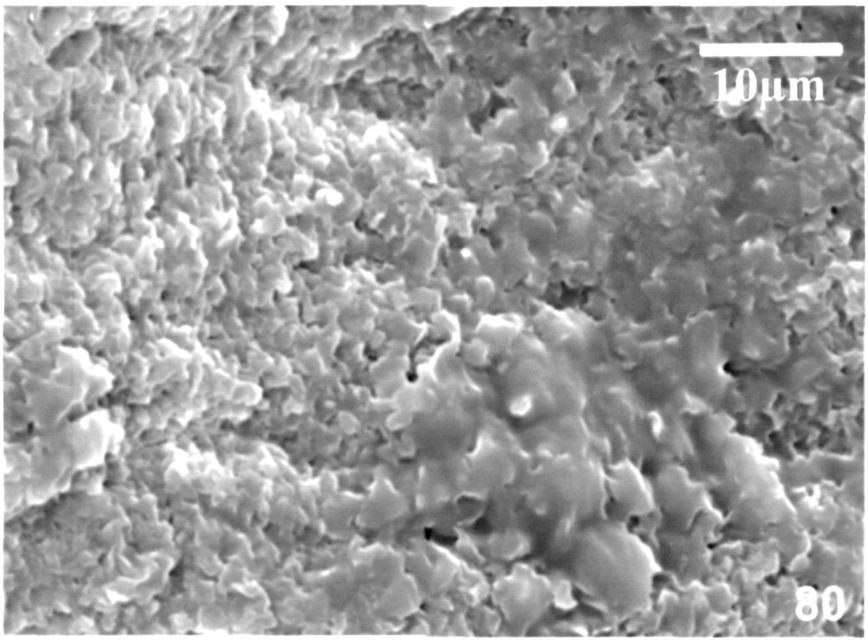


Figs. 77, 78: Scanning electron micrographs of *R. echinobothrida* treated with 12-amino-7, 17-dioxo-2-oxa-8, 16-diazatricylo [14.2.2.2 3, 6] tetraicosa-1 (20), 3, 5, 18, 21, 23-hexaene-12-carboxylic acid of *A. oxyphylla* (1 mg)

Fig. 77. Collapsed and retracted suckers leading to the rostellum to look elevated.

Fig. 78. Magnified view of the collapsed sucker.

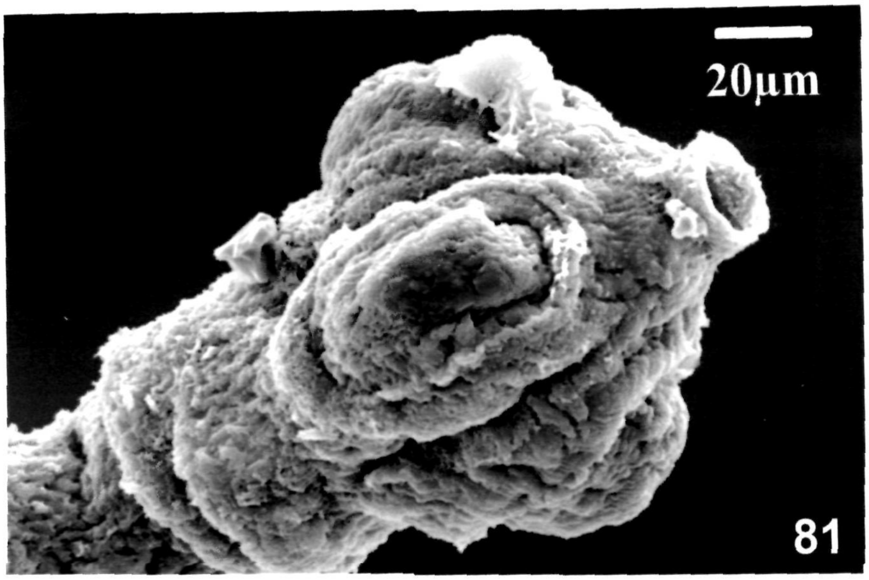


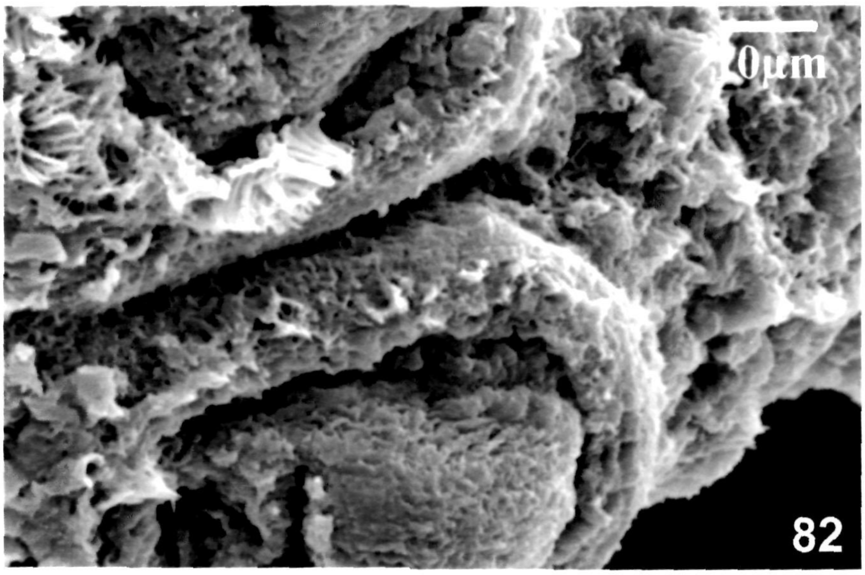


Figs. 79, 80: Scanning electron micrographs of *R. echinobothrida* treated with 12-amino-7, 17-dioxo-2-oxa-8, 16-diazatricylo [14.2.2.2 3, 6] tetraicosa-1 (20), 3, 5, 18, 21, 23-hexaene-12-carboxylic acid of *A. oxyphylla* (1 mg)

Fig. 79. Super-contorted and elongated proglottides with some almost breaking off from the main body.

Fig. 80. Microtriches fused together to form masses.

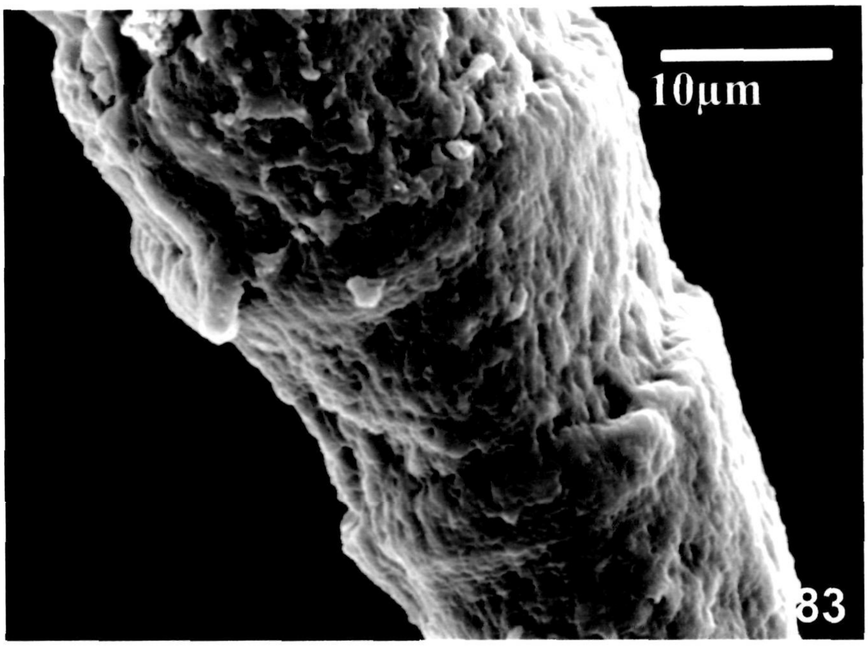


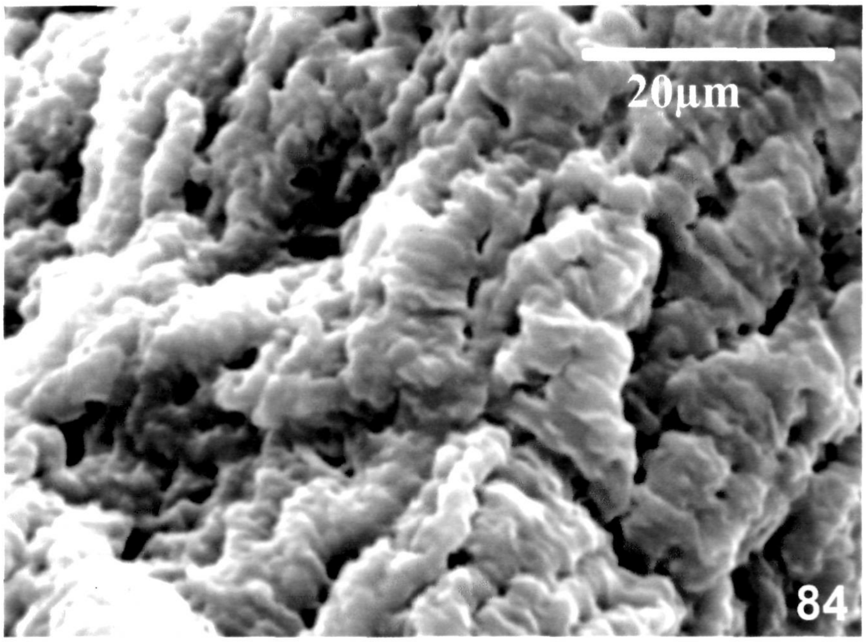


Figs. 81-82: Scanning electron micrographs of *R. echinobothrida* treated with virosecurinine of *S. virosa* (1 mg)

Fig. 81. Muffin-head appearance of the rostellum, along with elongation of suckers.

Fig. 82. Smooth rim of suckers due to falling off of hooks.

