ANTICESTODAL EFFICACY OF *ACACIA OXYPHYLLA* ON *RAILLIETINA ECHINOBOTHRIDA*: A LIGHT AND ELECTRON MICROSCOPIC STUDIES

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Summary

An ethanolic extract from the stem bark of *Acacia oxyphylla*, an indigenous anthelmintic plant among the Mizo tribes of north-east India, was tested *in vitro* to determine its anthelmintic activity on the fowl gastrointestinal cestode, *Raillietina echinobothrida*. The parasites were treated with varying concentrations, viz, 0.5, 1, 2, 5, 10, and 20 mg ml^{-1} of the plant extract. On assessment of the motility and mortality of the worms, a dose-dependent efficacy of the plant extract was clearly discernible. Light microscopy revealed distinct tissue damage in the subtegument and somatic muscle layer. In the scanning electron microscopy, extensive distortion and destruction on the surface fine topography of the tegument were evident. Thus, the present experiment provides the plausibility of *A. oxyphylla* stem bark as an anthelmintic agent.

Keywords: *Acacia oxyphylla*; anthelmintic; anticestodal; microscopy; *Raillietina echinobothrida*.

Introduction

Parasitic helminths remain the global concern in terms of prevalence and the concomitant threat they pose to human and animal health. As a matter of fact, more than a quarter of human population – approximately 2 billion people – is currently infected leading to major mortality and morbidity [1], and continue to be the most substantial cause of economic losses in livestock industry [2]. Discovery and commercialization of synthetic drugs, though great in number, have not ameliorated the persistent dilemma, primarily for two reasons: Firstly, there is rapid development of resistance in helminth parasites to all kinds of commercial drugs, old and new. Secondly, these drugs are far from access for the farmers, particularly native farmers who face the problem to afford and of regular supply [3]. Therefore, farmers are compelled to rely heavily on ethnomedicines for controlling helminthiasis for their livestock.

Considering the inevitable problems, there has been renewed interest on the evaluation of traditional helminthic remedies as an alternative to synthetic drugs and use of the well-established medicines are strongly advocated. Different kinds of plants and plant parts are employed in traditional medicines for the treatment of helminth infections. Thus, an earnest search for prospective anthelmintic phytomedicines has been considerably accelerated in recent years, reviving and assessing many traditional practices worldwide.

A large number of ethnomedicinal plants are already reported to have significant cestocidal property [4-7], while many of the traditionally claimed plants appeared to have no significant credibility as anthelmintics, and scientific evidence for the effectiveness of many in use remains to be investigated. Thus, it is evermore crucial to evaluate the validity of anthelmitic plants used in different traditional medicines.

Acacia oxyphylla Graham ex Bentham (synonyms: *A. caesia* Linnaeus, *A. intsia* Willdenow) is a leguminous perennial climbing shrub belonging to the Family Mimosaceae, and is native to south-east Asian countries. In different parts of India, the tender leaves are widely used in culinary preparation, and the stem for fencing agricultural fields. The powdered bark forms froth when rubbed with water and thus used as soap, while its decoction as lice killer [8,9].

The juicy extract from the stem bark is highly priced among the Mizo tribes inhabiting the remotest region of north-east India (located between latitude 21° 58' to 24° 34' N and longitude 92° 15' to 93° 25' E) as fish stupefying agent and as remedy to gastrointestinal infections [10]. However, there is still no scientific validation of any of its alleged medicinal properties. The present work, therefore, is an attempt to evaluate its anthelmintic efficacy, if any, on the common poultry tapeworm, *Raillietina echinobothrida* (Megnin, 1880).

Methods

Preparation of plant extract

The fresh stems of *Acacia oxyphylla* were collected from the nearby forest of Aizawl, Mizoram, India in July 2005. The stem barks were peeled off, thoroughly washed with deionized water, cut into small pieces, and dried in a hot air oven at 50ºC. The dried parts were crushed to fine powder and then refluxed with ethanol (100g/l) for 8 h at 60ºC, as described earlier [11]. The solution obtained was filtered through Whatman filter paper (No. 1) and then evaporated to complete dryness at 50ºC. The crude extract was obtained as a deep brown powdered material, which was then refrigerated at 4ºC until further use. The net yield from such extraction was 0.04%. 1 h prior to experimental assay, varying concentrations of the extract, viz, 0.5, 1, 2, 5, 10, and 20 mg ml^{-1}, were prepared by dissolving them in 0.9% phosphate buffered saline (PBS, pH 7-7.3), supplemented with 0.1% dimethylsulfoxide (DMSO).

