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RESEARCH ARTICLE

Anthelmintic activity of *Acacia oxyphylla* stem bark against *Ascaridia galli*

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Abstract

The anthelmintic activity of the ethanol extract of *Acacia oxyphylla* Graham ex Bentham (Mimosaceae) stem bark was tested against *Ascaridia galli* Schrank (Nematoda), the intestinal roundworm of domestic fowl. Different concentrations of the extract (0.5, 1, 2, 5, 10 and 20 mg/mL) were prepared in 0.9% phosphate buffered saline (PBS) with 1% dimethylsulfoxide (DMSO). *In vitro* treatment of the adult roundworms indicated concentration-dependent efficacy of the plant extract. Similar concentrations of a broad-spectrum antiparasitic drug, albendazole, were used as a standard reference. Control experiments consisted of nematodes maintained in 0.9% PBS with 1% DMSO. Albendazole was significantly effective ($P < 0.05$) at all concentrations tested in causing mortality of *A. galli*. However, the plant extract showed concentration-dependent efficacy only at the concentrations of 2, 5, 10, and 20 mg/mL. In order to ascertain the anthelmintic effect, scanning electron microscopy was performed, which indicated devastating structural alterations on the fine topography of *A. galli* treated with 20 mg/mL of the plant extract, when compared with that of the control specimen. Severe shrinkage of the cuticle, loosening and collapse of the lips, and extensive irregular wrinkles all over the body surface were very distinct on the plant extract-treated nematode. Moreover, high magnification of the cuticle revealed formation of a number of small swellings or blebs, which apparently marked the initiation of disintegration of the entire cuticle.

Keywords: Acacia oxyphylla*; albendazole; anthelmintic;* Ascaridia galli*; nematode; scanning electron microscopy*

Introduction

Nematodes constitute the most important gastrointestinal parasites of livestock animals and undoubtedly remain the major factor that compromises successful production resulting in heavy economic losses in animal-based industries. Although there have been remarkable achievements in the discovery and improvement of pharmaceutical anthelmintics, diseases due to nematode infections continue to be the greatest limiting factor in sustainable livestock production worldwide, primarily due to rapid evolution of drug resistance in these parasites to all classes of anthelmintics (Stear et al., 2007). In addition, global appreciation and general endorsement of organic farming pose serious restriction to the prophylactic use of synthetic drugs (Waller, 2003).

Ascaridia galli Shrank (Nematoda) is a roundworm parasitizing the small intestine of birds (McDougald, 2003), and is by far the most prevalent of all helminths infecting poultry (Permin et al., 1999). *A. galli* infections continue to be the most debilitating factor impeding poultry productivity, resulting in retarded growth, weight loss, diarrhea, poor absorption of nutrients, death and even the spread of fatal bacterial infections (Chadfield et al., 2001; Gauly et al., 2007).

Despite successful evaluations of a large number of traditionally used medicinal plants for anthelmintic activity against different helminth parasites, the global crisis of helminthic infestation is far from being ameliorated

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(Githiori et al., 2006). This is primarily due to the fact that although medicinal plants exhibit anthelmintic properties, their chemical nature, safety and, above all, mode of action remain poorly understood. Recent evidence has posited that even certain medicinal plants and their products are highly toxic to the host, and without any appreciable value in clinical and veterinary applications (Githiori et al., 2003; Botros et al., 2004; Hansson et al., 2005).

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. The Mizo tribes of north-east India use the juicy extract from the stem bark as a fish stupefying agent and as a remedy for gastrointestinal infections. In view of its traditional usage we had demonstrated that the crude ethanolic extract of the stem bark indeed exerted significant mortality effects associated with structural damage on the tapeworm, *Raillietina echinobothrida* Megnin (Roy et al., 2007), and inhibition of vital tegumental enzymes (Lalchhandama et al., 2007). The present work, therefore, is an attempt to further explore the possible broad-spectrum anthelmintic activity, if any, on the common poultry roundworm, *A. galli*.

Materials and methods

Plant material and preparation of the extract

The fresh stems of *Acacia oxyphylla* were collected from the nearby forest of Aizawl, the capital city of Mizoram, India, in July 2005. The specimens were identified by H.S. Thapa, plant taxonomist at the Department of Botany, Pachhunga University College, Mizoram University, India, and a voucher specimen (PUC-BOT-A 012) is maintained. The stem bark was peeled off, thoroughly washed with deionized water, cut into small pieces, and dried in a hot air oven at 50°C. The dried parts were crushed to fine powder and then refluxed with ethanol (100 g/L) for 8 h at 60°C, as described earlier (Roy et al., 2007). The solution obtained was filtered through Whatman filter paper (No. 1) and then desiccated to complete dryness at 50°C. The ethanol extract was obtained as a deep brown powdered material, which was then refrigerated at 4°C until further use. The net yield from such extraction was 4.4%. One hour prior to experimental assay, varying concentrations of the extract, 0.5, 1, 2, 5, 10, and 20 mg/mL, were prepared by dissolving them in 0.9% phosphate buffered saline (PBS, pH 7-7.3), supplemented with 1% dimethylsulfoxide (DMSO).