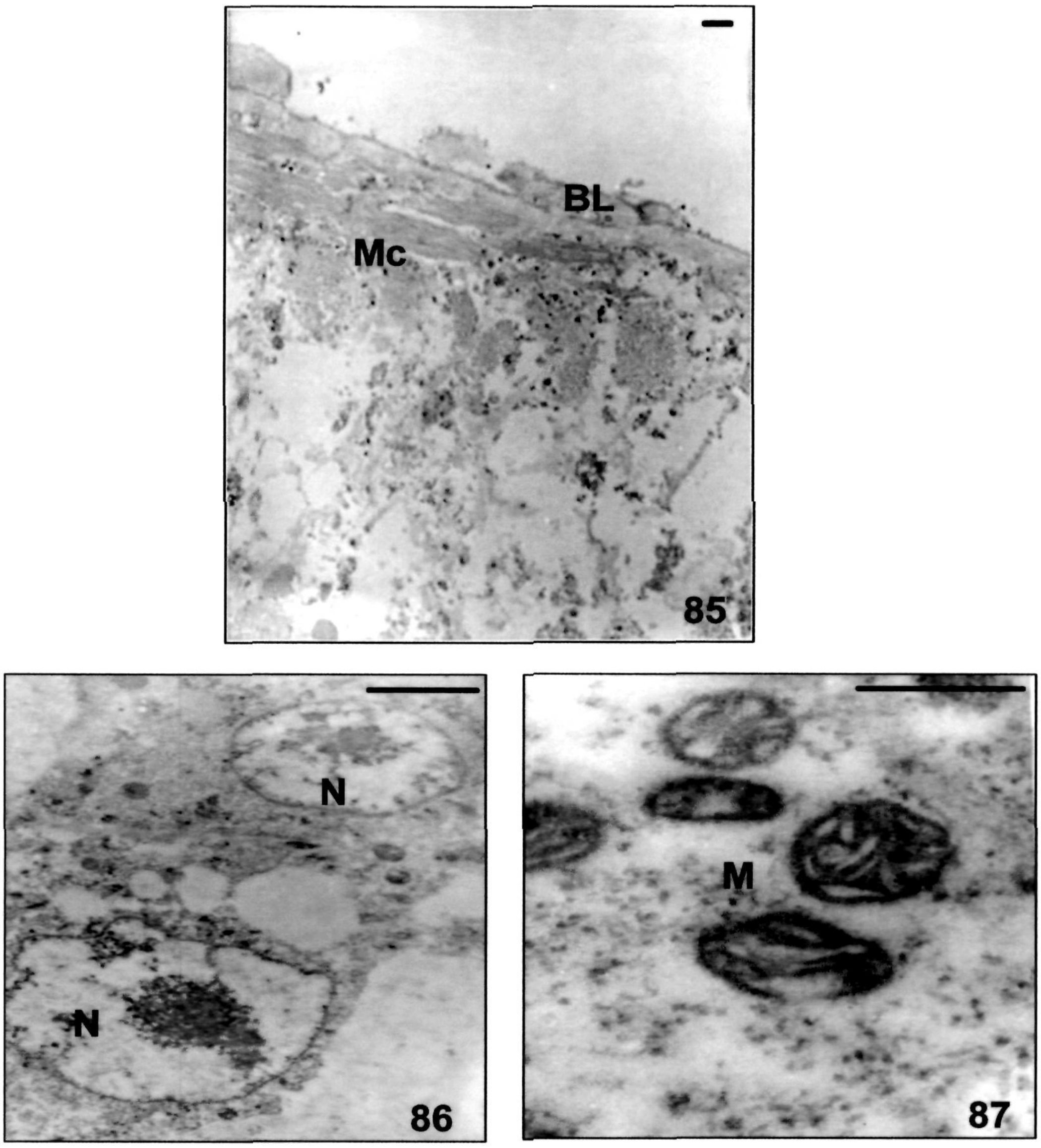




Figs. 83, 84: Scanning electron micrographs of *R. echinobothrida* treated with virosecurinine of *S. virosa* (1 mg)

Fig. 83. Deformed proglottides.

Fig. 84. Tortuous infoldings of tegument which has a glossy texture due to lack of microtriches.



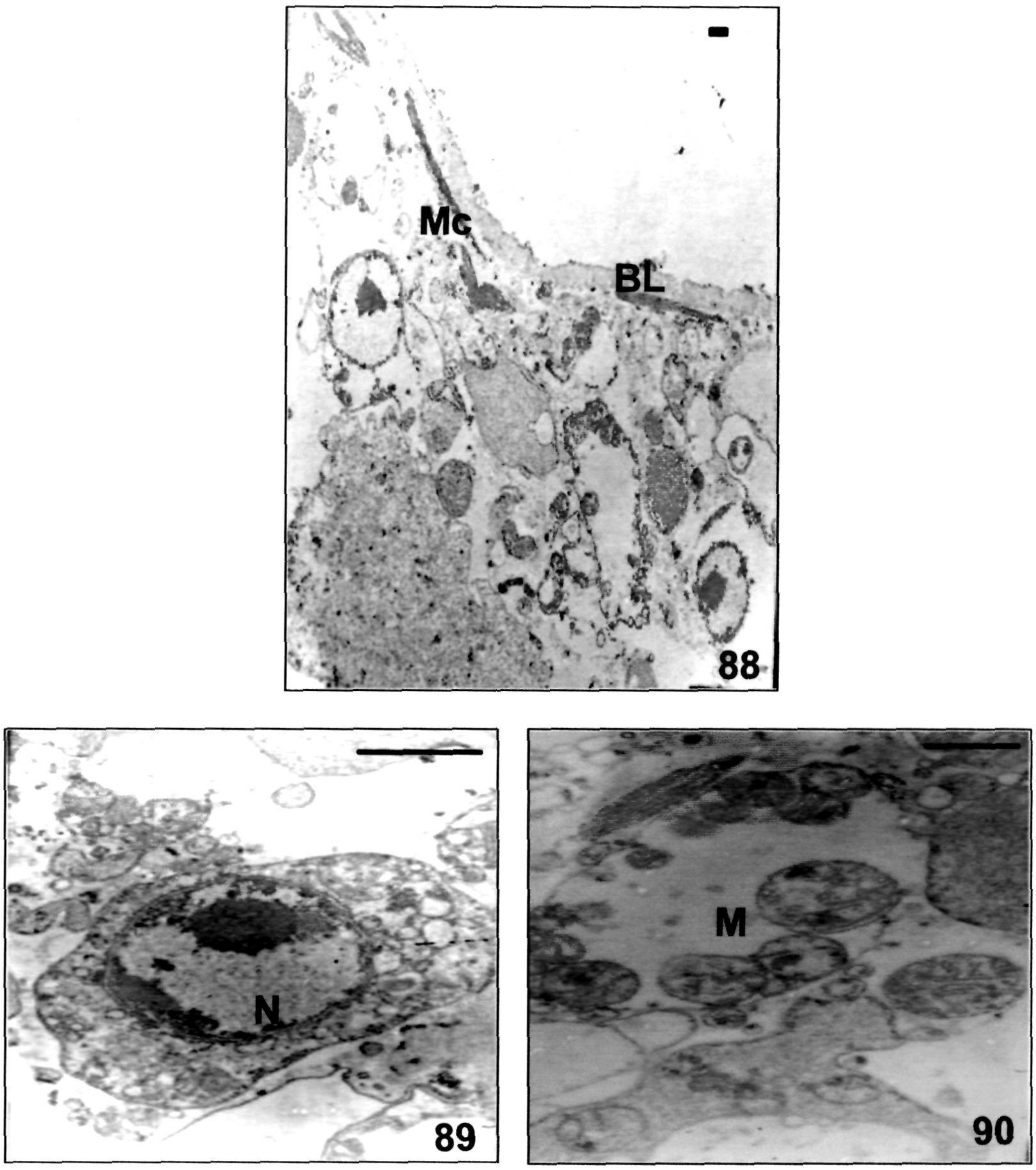
Figs. 85-87: Transmission electron micrographs of *R. echinobothrida* treated with Fraction F5-1d of *A. oxyphylla* (1 mg)

Fig. 85. Threads of basal lamina and disorganised muscle blocks.

Fig. 86. Distended nuclei within the cytons.

Fig. 87. Mitochondria almost normal with only slight damage.

All bars 0.5 μm



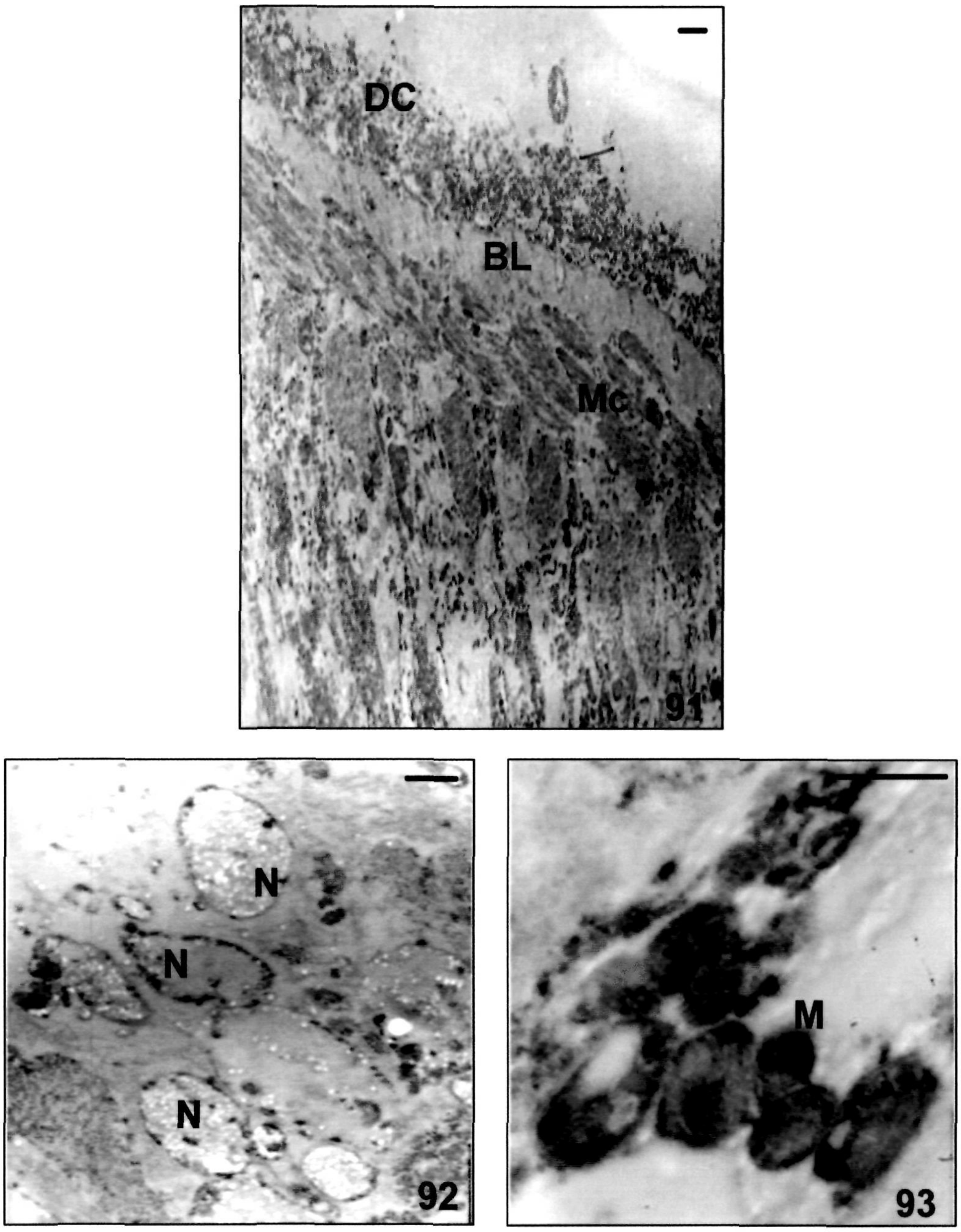
Figs. 88-90: Transmission electron micrographs of *R. echinobothrida* treated with 12-amino-7, 17-dioxo-2-oxa-8, 16-diazatricylo [14.2.2.2 3, 6] tetraicosa-1 (20), 3, 5, 18, 21, 23-hexaene-12-carboxylic acid of *A. oxyphylla* (1 mg)

Fig. 88. Stripped off tegument with highly swollen subtegumental cytons.