Chemicals and drugs

All the chemicals used were standard analytical grades, obtained from Merck, USA. Ethanol was supplied by Bengal Chemicals, Kolkata, India, and the reference drug albendazole is a product of GlaxoSmithKline Pharmaceutical Limited, India. Albendazole is a broad-spectrum anthelmintic drug highly effective for cestodes and nematodes [3].

Recovery and *in vitro* treatments of parasites

Live local fowls (*Gallus domesticus* Linnaeus) were obtained from the local abattoir in Aizawl, Mizoram, India. They were sacrificed and on immediate autopsy, live worms, *R. echinobothrida*, were recovered from the intestines. They were collected in PBS and then incubated at 37±1ºC. The fresh worms were directly treated with the different concentrations of the plant extract in PBS having 0.1% DMSO in separate Petri dishes. Similar treatment was performed for varying doses of albendazole as a reference drug, and one group is maintained in a

medium containing only PBS with 1% DMSO as control. Each incubation medium consisted of 5 replicates.

Motility, mortality, histology and SEM studies

Motility of the flukes were observed, time taken for paralysis and death was recorded, as previously described [11,12]. Dipping the parasites in tepid PBS (~45ºC) induced movement in sentient worms; if no movement occurred upon such stimulation death is confirmed. From each medium, individual worms were divided into two groups. One group is immediately fixed in Bouin's fluid and processed through double staining technique using eosin and haematoxylin for histology (sections cut at 8 µm thickness).

The second group was immediately fixed in 10% cold buffered formaldehyde at 4ºC at least for 4 h for scanning electron microscopy. The fixed specimens were dehydrated through ascending concentrations of acetone, and then specifically air-dried in tetramethylsilane following the standardized method of Roy and Tandon [13] for helminth parasites. After coating with gold and mounted on metal stubs, the tegumental integrity of the specimens were studied using a Jeol JSM 6360 scanning electron microscope at an electron accelerating voltage of 20 kV.

Data analysis

All data are presented as means plus or minus the standard error (SE) of the mean. Comparison of the mean values between the treated and control groups was made using Student's *t*-test, and probability value considered significant at $P < 0.05$.

Results

Dose-dependent anticestodal activity of the extract of *A. oxyphylla* and albendazole

Observations on the efficacy of the plant extract and the drug in terms of survivability of the parasites are presented in Table 1 and Figure 1. The results indicate that both the plant extract and albendazole indicated dose-dependent lethal cestocidal efficacy. *R. echinobothrida* maintained as control in only PBS *with* 1% DMSO survived very well up to 54.40 ± 0.91 hours. Those incubated with 0.5, 1, 2, 5, 10, and 20 mg of *A. oxyphylla* extract per ml of PBS showed complete loss of life in 53.40 ± 0.91, 45.48 ± 0.74, 31.97 ± 0.37, 15.94 ± 0.41, 10.28 ± 0.65 and 5.37 ± 0.47 hours, respectively. Actual death of cestode is always preceded by paralytic state with relatively early onset time such as 29.89 ± 0.43, 19.66 ± 0.19, 12.92 ± 0.37, 7.15 ± 0.33, 5.73 ± 0.41 and 2.42 ± 0.21. Though the time taken for each concentration to effectively kill the parasite is comparatively longer than that for the reference drug, the results are highly significant. Also the anticestodal activity of the plant extract increases with increased concentration, which was similarly noted for the drug.

Structural changes on *R. echinobothrida*

For histological and stereoscan observations, worms treated with 20 mg ml^{-1} of *A. oxyphylla* extract were chosen as the most extensive alterations were shown at this concentration in comparison with the control worms. Scanning electron micrograph of the morphological organization of the control worm revealed a normal body structure of the cestode (Figure 2). The anterior lobed extremity is the scolex. Four suckers are located radially around the proximal end of the scolex. Each circular sucker is marked with rows of short but thick pointed hooklets or spines. Centrally around the four suckers is located a rostellum possessing circularly arranged double set of hammer-shaped hooks. The entire body covering called tegument is densely covered with arrays of special microvilli called microtriches, giving the surface a velvety appearance. The microtriches showed variations in their distribution on different segments or proglottids along the body proper or strobila. Those in the neck region are thin, slender with pointed ends, and uniformly distributed; while those in the

strobilar surface (from immature to gravid proglottids) are thick, elongated, conical, wider at the base and slightly tapering towards the tip.