Fig. 89. Vacuolated cyton with chromatin clumping in the nucleus.

Fig. 90. Mitochondria few and mostly acristate.

All bars 0.5 µm



Figs. 91-93: Transmission electron micrographs of *R. echinobothrida* treated with virosecurinine of *S. virosa* (1 mg)

Fig. 91. Dissolved microtriches with remnants of distal cytoplasm; intact basal lamina and muscle blocks, and electron lucent subtegument.

Fig. 92. Bloated nuclei with no distinct nucleoplasm.

Fig. 93. Deformed mitochondria with few cristae.

All bars 0.5 μm

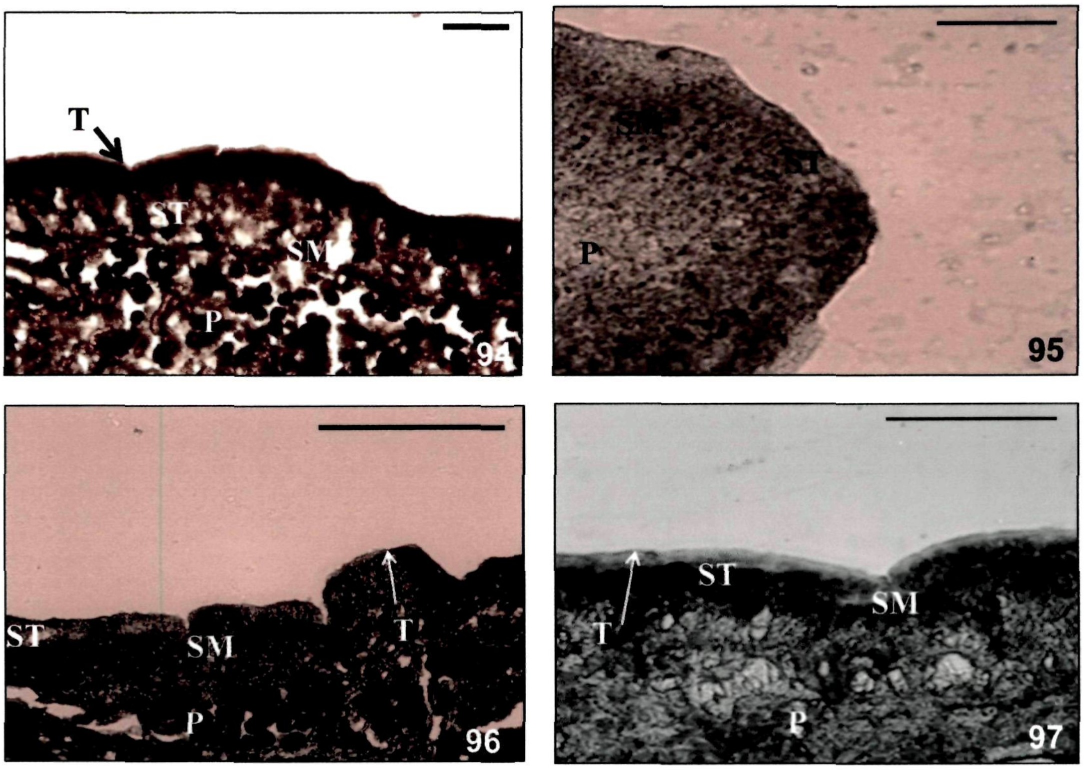
Table 12: Histochemical localization of ATPase, AlkPase and AcPase in various structures of *Raillietina echinobothrida* treated with active principles F5-2d and virosecurinine, and reference drug praziquantel

Control/Treated cestode with	ATPase				AlkPase				AcPase			
	T	ST	SM	P	T	ST	SM	P	T	ST	SM	P
Control	++++	++++	+++	+++	++++	+++	+++	++	++++	++	++	++
F5-2d (1 mg/ml)	NP	++	+	-	NP	+	+	-	NP	-	+	-
Virosecurinine (1 mg/ml)	+	+	++	++	NP	-	+	-	+	+	+	+
PZQ (0.001 mg/ml)	-	+	+	-	-	+	-	-	+	+	-	-

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

T-Tegument; **ST**-Subtegument; **SM**- Somatic musculature; **P**- Parenchyma; **NP**- Structure not present

++++ Very intense activity, +++ Intense activity, ++ Moderate activity, + Mild activity, - No activity



Figs. 94-97: Adenosine triphosphatase (ATPase) activity in *R. echinobothrida*, frozen sections

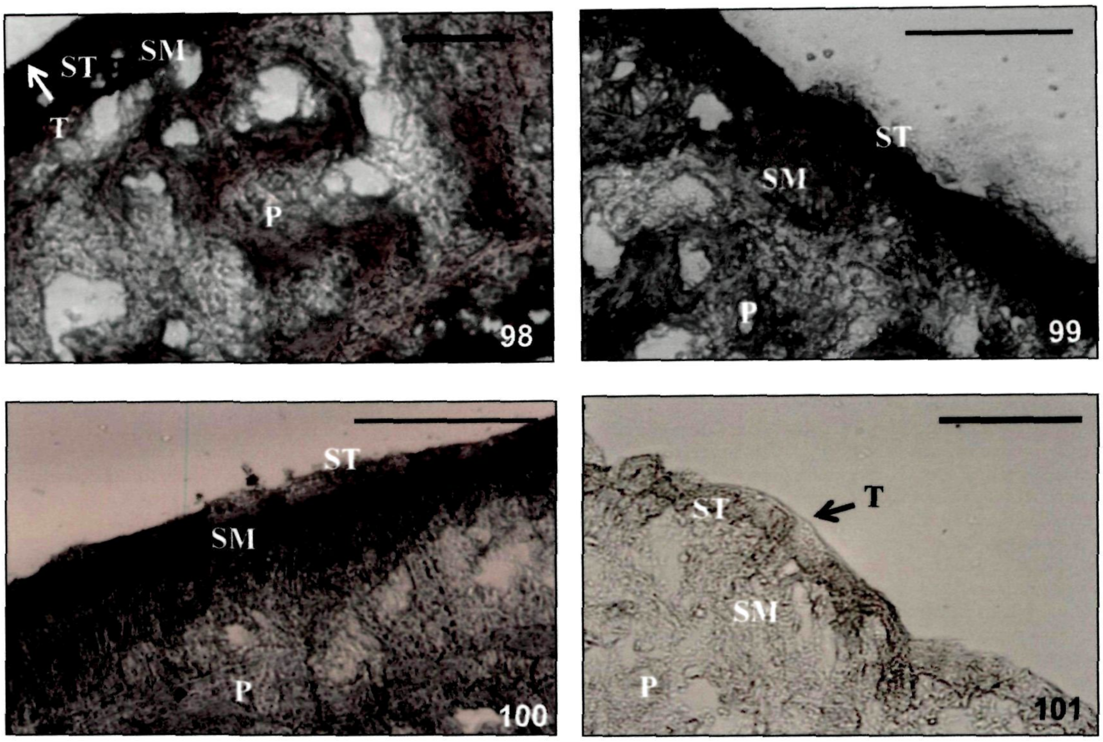
Fig. 94. Control (T- tegument, ST- subtegument, SM- somatic musculature, P- parenchyma) section showing high ATPase activity

Fig. 95. 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 treated parasite section showing very low enzyme activity in somatic musculature and parenchyma

Fig. 96. Virosecurinine treated parasite section showing low stain intensity in tegument and subtegument

Fig. 97. Praziquantel treated section showing diminished enzyme activity in tegument, somatic musculature and parenchyma

All bars 50 μm



Figs. 98-101: Alkaline phosphatase (AlkPase) activity in *R. echinobothrida*, frozen sections

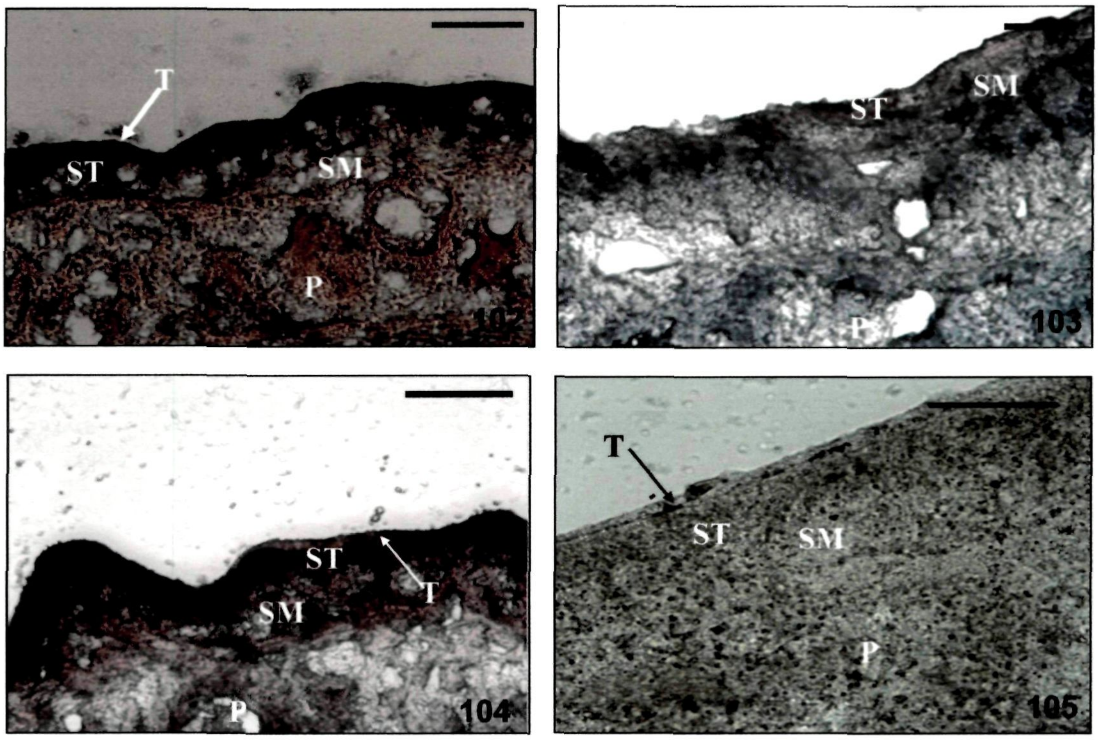
Fig. 98. Section of control worm showing intense staining for AlkPase

Fig. 99. 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 treated section showing diminished enzyme activity in subtegument and muscles

Fig. 100. Virosecurinine treated section showing no enzyme activity in subtegument and parenchyma

Fig. 101. Praziquantel treated section showing a decline in enzyme activity

All bars 50 µm



Figs. 102-105: Acid phosphatase (AcPase) activity in *R. echinobothrida,* frozen sections

Fig. 102. Section of control worm showing high AcPase activity

Fig. 103. 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 treated section showing very mild enzyme activity in somatic musculature

Fig. 104. Virosecurinine treated section showing reduced enzyme activity

Fig. 105. Praziquantel treated section showing no activity of AcPase in muscles and parenchyma

All bars 50 µm

Discussion

In vitro

Results obtained from the present investigation indicate that *S. virosa* and *A. oxyphylla* indeed have anthelmintic efficacy on poultry cestode *R. echinobothrida*. When treated with the ethanol extract and acetone and methanol fractions of these plants, the parasites entered into a state of paralysis soon after incubation which is consistent with other *in vitro* studies on cestocidal and trematocidal activities (Roy and Tandon, 1996; Tandon *et al.*, 1997; Roy *et al.*, 2007 a, b, 2008, 2009). Thereafter, they proceeded towards death maintaining a direct relation between the concentrations of the plant extracts and the timings taken to attain mortality. However, the parasites incubated in the lowest dosage of plant extracts took a significant amount of time to get paralysed or arrive at mortality. As it happens with natural products, the most biologically effective substances constitute only a minor proportion of the plant extract. There are many undesirable compounds or extraneous residues present in the extract, so that at low concentrations, the biological effect is greatly diminished (Mwambete, 2009). Therefore, consumption of large quantity of extract is generally necessary to induce a substantial pharmacological effect. Thus, even though incubation of the parasites in lower doses of the plant extracts took a longer stretch of time for attainment of mortality, paralysis did occur within a time period that could imply the possibility of weakening of the hold of the parasite in the host's gut and its final expulsion from the host by gut peristalsis. So, it is assumed that at lower dosages, the plant preparations may function as vermifuges. A number of ethno medicinal plants used by the neighbouring Naga tribes have been demonstrated to show similar anticestodal activity including *Psidium guajava*, *Houttuynia cordata* and *Lasia spinosa*, while many of the traditionally acclaimed plants did not show significant efficacy (Temjenmongla and Yadav, 2005).

A higher efficacy was noted for the methanol fraction of *S. virosa* instead of the acetone fraction, even though the latter gave acceptable results both in terms of motility and survival of the worms. Ethanol extract, methanol and acetone fractions of plant extracts have been reported to be most active as anthelmintics rather than other extracts made in other polar solvents like ethylacetate, diethyl ether, etc. (Barrau *et al.*, 2005; Iqbal *et al.*, 2006; Ademola and Idowu, 2006; Fujimaki *et al.*, 2005). In a similar kind of *in vitro* experiment, Lalchhandama (2008) showed that the ethanol extract of root peel of *Millettia pachycarpa*, an anthelmintic medicinal plant of Mizoram, had maximum cestocidal efficacy as compared to its methanol and acetone fraction. Relative analysis of the motility and mortality of the two plant extracts revealed *A. oxyphylla* to be a faster acting anthelmintic as compared to *S. virosa*. The acetone fraction of the former proved to be the most effective with ethanol extract coming moderately close. On the other hand, the methanol fraction of *S. virosa* showed earlier paralytic effects on the parasites as compared to the acetone fraction or ethanol extract. Therefore, it may be speculated that *S. virosa* can cause considerable inhibition of the muscle movements of the parasites leading to their expulsion from the host intestine. However, it seems that *A oxyphylla* can bring about the same effect at a much shorter interlude.

Scanning electron micrographs of the present study showed that both the plant extracts (*A. oxyphylla* and *S. virosa*) caused morphological alterations on the tegument of *R. echinobothrida.* Control cestodes exhibited smooth surfaces with finely arranged microtriches all over the body whereas, plant extracts' treatment caused the microtriches on the parasite surface to clump, giving the tegument a coarse appearance. Similar kind of observations was also recorded in *R. echinobothrida* treated with crude extract of *M. pachycarpa* (Roy *et al.*, 2008). While the fastest acting fraction (methanol fraction) of *S. virosa* caused lesions and cracks around the suckers of the tapeworm, the fastest acting fraction (acetone fraction) of *A. oxyphylla* caused the scolex to go completely twisted and contorted. Thus, the two plants exerted two different types of effects on the test parasite. Incubation of *Fasciola gigantica* in *Siwa propolis* caused blebbing and lesions on the surface, and the tegument was marked by a number of pits caused by the loss of spines (Hegazi *et al.*, 2007). Surface changes to the adult fluke *Fasciola hepatica*, when treated with genistein, comprised swelling and blebbing, especially in the posterior region, and there was particular disruption to the spines, accompanied by some spine loss (Toner *et al.*, 2008). Same kind of destructive effects were witnessed in *R. echinobothrida* as well on treatment with genistein (Tandon *et al.*, 1997).

The main injury observed in the tegument of the worms was the widespread vacuolization. Further and ensuing the degeneration of the syncytial matrix and the extensive swelling of the inner membrane folds, countless large vacuoles appeared. Most parenchymal cells suffered from necrosis, as indicated by their swelling, increased electron-lucency, and absence of their mitochondria and nucleoli. Furthermore, the mesenchymal matrix endured widespread vacuolative degeneration. On the other hand, the injury assaulting the tegument-forming cells was the cytoplasmic lysis. Additionally, and other than the disappearance of the nucleoli and significant dilatation of the nuclear membrane, the nuclei of the tegumental cells showed loss of nucleoplasm due to rupture of the nuclear membrane at many places. Cyclosporin A (CsA), an immunosuppressive component, when tested against the tapeworm *Hymenolepis microstoma in vitro*, the initial foci of drug damage were the parasite surface and mitochondria in the syncytium (McLauchlan *et al.*, 2000). Changes many a times have been noticed in the tegument and in the intracellular material of the parasites as in the case of *H. fraterna* when treated with anthelmintics like taenifugin (Stoitsova *et al.*, 1992). Blebbing of surface along with accumulation of membrane fragments over the microthrix tips and erosion of the microthrix layer have also been observed with anticestodal drugs as observed in the present study involving *A. oxyphylla* and *S. virosa*. Cysteine proteinases isolated from papaya (*Carica papaya)* can cause digestion of the tegumental surface of *Rodentolepis microstoma* as proven by Stepek *et al.*, (2007). The strobila of this cestode comprises of a chain of proglottids which, on incubation with papaya latex or stem bromelain, became smaller and erupted in blisters over the surface.

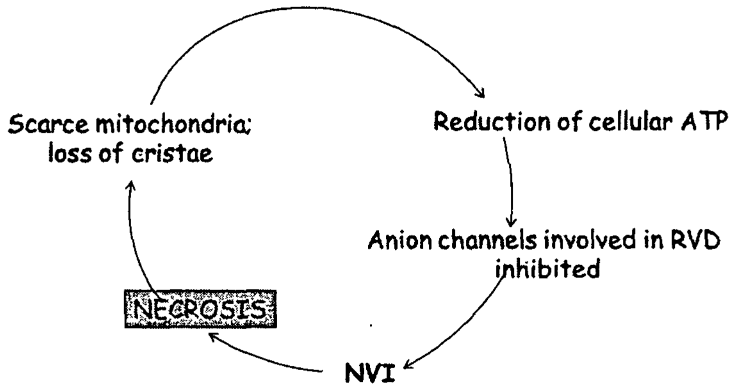
In the present study, the disorganization seen due to the action of both the plants in the parasites was primarily of plasma and organelle membranes with the net result being a necrotic volume increase (NVI) within the cells until cell membrane rupture which gave rise to cytolytic release of internal parasite substances especially associated with nuclear materials, resulting in tissue injury and inflammation and lysis /dissolution (Toner *et al.*, 2008; Roy *et al.*, 2009). Also the cell–cell contacts and the muscular fibres appeared disorganized in many areas. Differentiated cells disappeared, and cellular residues were present in large parts of the tissue. Many nuclei exhibited a high degree of chromatin condensation, the cytoplasm in many cells was largely vacuolized, large numbers of lipid droplets were visible and mitochondria when present, mostly appeared electron lucent and vesicle-like. Erosion of microtriches, distortion and disruption of mitochondria, nucleus, nucleolus, nuclear membrane, basal lamina, and tegumental vacuolization in the distal cytoplasm leading to scar formation in the surface was also noticed in *R. echinobothrida* treated with *M. pachycarpa* (Roy *et al.*, 2008).

The microtriches in the tegument are thought to serve two functions. Firstly, the tapeworms do not possess a gut and must absorb all of their nutrients across the surface tegument. The microtriches greatly increase the surface area of the parasite, and can be seen as an adaptation to maximise the amount of nutrient absorption in the parasite (Smyth, 1996). This is supported by the finding of microtubules in the shaft of the microtriches. The tegumental erosion noticed in this study thus might have caused severe nutrient deficiency within the parasite. Secondly, the spine-like character of the microtriches of the scolex probably helps the parasite maintain its position in the gut (Lalchhandama, 2008). This can be more clearly seen by comparing the microtriches found in different regions of the parasites body. It has been noted in many species that the microtriches found covering the scolex, the attachment organ of the parasite, are much longer than those covering the strobila, and in some species show special adaptations. For example, the microtriches covering the strobila of *E. granulosus* have been found to be curved with hooks or sometimes even barbs (Thompson *et al.*, 1982). So, depletion of the microthrix layer, as observed, can cause the parasites to lose their hold to the host gut.

Disruption and dissolution of parasitic tegument/cuticle in the presence of different plant extracts is not new. It was first suggested in the late 1870s (Wurtz and Bouchut, 1879) and then shown convincingly in the last century, first with extracts of figs (Robbins, 1930) and then pineapples and papain on *Ascaris suum* (Berger and Asenjo, 1939, 1940). The same authors found that the tegument of porcine acanthocephalan parasite *Macracanthorynchus hirundinaceus* disappeared in the presence of fresh pineapple juice (Berger and Asenjo, 1939). The observations that cysteine proteinases actually damage cestodes (*R. microstoma*, as mentioned earlier) and acanthocepahalans, raise the possibility that there may be common target sites in proteins in the nematode cuticle and the cestode and acanthocephalan teguments (Behnke *et al.*, 2008).