Table 1: Dose-dependent effect of the crude extract of *A. oxyphylla* stem bark in comparison with that of albendazole on *R. echinobothrida*.

Test group	Dose (mg ml^{-1})	Time in hour taken for		Strudent's *t*-test
		Paralysis	**Death**	
Control	0		54.78 ± 0.65	
A. oxyphylla extract	0.5	29.89 ± 0.43	53.40 ± 0.91	N.S.
	1	19.66 ± 0.19	45.48 ± 0.74	$P < 0.05$
	2	12.92 ± 0.37	31.97 ± 0.37	$P < 0.05$
	5	7.15 ± 0.33	15.94 ± 0.41	$P < 0.05$
	10	5.73 ± 0.41	10.28 ± 0.65	$P < 0.05$
	20	2.42 ± 0.21	5.37 ± 0.47	$P < 0.05$
Albendazole	0.5	17.15 ± 0.43	27.50 ± 0.63	
	1	12.03 ± 0.33	18.87 ± 0.32	$P < 0.05$
	2	8.95 ± 0.42	11.78 ± 0.46	$P < 0.05$
	5	3.38 ± 0.48	8.91 ± 0.78	$P < 0.05$
	10	1.25 ± 0.27	3.79 ± 0.54	$P < 0.05$
	20	0.68 ± 0.55	1.49 ± 0.62	$P < 0.05$

Values are expressed as mean $\pm$ SEM (*n*=5); $P < 0.05$, significant and N.S., not significant compared to the control.
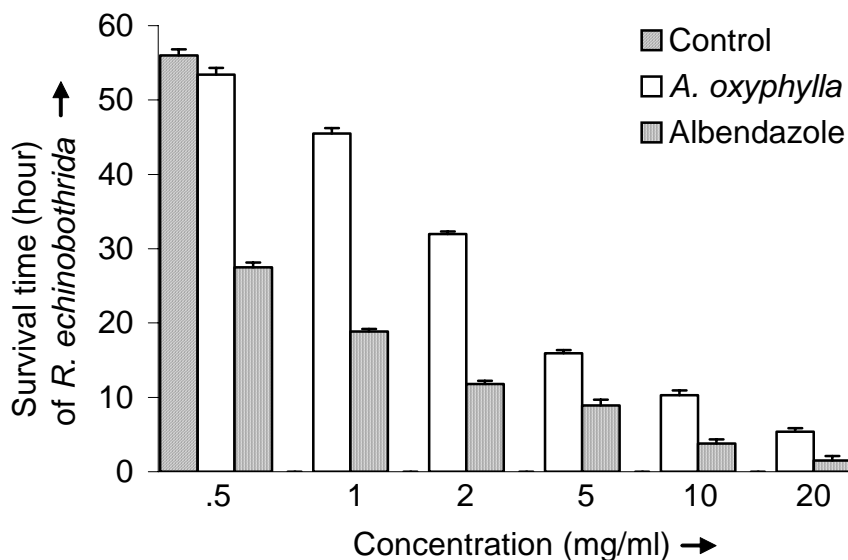