The location of important cellular elements in the subtegumental cytons, away from the outer surface of the parasite is an important adaptation to a parasitic lifecycle adopted by all of the parasitic platyhelminths to avoid immunological attack by host. However, when the tegument gets eroded, these cellular components get exposed to host attack and dissolve causing high metabolic stress. The present study revealed that, *in vitro*, the plant botanicals from *A. oxyphylla* and *S. virosa* caused substantial damage to the surface of parasitic cestodes (*R. echinobothrida*), which resulted in a significant reduction in the physical activity of the parasites. Despite the lack of speed in attaining worm mortality, *S. virosa* possessed good efficacy against the cestode, as shown by its apparent effect on the tegument, perhaps indicating a different mechanism of action. However, in most cases, the *A. oxyphylla* stem bark appeared to have the greater potency.

The interaction of the tegumental surface membrane with the underlying cytoskeleton may have been disrupted at focal points in the microtriches, resulting in a loss of the membrane anchoring required for the structural integrity of the tegument. Vacuolization of the tegument was observed as an effect of several chemotherapeutic agents as seen in other studies (Mehlhorn *et al.*, 1981; Kohn *et al.*, 1982; Shaw and Erasmus, 1983, 1987; Popiel and Erasmus, 1984; Shaw, 1990; Xiao *et al.*, 2002; Taha, 2007). Mehlhorn *et al.* (1981) observed that vacuolization of the tegument leads to disruption of the apical tegument layer which eventually causes the parasite to die. Shaw and Erasmus (1983) assumed that the vacuolization and the loss of tegumental matrix are because of the formation of blebs. In many studies with cestodes, the intracellular organelles were changed in structure, showing increased electron density or lucency in some areas and oedema in other areas. Apart from causing vacuolization and disruption of the tegumental matrix, the plants caused lytic degeneration to the tegumental cells and swelling and release of materials from the parenchymal cells. Similar results were also obtained under the influence of antibilharzial agents (Shaw and Erasmus, 1983, 1987; Xiao *et al.*, 2002; Taha, 2007). Since the cells clearly showed signs of necrosis, we can deduce that they might have lost control of ion flux resulting in reduction in concentration gradients of Na^+, K^+, Ca^{2+}, Mg^{2+}, as well as changes in osmotic pressure. During apoptosis, mitochondria remain functionally intact, providing the necessary energy (Okada *et al.*, 2004). But during necrosis, they face NVI as anion channels involved in regulatory volume decrease (RVD) are inhibited due to subsequent reduction of cellular ATP and elevation of cytosolic Mg^{2+}, lose cristae and eventually disintegrate (Okada *et al.*, 2004), as was noted in this study.



Probable steps leading to loss of mitochondria because of necrosis

Granular endoplasmic reticulum (GER) is believed to be the site of protein synthesis including enzymes and hormones. So, its vesiculation reflects poor cellular ability to synthesize proteins needed by cells in the process of wear and tear. Also, the mitochondria, in addition of being the sites of cellular energy, they are indirectly concerned with the synthesis of amino acids. Therefore, it is not surprising that cell respiration is inhibited if mitochondria lose their regular organization (Stringer *et al.*, 1971) and protein synthesis will be affected because of lack of energy (Robbins and Angell, 1976).

Another common response observed after the drug treatments in the present study is the appearance of myelin-like membranous whorls or vesicles. The production of blebs and membranous whorls is regarded to be an emergency repair mechanism utilized by the worms to replace damaged surface membranes (Shaw and

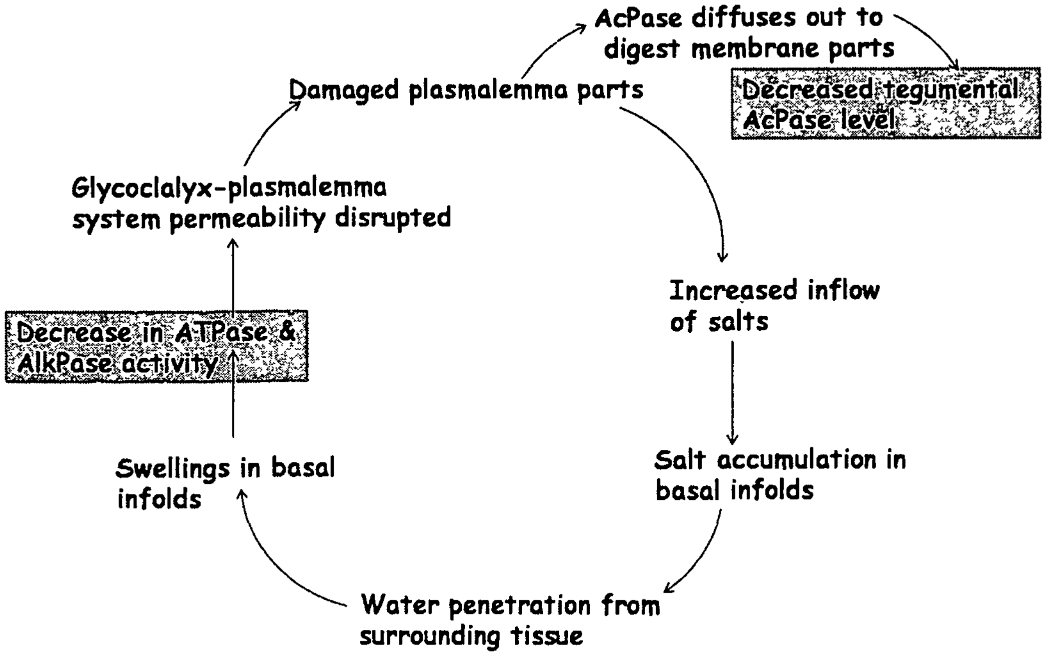
Erasmus, 1983). During the investigations, membranous whorls were noticed interspersed throughout the parenchyma of the *A. oxyphylla* extract-treated parasite. The formation of membranous whorls and blebs has also been observed on the tegument of worms treated with other anthelmintics (Voge and Bueding, 1980; Mehlhorn *et al.*, 1981; Kohn *et al.*, 1982; Amin & Mikhail, 1989; Shaw & Erasmus, 1983, 1987; Fallon *et al.*, 1996; Mostafa and Soliman, 2002; Shaohong *et al.*, 2006; Taha and Soliman, 2007). Shaw and Erasmus (1983) also noted large, membranous whorls and small, lucent vesicles in the gastrodermis of *S. mansoni* treated with PZQ *in vivo*. This phenomenon also occurred following worm starvation in the gastrodermis of *P ohirai* (Fujino and Ishii, 1978) and some other trematodes (Bogitsh, 1973, 1975).

The active principles of the two plants in the present study greatly intensified the damaging effects on the tegument of the parasite at concentrations lower than that of the crude plant extracts. Both the final fractions (F5-1d and F5-2d) of *A oxyphylla* exerted extreme adverse effects on the parasite, but 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) seemed to be a more powerful agent in terms of anthelmintic efficacy. On the other hand, virosecurinine seemed to be comparatively less active as noted from the surface alterations observed through SEM.

Intense ATPase, AlkPase and AcPase activities were observed in the tegument and subtegument areas of control *R echinobothrida* while these enzymes were noted to diminish in the parasites treated with both the plant extracts and their active principles. Cytochemical studies at the ultrastructural level have indicated that phosphatases are localised in or on the tegumentary brush border of cestodes (Rothman, 1966; Lumsden *et al.*, 1968). It has been suggested that these enzymes act in digestive capacity, hydrolyzing phosphate esters to which the cell may be virtually impermeable (Read, 1966; Lumsden *et al.*, 1968). Phosphatases are important tegument proteins whose association with membrane transport have been implied by their ubiquitous presence in tissues of secretory or absorptive function. The view that physiological transport activity is mediated directly by enzymes located on or within cytomembranes (Mitchell, 1961), has received much support in recent years. Nucleotides are converted to their nucleoside derivatives prior to passing through the surface membrane of parasites. Depending on the relative permeability of the surface membrane to phosphorylated compounds, hydrolysis of phosphate esters at the strobilar surface could facilitate the subsequent passage of the organic portions of the molecule across the plasmalemma (Lumsden *et al.*, 1968).

Phosphatase functions in regulating metabolic processes, with alkaline phosphatase and adenosine triphosphatase taking part in active transport through cellular membranes and acid phosphatase (lysosome marker) indirectly providing information regarding intracellular digestion processes. In many parasites, the presence of phosphatase is regarded as the indicator of the areas responsible for secretion and excretion activities and the absorption of nutritive components. High activities of both acid and alkaline phosphates have been confirmed in the tegument, subcutaneous cells and parenchyma of tapeworms (Arme,1966; Niemczuk, 1993). Arme (1966) and Niemczuk (1993) established a clear dependence of the activity of these enzymes on the degree of maturity of tapeworm segments with greater activity observed in mature segments. The alkaline phosphatase in the mammalian small intestine is believed to participate in the degradation of organic phosphate to free phosphate. Although there is no lucid evidence, the enzyme has been known to act in the substance transportation (Kaplan, 1972). Dusanic (1959) observed alkaline phosphatase activity in *Schistosoma mansoni* and reported that alkaline phosphatase supports the absorption and secretory functions of the parasites. Among the majority of nematodes, the activity of acid phosphatase in the tegument is high and is correlated with the absorption of glucose through the body wall (Maki and Yanagisawa, 1980). According to Skotarczak (1987), both enzymes play an important role in the metabolic processes of helminth embryos, and their activity depends on the metabolic intensity. These enzymes are believed to be responsible for the transport of substances across the walls of excretory canals and also in ionic regulation.

It is widely speculated that when there is a decrease in the tissue ATPase activity, the permeability of the glycocalyx-plasmalemma system is disrupted causing the plasmalemma parts to get damaged. This in turn causes the AcPase to diffuse out to digest the damaged membrane parts leading to a decrease in the tegumental AcPase levels. Damage of the parasite membrane also causes an increased inflow of salts from the external media which accumulates in the basal infolds (Fairweather and Boray, 1999). To compensate, there is penetration of water from the surrounding tissue leading to swellings in the basal infolds (as observed in *A oxyphylla* ethanol extract treatments) and vacuolization of tissue (Anderson and Fairweather, 1995; Dasgupta *et al.*, 2010). In treatments with the two plant extracts, AcPase diminished the most followed by ATPase in case of *A. oxyphylla*-treated parasites while AlkPase declined most in case of *S virosa*-treated parasites. This proved that there was extensive digestion of the tegument in *A oxyphylla*-treated parasites (in tune with observations made by electron microscopy) by tegumental AcPase which eventually leaked out causing a drop in its level, while in case of *S. virosa*-treated parasites, the tissue pH was majorly disrupted due to lowering of the AlkPase level because of which most of the damages were interior in the subtegumental level (as also observed through transmission electron microscopy). The active principles of the plants also produced results corroborating with the results obtained through the crude plant extracts as observed by histochemical staining. AlkPase activity was reduced majorly with virosecurinine, and AcPase activity was reduced most with F5-2d. The reference drug PZQ also showed the highest decrease in AlkPase and most of the damages observed were tegumental than internal organs.



Probable route/causes of damage of tegument resulting from decline in the levels of tegumental enzymes

Treatment of *R. echinobothrida* with the plant extracts and PZQ led to the decrease in the levels of certain free amino acids and increase in the levels of others. Protein metabolism in parasites is less well known than carbohydrate metabolism, but it may be considered more important because all animals require nitrogen in their metabolism. Compared with protein amino acids, the free amino acid fraction in tissues is small and probably represents several independent pools (Barrett, 1991). Free amino acid pool is considered to be the protein precursor of the amino acid pool and is thus a very important prelude to study of protein metabolism. Parasitic helminths show a wide range of free amino acid levels, with the highest levels being found in marine monogeneans (Arme, 1977). Amino acids of late have emerged as potential targets for anthelmintic drug development, as they are a major constituent of all biological materials. The latest drug designs have exploited the differences between the pathways of amino acid metabolism in helminth parasites and their mammalian hosts.

Scanty information is available regarding the amino acid metabolism in helminths. There are published reports on the free amino acid (FAA) pool of several cestode species (Chappell and Read, 1973; Soutter *et al.*, 1980). Most of the helminths studied to date have been found to excrete significant amounts of nitrogen in the form of amino acids, peptides or proteins. The excretion of amino acids can provide a means of detoxifying ammonia that is known to have a neurotoxic effect and also to cause various neurological disorders (Cooper and Plum, 1987; Campbell, 1991), of which paralysis of the worm may be one such manifestation.

In cestodes, the size of the free amino acid pool ranges from 100 to 400 mg per 100 g fresh weight, the highest values being found in the tapeworms of sharks (Simmons, 1969). In *Hymenolepis diminuta*, the major free amino acid is reported to be alanine (Daugherty, 1952; Foster and Daugherty, 1959; Webb, 1986), although the free amino acid pool of *H. diminuta* seems to be, at least to a certain extent, in equilibrium with the external amino acid pool (Arme and Read, 1969). *H. diminuta* is capable of volume regulations in hypotonic fluids and an increased efflux permeability of the brush border of *H. diminuta* to amino acids was noticed during hypotonic incubations, thus establishing the fact that free amino acids may be involved in osmotic regulation (Lussier *et al.*, 1978; Wack *et al.*, 1983) as there is always a danger that high levels of a single osmolyte will disrupt protein structure.

The problem of amino acid absorption by the helminth parasites have been recorded by several authors. The studies of Daugherty (1957 a, b), and of

Daugherty and Foster (1958) demonstrated active transport of certain amino acids (labelled methionine and cystine) into *Hymenolepis* and *Raillietina*. Read *et al.* (1960) declared that either valine or leucine competitively inhibits permeation of the other, and that certain other amino acids inhibit permeation of valine or leucine. Since proline is apparently involved in protein synthesis and the formation of collagen, it is expected that considerable amount of exogenous proline will be absorbed by the parasites. In the present investigation, treatments with *A. oxyphylla* revealed considerable decrease of Gly, Ala, Cys, Arg and Glu, whereas, Pro, Ser and Val levels remained unchanged. This simultaneous accumulation of Pro with an absence of Ala could be due to competition for substrate of the enzymes in the Ala and Pro biosynthesis. Ala and Pro are known to be the true metabolic end products of carbohydrate metabolism. There is evidence that alanine, which was present in greatest quantity in all samples, may be an end-product of glucose catabolism (Roberts, 1983). Wack *et al.* (1983) found substantial quantities of label in alanine when *H. diminuta* was incubated in 14C-glucose. It appears that alanine is formed from pyruvate by the action of alanine aminotransferase, glutamate being the amino group donor (Wack *et al.*, 1983). Cestodes treated with crude extract of *S. virosa* revealed a decrease in Gly (50%) and Glu levels; whereas an increase in the levels of Pro, Ala and Cys was recorded as compared to control ones. Arg levels remained unchanged just like PZQ-treated parasites. Thus, there was an increase in the end products of carbohydrate metabolism on treatment with *S. virosa* (Roberts, 1983). As Arg and Pro are the kind of amino acids that are different in helminths and mammals, this point of energy metabolism can be exploited in drug design against intestinal parasites. Though Val levels remained unaltered in *A. oxyphylla*-treated parasites, it decreased by about 50% in *S. virosa*-treated parasites and both the plant treatments brought about a significant decline in the Leu/Ile levels indicating that other amino acids somehow inhibited the permeation of the two amino acids in case of *S. virosa* treatments. Glutamic acid, a common substrate of glutamine, arginine and proline and the primary NH_4^+ acceptor, as well as the product of ammonia assimilation, showed a considerable decline in both the plant extract-treated parasites.

Parasites treated with the plant extracts and PZQ showed a significant decline in the levels of calcium and magnesium and a relevant increase of the same in the incubation media. Bricker *et al.*, (1983) demonstrated the correlation between vacuolization of the parasite tegument and the concentration of Ca^{2+}. The Ca^{2+} ATPase is found to be unable to control the rise of Ca^{2+} ion concentration brought about by PZQ. It has also been postulated that different calcium channels might be involved in the impairment of the calcium homeostasis within the parasites. *In vitro* disruption of the bilayer structure of synthetic phospholipid vesicles (phosphatidylserine, phosphatidylethanolamine) can be induced in the presence of calcium by PZQ resulting in the formation of hexagonal structures. The appearance of such hexagonal structures in the tegumental membranes may thereafter facilitate Ca^{2+} entry into the worms leading to a disturbance of Ca^{2+} homeostasis and overall changes in membrane integrity. Probably, interactions between negatively charged phospholipids, Ca^{2+} ions and the electrically neutral praziquantel are responsible for the perturbations of membranes. Therefore, any significant alterations induced by a chemotherapeutic agent in the Ca^{2+} influx/efflux of a cestode or trematode system can be critically disturbing for the normal contraction of the parasite musculature. Tetas and Lowenstein (1963) have demonstrated that a number of divalent cations, including calcium, copper, magnesium, and manganese, catalyze the hydrolysis and transphosphorylation of phosphate esters in the absence of the appropriate enzymes. So, alterations in the levels of these elements can have a devastating effect on the transport system of cestode parasites. Therefore, it is highly feasible to assume that similar cestocidal activity of the two test plants is attributable to similar internal alterations brought about by their active principles.

In vivo

In the present study, a noteworthy decrease was noted in the post treatment EPG count as compared to the pre-treatment EPG count in all treatment groups. To evaluate the effect of plant extract against intestinal helminth parasites, the number of eggs excreted in faeces has been used as the endpoint (Akhtar *et al.*, 1982; Gathuma *et al.*, 2004). A useful anthelmintic effect can be obtained from any ethno veterinary medicine preparation if the level of reduction in faecal egg count of treated animals is in the order of 70%. This is the criterion that has been used by scientists to evaluate the anthelmintic properties of a number of plants to treat small ruminants (Githiori *et al.*, 2002, 2003, 2004). The EPG counts obtained in the present study have shown more than 70 % reduction post treatment with *A. oxyphylla* and near 70% reduction with *S. virosa* treatments. A daily treatment with ethanol extract of *Canthium manni* (Rubiaceae) at 5600 mg/kg, showed a 75% decrease in fecal egg count and 84% reduction in worm burden with 7 days of treatment of rodent (Wabo *et al.*, 2009). A 600 mg/kg treatment with extract of stem bark of

Sacoglottis gabonensis was extremely effective, but exceedingly toxic, with mice showing signs such as depression, drowsiness, unsteady gait and paralysis of the hind limbs, dyspnoea, coma and death apparent within 1-2 min following intraperitoneal injection (Nwosu *et al.*, 2008).

The results in the present study indicate that the efficacy of the plants is dose- dependent. Lower worm counts after treatment periods as recorded in the present study may be owing to loss of worm and/or process of destrobilation (Dixon and Arai, 1991). It is known that the cestodes usually undergo the process of destrobilation if exposed to any anthelmintic drug or if owing to any physiological stress in the intestine. The worm recovery rate decreased subsequently on increasing the dose of the plant extracts. Similar results were also recorded when *H. diminuta* was subjected to *in vivo* treatments with the crude extracts of *Trifolium repens* and *Psidium guajava,* two traditionally used medicinal plants of Northeast India (Tangpu *et al.*, 2004; Temjenmongla *et al.*, 2006). Low worm burdens have been observed post treatment with other ethno veterinary medicine preparations against cestode-infected hosts including humans (Desta, 1995), rodents (Galal *et al.*, 1991; Vishnyauskas *et al.*, 1993; Bogh *et al.*, 1996; Ghosh *et al.*, 1996; Molgaard *et al.*, 2001) and ruminants (Akhtar and Riffat, 1986). The recovery of low number of worms on necropsy after treatment and reduction in EPG count further substantiates the claim regarding the anticestodal efficacy of the two plant extracts. Thus, the extracts of the two plants showed major anthelmintic efficacy at dosages of 50 mg/kg (*A. oxyphylla*) and 1500 mg/kg body weight (*S. virosa*).

The results obtained in the present investigation through *in vitro* and *in vivo* experiments clearly indicate that the stem bark of *A. oxyphylla*, leaves of *S. virosa* and their active principles act as anthelmintic, as they are responsible for destruction and degeneration of tegument and cellular organelles, reduced activity of vital tegumental enzymes and trace elements, and disruption in the levels of various free amino acids within the tested cestode parasites. However, the mechanism underlying the exact mode of action that brings about the cestocidal activity is not clear; therefore, further detailed investigations with the active principles are required to know the exact mechanism of action of the anthelmintic components of the plants. In this context, standby alternatives will have an important role to play, but more importantly, with investment in improving the delivery and formulations of available plant botanicals, they can be developed into effective anthelmintics.