Figure 1. Time taken for different doses of *A. oxyphylla* extract to cause significant mortality on *R. echinobothrida* using albendazole as a reference anthelmintic. Columns represent values as mean ± SEM and error bars as the corresponding SEM.
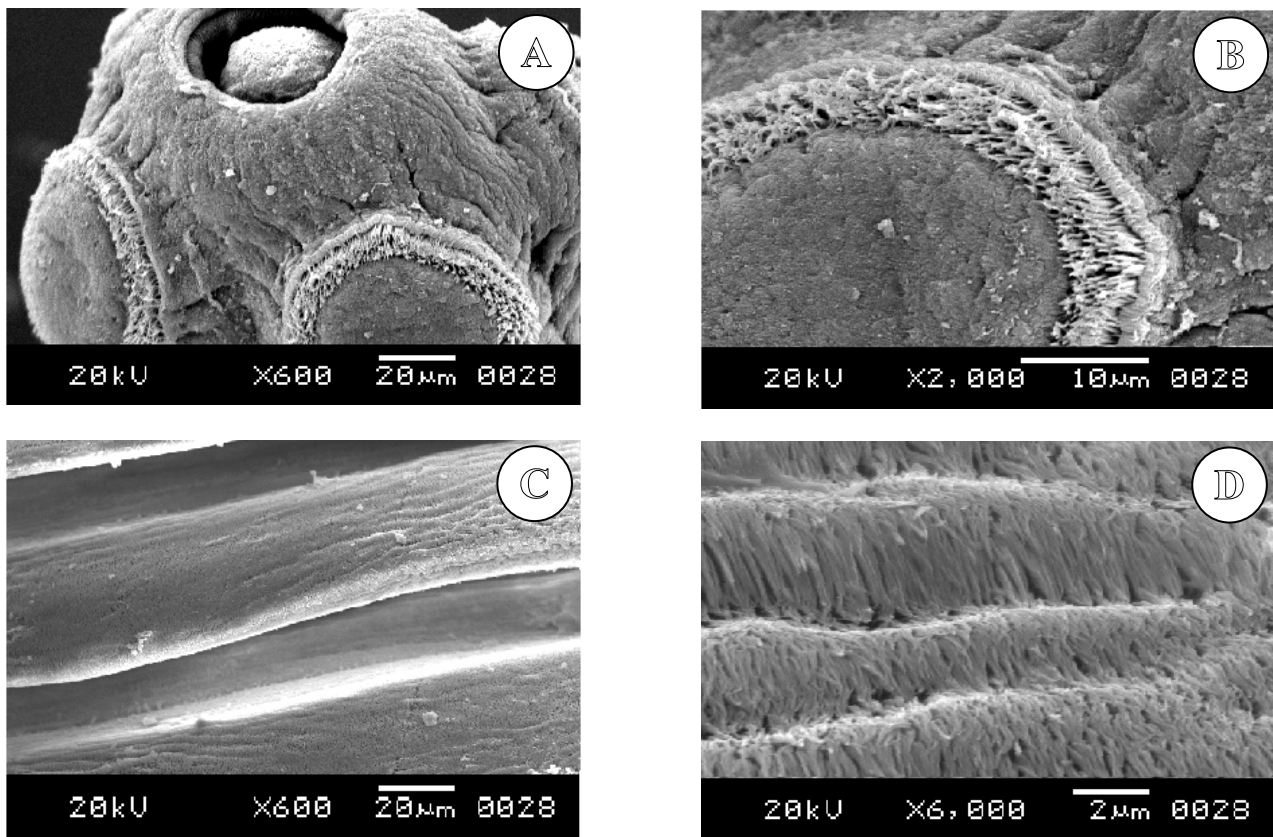
Figure 2. Scanning electron micrographs of an untreated control *R. echinobothrida*. A. The scolex showing apical rostellum surrounded by suckers. B. A sucker with its marginal rows of spines or hooklets. C. The body proper or strobila consisting of proglottids, which are entirely covered with a soft tegument. D. Cascades of microtriches on the tegument.

The cestode treated with the plant extract showed irrevocable destruction throughout the general topography of the body (Figure 3). The scolex appeared greatly distorted with the suckers extensively shrunken. Some vesicular secretions probably due to extreme stress were evident within the suckers. Spine around the suckers were sharply crooked. The tegument at intervals were extensively degenerated with formation of wrinkles. Surface erosion occurred at may regions. The fine filamentous nature of the microtriches were completely lost and were clumped into irregularly distributed clustered protuberances on the tegument.

Histology on an untreated cestode showed the body entirely surrounded by the tegument, followed by the underlying subtegument and the basal membrane (Figure 4). The basal membrane is connected to a thick muscle layer. Body cavity is completely lacking, instead filled with parenchyma cells. In this are distributed anatomical structures such as reproductive organs, excretory canals, nerve fibres and gland cells. The plant extract treated cestode showed definitive degeneration in the muscular layer, with formation of vacuoles at several points (Figure 4). The tegumental integrity was also destroyed such that the tissue showed signs of cracking. The meshy network of the internal parenchymatal cells was also extensively disrupted.
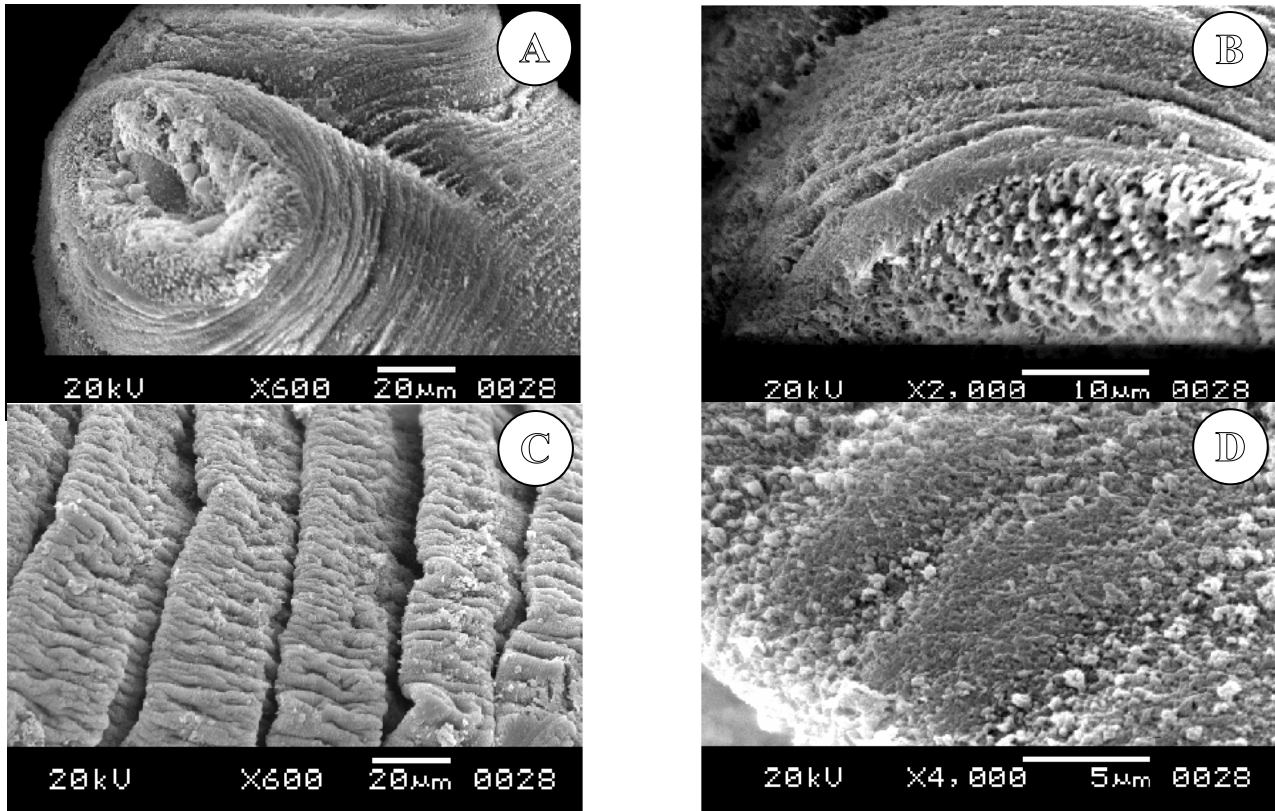
Figure 3. Scanning electron micrograph of *R. echinbothrida* treated with 20 mg ml^{-1} of the ethanol extract of *A. oxyphylla* stem bark. A. Shrunken scolex with vesicular secretion inside the sucker. B. A sucker with deformed spines, which appeared bent outwardly. C. Abnormal tegumental folds and surface erosion. D. Microtriches represented by clumps of dishevelled protuberances.
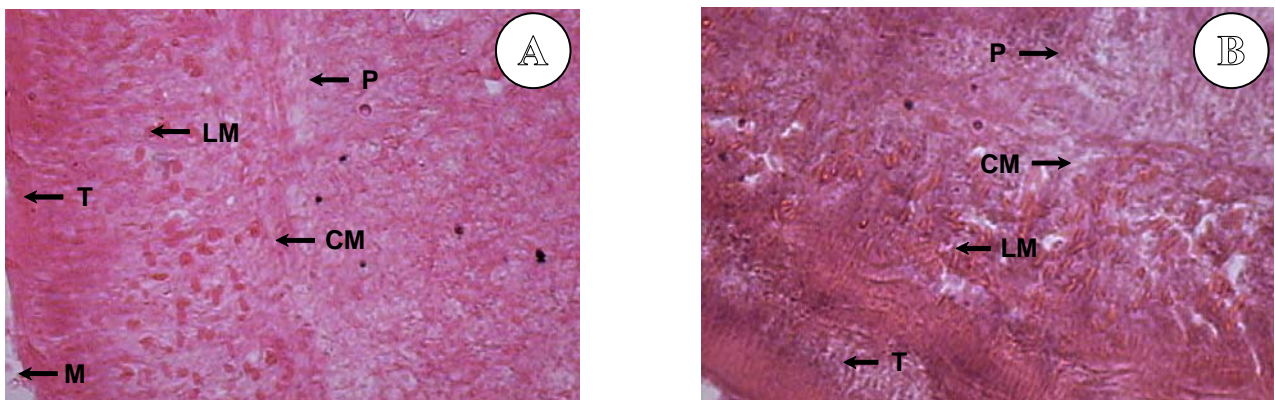