ABSTRACT

The present study involves an investigation on the anthelmintic efficacy of *Acacia oxyphylla* Graham ex Bentham (Family: Leguminosae) and *Securinega virosa* (Roxb. ex Willd.) Baill. (Family: Euphorbiaceae). The aqueous concoction of the plants (stem bark of *A. oxyphylla*, leaves of *S. virosa*) is taken by the Lushai tribe in Mizoram, a state in Northeast India, to cure intestinal worm infections. The *in vitro* activity of the extracts of the two plants and their active principles was tested against the poultry cestode, *Raillietina echinobothrida*. Alterations in the motility of the parasite, structural changes (both morphological and ultrastructural), changes in important enzymes associated with the tegument, free amino acid pool and vital trace elements were taken as parameters for the study. The plants were also tested *in vivo* against the rodent parasite *Hymenolepis diminuta* maintained in adult Wistar rats. Changes in EPG count pre-treatment and post treatment with the plant extracts and the percentage recovery of worms at the end of experimental period were used as parameters for *in vivo* experiment.

1. *In vitro* studies

a) *Motility and mortality studies: In vitro* treatment of the parasite with the plants' extracts, their fractions, and the reference drug praziquantel (PZQ) led to complete immobilization of the parasite followed by death. On exposure to the test solutions, the worms proceeded from a state of vigorous movements to a relaxed condition in which they continued to remain till they attained paralysis. The time taken for paralysis showed a

dose-dependent effect as paralysis occurred at a shorter period of time for higher concentrations of test materials while for lower doses it took a much longer period of incubation. 10 mg/ml dose of crude extract of *A. oxyphylla* took 3.50 ± 0.12 h to cause paralysis in the parasite, which was comparable with the time frame of onset of paralysis in parasites treated with 0.001 mg/ml dose of PZQ. Onset of paralysis within a time span similar with 0.001 mg/ml dose of reference drug occurred at 3.76 ± 0.11 h, with 25 mg/ml dosage of *S. virosa*, demonstrating *A. oxyphylla* to be a faster acting anthelmintic.

b) *Surface topography and ultrastructural studies:* Stereoscan observations on control *R. echinobothrida* revealed normal contour of scolex having four suckers and a rostellum having spines. Trunk of the parasite comprised of a chain proglottides having fine microtriches arranged in a specific direction. On exposure to crude extract of *A. oxyphylla* and its fractions, proglottides became smaller and the surface erupted into blisters all over. Distortion and deformation of scolex was also prominent. A similar kind of distortion and deformation was observed in parasites exposed to *S. virosa* crude extract and its fractions as well. Transmission electron microscopic observations on the control worms revealed the typical cestode morphology with respect to the tegument, subtegument and inner parenchyma. The tegument of *A. oxyphylla* (crude and fractions of crude extract) treated worms was mostly stripped right down to the basal lamina and the remnants of the basal infolds left behind were highly swollen. There was an increase in electron-lucency of the background due to both glycogen loss and vacuole formation and the subtegument showed immense vacuolization and accumulation of debris. There was very little amount of GER remaining with no evidence of Golgi complexes. The parenchymal cytons of the treated parasites showed complete loss of connections with the surrounding parenchyma and the chromatin in the nucleus appeared clumped into large areas of electron-dense heterochromatin. Disintegrated muscle blocks, vacuolated tissue material, distorted subtegumental cytons and loss of cell organelles were some of the major damages seen. When cestodes were treated with the extract of *S. virosa* and its fractions, the distal cytoplasm was perforated and no tegumental discs or mitochondria were visible. The cytoplasm was observed to stream down into the subtegument basal lamina in the form of thin laminar processes. The lamina itself had vacuoles which went down to penetrate and cause disintegration of the subtegumental cytons (making them hardly discernible) between the muscle blocks. Here too the degree of damages was noted to be dose dependent.

c) *Histochemical localization and biochemical analyses of tegumental enzymes*: On treatment with *A. oxyphylla* and *S. virosa* extracts, and PZQ, a decline in the levels of important tegumental enzymes, namely, adenosine triphosphatase, alkaline phosphatase and acid phosphatase was observed histochemically in various structures within the parasite. The same was confirmed with biochemical tests where a decline in the total and specific activities of these enzymes was observed. While control worms showed the highest activity for ATPase followed by AlkPase and AcPase, the treated worms went on to show decrease in the activities of all the three enzymes. AcPase activity declined most (97%) in the parasites, as compared to ATPase (72%) and AlkPase (90%), when treated with *A. oxyphylla*. On treatment with *S. virosa*, AlkPase activity was noticed to decline most (96%), as compared to ATPase (91%) and AcPase (34.5%).

d) *Free amino acid pool*: The free amino acid pool of the control and the treated worms was evaluated using mass spectrometry. Out of the 20 amino acids detected, Gly was found to be at the maximum level, followed by Ala, Ser, Pro, Val, Thr, Cys, Ile/Leu, Asn, Asp, Gln/Lys, Glu, Meth, His and Phe, respectively, in a decreasing order. Parasites treated with plant extracts and reference drug showed a marked decrease in the levels of Meth, Ile/Leu, Phe, Asn Gly, and Tyr. *A. oxyphylla*-treated parasites showed an increase in the level of Thr while *S. virosa* showed an increase of Ala, Pro, Cys, Thr and Trp.

c) *Trace elements studies*: On exposure to the plants' extracts and PZQ, the parasites were seen to lose high amounts of calcium and magnesium ions into the treatment medium as compared to the control groups which retained high levels of the two elements.

2. *In vivo* studies

Each acclimatized adult rat was infected with 5 cysticercoids of *H. diminuta* and later treated with single doses (25 mg and 50 mg/kg body weight for *A. oxyphylla*, and 750 mg and 1500 mg/kg body weight for *S. virosa*) of the two plants' extracts and PZQ, for three days. A significant decline in the post treatment egg count was noticed in all the treatment groups when compared to the pre-treatment egg counts, except for the group treated with 750 mg of *S. virosa* (16.8%-18.2% reduction only). On autopsy, 50 mg *A. oxyphylla* and 1500 mg of *S. virosa* treated groups showed 87% and 83% decline in worm burden, respectively. PZQ reduced the worm recovery rate by 92%. However, 25 mg of *A. oxyphylla* and 750 mg of *S. virosa*/kg dose brought down the worm burden by 80% and 40%, respectively.

3. Active principles testing:

a) *Motility and mortality studies*: The active component of *A. oxyphylla* was isolated from the crude extract of the plant and was identified 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. Virosecurinine, the active component of *S. virosa* was obtained commercially. When *R. echinobothrida* was incubated in the active principles of the two plants, there was a decline in the motility of worms resulting eventually in their death, taking 5.75 ± 0.1h and 8.9 ± 0.01 h for 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 and virosecurinine, respectively.

b) *Surface topography and ultrastructural studies:* Scanning electron microscopy revealed total depletion of microtriches which fused together to form masses. Proglottides were noted to be super-contorted and seemed to be breaking off from the main body at many places due to extreme shrinkage. The suckers in the scolex collapsed and retreated back, pushing the rostellum forward. Transmission electron microscopic observations showed that treatment with active compounds caused the tegument to strip off and the nuclei of subtegumental cytons to swell up severely. Mitochondria were found sparsely in the parasite body and were mostly acristate.

c) *Histochemical localization of tegumental enzymes:* Histochemically, the control worms showed intense activity for all the three tegumental enzymes (ATPase, AlkPase and AcPase) while the treated worms portrayed a diminished or no activity of the enzymes in different structures of the body.

The active principles (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 and virosecurinine) present within the plants seem to bring about all the alterations noticed in the present study by acting transtegumentally and as the tegument of cestodes happens to be the main interface of nutrition, the resulting effect is the instant paralysis and ultimate death of the parasite.

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Peer-reviewed publications:

- **Dasgupta S and Roy B.** (2010). Antiparasitic activity of methanolic extract of *Acacia oxyphylla* (Leguminosae) against *Raillietina echinobothrida*. Journal of Parasitic Diseases (Accepted, May 2010).
- **Dasgupta S, Roy B and Tandon V.** (2010). Ultrastructural alterations of the tegument of *Raillietina echinobothrida* treated with the stem bark of *Acacia oxyphylla* (Leguminosae). Journal of Ethnopharmacology. 127 (2): 568-571.
- **Roy B, Dasgupta S and Tandon V.** (2009). Ultrastructural observations on *Fasciolopsis buski* and its alterations caused by shoot extract of *Alpinia nigra*. Microscopy Research and Technique. 72 (2): 60-66.
- **Roy B, Dasgupta S and Tandon V.** (2008). Ultrastructural observations on tegumental surface of *Raillietina echinobothrida* and its alterations caused by root-peel extract of *Millettia pachycarpa*. Microscopy Research and Technique. 71 (11): 810-815.
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- **Dasgupta S, Roy B and Manivel V. (2010).** *Raillietina echinobothrida*: an investigation on functional characteristics after treatment with *Acacia*

oxyphylla and *Securinega virosa.* International Journal for Parasitology (Communicated).

- **Dasgupta S, Roy B, Manivel V and Parameswaran PS. (2010).** Surface topographical and ultrastructural alterations induced by compound isolated from *Acacia oxyphylla.* Journal of Ethnopharmacology (Communicated).

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Ethnopharmacological communication

Ultrastructural alterations of the tegument of *Raillietina echinobothrida* treated with the stem bark of *Acacia oxyphylla* (Leguminosae)

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Ethnopharmacological relevance: The stem bark of *Acacia oxyphylla* Graham ex Bentham is used as an anthelmintic by the natives of Mizoram (North-East India).
Aim of the study: The present study was performed to evaluate whether or not the plant-derived components caused any ultrastructural changes in the tegumental interface of the parasite.
Materials and methods: The test parasite *Raillietina echinobothrida*, the cestode of domestic fowl, was exposed to the ethanolic crude extract and acetone fraction of stem bark of *Acacia oxyphylla* for varying concentrations and time duration and processed for transmission electron microscopy as soon as paralysis set in the treated parasites.
Results: Treatment with crude alcoholic extract and its acetone fraction revealed complete inactivation and flaccid paralysis of the cestode, which was soon followed by death. The treated parasites also exhibited intense vacuolization of the tegumental layers along with complete disorganization and/or erosion of microtriches.
Conclusions: Considerable structural alterations in the treated parasites are suggestive of an efficient vermicidal activity of the *Acacia oxyphylla* stem bark-derived botanicals against cestodes.