Figure 4. Light micrographs of semithin sections of mature proglottids of *R. echinobothrida*. A. Control cestode showing well-defined tegument (T) with microtriches (M), the underlying circular (CM) and longitudinal muscles (LM), and the internal parenchyma tissue (x 20). B. Cestode treated with *A. oxyphylla* extract showing formation of vacuolated regions in the tegument and muscle layers, the fine network of parenchyma appeared obliterated (x 20).

Discussion

On assessment of the survivability, histology and scanning electron micrographs, it become apparent that the crude extract of *A. oxyphylla* indeed possessed anthelmintic property against the test parasite, *R. echinobothrida.* The primary evaluation for anticestodal efficacy generally involves observations on the loss of spontaneous movement and/or complete immobilization of the worms upon exposure to the plant extracts in *in vitro* studies, and following, several ethomedicinal plants have been established for their anthelmintic potency [11-19]. A closely related species of the present investigation, *Acacia auriculiformis* reportedly caused significant cestocidal activity [20].

The present study also provides the evidence for anthelmintic property of the extract of *A. oxyphylla* stem bark as used by the Mizo tribes in terms of paralytic and mortality effects. The dose-dependent effect of the plant extract indicated potent necrotic impact on the cestode which was comparable to that of the standard drug albendazole. A number of ethnomedical plant used by the neighbouring Naga tribes had 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 [21].

The different concentrations of the plant extract applied in the experiment exerted significant lethal effect ensuing paralysis. The standard drugs macrocyclic lactones act via paralytic effect on the parasites within the host intestinal tract. The immobile worms are simply swept away by the peristaltic movement of the host along the faeces [22]. It can be inferred from this that the plant material tested herein exhibit significant anthelmintic activity at all level tested.

The parasite tegument has been ascertained as the principal target site of different classes of synthetic drugs and natural anthelmintic products [18,23]. Drugs like albendazole and its related compounds are known to enter the parasite tegument through simple diffusion and then cause disruption of the tegumental and muscle layers [24,25]. Morphological and structural changes caused by anthelmintic agents on different helminths with respect to tegumental deformity have been well documented [26-28]. *R. echinobothrida* treated with the plant extract (20 mg ml^{-1}) clearly showed permanent damages in the contour of the microtriches and disorganization of tegumental appearance; while the microtriches were severely deformed and clumped, the tegument surface exhibited extensive distortion in the form of vacoulization and shrinkage. Tandon et al. [11] had firmly demonstrated similar destruction using crude tuber root extract of *Flemingia vestia*. Further, the tegumental enzymes appeared to be the vital, if not primary, target of genistein, an anthelmitic compound from *F. vestia* [29]. The same kind of deformity observed in the present experiment may also be attributed to such action. Therefore, the degenerative effects are clearly consistent with conform the anthelmitic effects demonstrated for other anticestodals.

Even though the crude extract of *A. oxyphylla* apparently indicated efficacious anticestodal activity in the present observations, the precise mechanism by which the lethal action is exerted is not clear. Further, biochemical and molecular analyses are required to form concrete inferences to the actual mode of action and to determine the specific active compound(s) responsible for such anthelmintic activity.

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