1. Introduction

Acacia spp. (Family Leguminosae) are purported to be effective as anthelmintic, anti-bacterial and antifungal agents. The most pharmacologically active components of the plants of this genus are the condensed tannins (CTs), a class of phenolic secondary metabolites that are relatively stable in the digestive tracts of animals and rarely have toxic effects (Haring et al., 2007). CTs with proven anthelmintic activity have been reported from several species of *Acacia*, viz. *Acacia mearnsii*, *Acacia nilotica* and *Acacia karroo* (Cenci et al., 2007; Kahiya et al., 2003) and are known to inhibit endogenous enzyme activities (Iqbal et al., 2002; Wang et al., 1996). The aqueous concoction of the stem bark of *Acacia oxyphylla* is used to control gastrointestinal worm infection by the people in North-East India, particularly by the natives of the state of Mizoram. Roy et al. (2007) have recently given scientific credence to the plant by proving its anticestodal activity through scanning electron microscopy. Severe erosion of the tegument along with distortion of suckers was observed in the model cestode parasite *Raillietina echinobothrida* after treatment with the ethanolic extract of the plant. In respect of cestode parasites (tapeworms) that lack a conventional digestive system, the body surface or the tegument serves as an important interface between the parasite and the microenvironment of the host. In view of this, the present study was taken up to further validate the anthelmintic efficacy of the plant by ascertaining if the plant-derived components caused any alterations in the tegumental architecture of the parasite at the sub-cellular level.

2. Materials and methods

2.1. Preparation of extracts

The fresh stem bark of *Acacia oxyphylla* (voucher number PUC-BOT-A012, deposited at Department of Botany, Pachhunga University College, Mizoram University) was collected from Aizawl, Mizoram. The crude ethanol extract and its acetone fraction were made following standard protocol as described elsewhere (Roy et al., 2008). In all 2.0 g crude ethanol extract was obtained from 100 g dried material which gave about 0.4 g of acetone fraction. Both the extract and the acetone fraction were stored at 4 °C until further use.

2.2. Treatment

Adult *Raillietina echinobothrida*, collected from the intestine of freshly sacrificed domestic fowl, were used as the model test

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parasite. The live worms were treated in vitro with various concentrations (5, 10 and 25 mg/ml physiological buffered saline, PBS) of the ethanol extract and acetone fraction of the stem bark, and 0.01 and 0.001 mg praziquantel (PZQ, the broad-spectrum reference drug)/ml PBS of as described earlier (Roy et al., 2008) along with simultaneous maintenance of controls in PBS with 0.1% dimethylsulphoxide (DMSO).

2.3. *Electron microscopy*

On exposure to 10 mg/ml of the plant extracts, paralysis in worms was observed to ensue within a time span that was comparable with the paralysis timings of worms treated with PZQ. Thus, this concentration was selected to carry on further ultrastructural studies on the parasite. The mature proglottides of the control and treated parasites were fixed in modified Karnovsky's fixative, followed by post-fixation in 2% OsO_4 buffered in 0.2 M sodium cacodylate as per the standard procedure (Roy et al., 2009). The ultra-thin sections were viewed in a JEM 100CX II (JEOL) transmission electron microscope operated at 80 kV.

2.4. *Data analysis*

Unpaired Student's *t*-tests were performed and data are expressed as the mean ± S.E.M. of multiple experiments. Values of $p < 0.05$ were considered statistically significant.

3. Results

On exposure to the various concentrations of the plant extract, the tapeworms showed an onset of paralytic state: the time required for the parasites to attain paralysis has been detailed in Table 1. While treatment with 25 mg/ml concentration of the crude alcoholic extract induced loss of motility and consequent paralysis in 1.93 h, the 5 and 10 mg/ml doses took about 4.93 and 3.5 h, respectively, indicating a dose-dependent effect of the plant material; in comparison, the 0.001 mg/ml dose of the reference drug caused paralysis to set in after 3 h post-incubation. The observations indicate significant difference (at $p < 0.05$) between *Acacia oxyphylla*-treated group and the control group.

The control worms revealed typical cestode ultrastructure with respect to the tegument and inner parenchyma. The outer plasma membrane of the control worms has abundant microtriches covered with a fuzzy glycocalyx coat. The distal cytoplasm is electron-dense with its secretory bodies and has an abundance of granular endoplasmic reticulum and other cell organelles including mitochondria with prominent cristae. The basal lamina and the subtegumental muscles are well organized (Fig. 1). In worms treated with the crude stem bark extract of *Acacia oxyphylla*, degenerative changes were noticeable in the tegumental ultrastructure; the tegument appeared as stripped right down to the basal lamina and the remnants of the basal infolds left behind were highly swollen (Fig. 2). The degeneration caused by the acetone fraction of the stem bark was more pronounced than that due to the alcoholic extract. The tegument appeared to be completely sloughed off leaving an exposed basal lamina that was only present in remnants and there was release of underlying structures to the outside at the basal lamina (Fig. 3). The circular muscle layer showed intense degradation and was loosely stacked up with loss in continuity; mitochondria were seen to disappear from the apical zone and those encountered were devoid of cristae. There was very little amount of granular endoplasmic reticulum (GER) remaining with no evidence of Golgi complexes. Worms treated with PZQ also

Table 1
Efficacy of crude stem bark extract of *Acacia oxyphylla* and reference drug praziquantel: effect on *Raillietina echinobothrida*[*] (number of worms in each test medium, n = 6).

Test material	Concentration (mg/ml)	Paralysis (h)	Death (h)
Crude extract-Ethanol	5	4.93 ± 0.12	7.70 ± 0.07
	10	3.50 ± 0.12	5.53 ± 0.10
	25	1.93 ± 0.27	3.60 ± 0.26
Acetone	5	2.25 ± 0.08	4.19 ± 0.09
	10	1.70 ± 0.25	2.71 ± 0.12
	25	1.30 ± 0.13	1.87 ± 0.12
Praziquantel	0.01	0.5 ± 0.01	7.3 ± 0.15
	0.001	3.0 ± 0.14	0.8 ± 0.21

[*] Control worms survived for 72 ± 0.05 h in the incubation medium. $p < 0.05$ vs. control value, Student's *t*-test. Values are mean ± S.E.M.

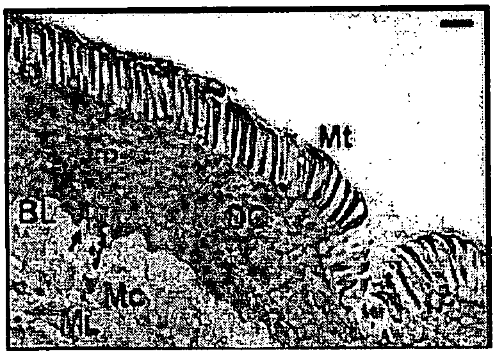

Fig. 1. Tegument of *Raillietina echinobothrida*. Control tegument with intact microthrix layer; distal cytoplasm (DC) electron-dense with tegumental discs (TD); non-disrupted basal lamina (BL) with basal infolds (arrows) and well organized subtegumental circular and longitudinal muscle blocks (Mc), (ML).

Fig. 2. Tegument of *Raillietina echinobothrida*. Stripped-up tegument (arrowheads) of parasite treated with acetone fraction showing intense degradation of longitudinal and circular muscle layers (Mc); breaking up of tegumental cells (arrows).

Fig. 3 Tegument of *Raillietina echinobothrida*. Ethanolic extract-treated parasite tegument is sloughed off down to the inner plasma membrane which shows a swelling in the basal infolds (arrowheads). Also seen is loss of organization in the muscle blocks (thick arrows) and a trail of glycogen depletion seeping in from outside to inside (thin arrows).

showed major damage to the tegument with the microthrix layer damaged at many places and the muscle stacks showing degeneration (Fig 4). The mitochondria showed either a loss or a complete lack of cristae altogether. An overall increase in electron lucency of the background and vacuole formation was observed for all treatment cases.

The tegumental syncytium of control worms is well connected with the subtegumental cytons through cytoplasmic bridges and the nuclei in these cytons reveal prominent nuclear membranes and dense nucleoli while the nuclei encountered in the plant extract-treated parasites were electron lucent due to loss of nucleoplasm, and the nucleoli were diffused in appearance. The parenchymal cytons of the acetone fraction-treated parasite showed complete loss of connections with the surrounding parenchyma and the chromatin in the nucleus appeared clumped into large areas of electron-dense heterochromatin. However the effect of PZQ on the subtegumental region was mostly limited to vacuolization of the cytons and the surrounding parenchyma.

Fig 4. Tegument of *Raillietina echinobothrida*. Depletion of tegument (arrowheads) and intense loss of glycogen (arrows). All bars 0.5 μm.

4. Discussion

The present study showed a dose-dependent efficacy of the crude plant extract as an increase in the concentration of the test material caused more pronounced destructive effect on the tegumental surface of the parasite as also observed earlier by Roy et al. (2007). Compared to the ethanol extract, treatment with the acetone fraction showed more severe deleterious effect even in lower concentration.

Following *in vitro* incubation with various concentrations of the crude extract of *Acacia oxyphylla*, *Raillietina echinobothrida* showed severe tegumental damage. The basal lamina of the tegument is speculated to function as the site for active mitochondrial biogenesis (Smyth, 1996). A progressive disappearance of mitochondria and erosion of the basal lamina consequent to the exposure to the test materials as observed in the present study are indicative of their derogatory effect on the parasite. Some of the responses seen, like stripping of tegument, increase in electron lucency of the mitochondria and surrounding parenchyma are typical of a generalized stress response and have been described in other helminthes under drug insult as well (Buchanan et al. 2003; O'Neill et al., 2009).

In the PZQ-treated parasites also most of the damage induced was tegumental; the subtegumental degeneration was on a much lesser scale than the plant extract-treated ones. The ultrastructural changes such as clumping of chromatin into heterochromatin, a decrease in the amount of GER and disappearance of Golgi complexes are indicative of protein synthesis inhibition (Stitt and Fairweather 1996). Changes observed in mitochondria may have an impact on energy production also (McConville et al. 2008). Botanicals like artemisinins are known to cause a collapse of the membrane potential of mitochondria leading to their swelling and inhibition of electron transfer and oxidative phosphorylation (Li et al. 2005; Wakabayashi and Karbowski, 2001). The degenerative changes observed in the mitochondria as well as their disappearance from the parasite tissue may lead to a faster approach in the parasite towards paralysis and death.

The *Acacia oxyphylla* extract caused swelling of the basal infolds and intense vacuolization of the tegument as observed herein. The swelling of the basal infolds could have an osmotic basis due to impairment of energy-dependent ion pumps (Anderson and Fairweather 1995; Meaney et al. 2004). Further vacuolization of the tegument has been known to be induced by a triggering of calcium ion flux, for example by anthelmintics like PZQ (Jiraungkoorskul et al. 2006).

5. Conclusion

Structural alterations and deformity in the tegumental interface of the parasite subsequent to the exposure to the test plant extract and fraction indicate towards the vermicidal potential of *Acacia oxyphylla*. To ascertain which components of the test plant material are causing the aforementioned tegumental destruction, and what is the exact mode of action, further studies involving identification and testing of the plant active principles is required.

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