Chemicals and drug

All the chemicals used were of standard analytical grades, obtained either from Merck or SD Fine-Chemicals, Mumbai, India, except where otherwise stated. Ethanol was supplied by Bengal Chemicals, Kolkata, India, and the reference drug albendazole is a product of GlaxoSmithKline Pharmaceutical, India.

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 necropsy, live adult roundworms, *A. galli*, were recovered from the small intestines. All the experiments were carried out in accordance with the guidelines laid down by the Institutional Animal Care and Use Committee. The worms were collected in 0.9% PBS and then kept at 37° ± 1°C in an automated glass-chambered incubator. The fresh worms were directly introduced to the different concentrations (0.5, 1, 2, 5, 10, and 20 mg/mL) of the plant extract in separate Petri dishes maintained at 37° ± 1°C. Similar treatment was performed for varying doses of albendazole, prepared by serial dilution of the prescribed concentration (20 mg/mL). The control experiment consisted of nematodes maintained in a medium containing only PBS with 1% DMSO. Each incubation medium consisted of 5 replicates. Vigilant observations were made on the physical activity of the nematodes and time taken to attain death was recorded following the method of Tandon et al. (1997). Death was substantiated when complete immobility was noted upon dipping the parasites in tepid PBS (~45°C) that induced movement in sentient worms. Data were presented as means plus or minus the standard error (SE) of the mean. Statistical analyses were performed using Biostat 2007, a product of AnalystSoft, Vancouver, Canada. Comparison of the mean values of the experimental treatments against those of the control groups was made using unpaired Student's *t*-test, and the level of probability considered significant when $P < 0.05$.

Scanning electron microscopy

A set of worms from each of the plant extract-treated and control media was thoroughly washed in PBS and then fixed in 4% or 10% neutral phosphate buffered formaldehyde at 4°C at least for 24 h. After post fixation in 1% buffered osmium tetraoxide for 1 h, the worms were washed again with PBS. Subsequent dehydration was carried out through ascending concentration of acetone up to pure acetone. Following the standardized method of Roy and Tandon (1991) specific for helminth parasites, the specimens were treated with tetramethylsilane for 10 min and then allowed to dry at room temperature (25°C). The dried specimens were placed on metal stubs and sputter-coated with gold in a fine-coat ion sputter, JFC-1100 (JEOL), and finally observed under scanning electron microscope (LEO 435 VP) at an electron accelerating voltage of 20 kV.

Results

A. galli in the control group did not show any sign of loss of physical activity, and survived sturdily for 84.83 h ± 0.89 h in the medium composed of 0.9% PBS with 1% DMSO at 37° ± 1°C. The nematodes were persistently active, but once their movement ceased, death ensued abruptly.

Table 1 presents the response in physical activity of *A. galli* upon treatment with albendazole. Albendazole was found to be a highly effective nematocide exhibiting profound dose-dependent activity at all concentrations tested. Significant mortality was observed at 55.17 h ± 1.04 h in the lowest concentration (0.5 mg/ mL), and at 5.31h ± 0.77 h in the highest concentration (0.5 mg/mL).

The extract of *A. oxyphylla* stem bark also indicated concentration-dependent efficacy on the nematode. However, at lower concentrations, i.e., 0.5 and 1 mg/mL, of the plant extract, the nematodes did not show any significant mortality with respect to the control. At concentrations of 2, 5, 10 and 20 mg/mL, the extract indicated significant nematocidal efficacy at 79.84 ± 0.94 h, 62.58 ± 0.81 h, 44.45 ± 0.92 h and 32.78 ± 1.14 h, respectively.

Scanning electron microscopy of the untreated nematode revealed a prominent triangular mouth at the extreme anterior end, which is surrounded by three conspicuous denticulate lips having smooth cuticle and appeared anchored to one another. Prominent cuticular protuberances named labial papillae are situated on the lips, one on each of the latero-ventral lips and two on the dorsal lip; these papillae are sensory in function (Figure 1A). The surface fine topography of the body showed a smooth cuticle characterized by a series of continuous transverse annulations with distinct striations extending from the cephalic region to the posterior end of the body (Figure 1B). The striations were of fine transverse grooves in the form of parallel concentric rings running completely around the cylindrical body. Annulations were deep transverse grooves occurring at regular intervals giving the body a segmented appearance (Figure 1C).

Table 1. Concentration-dependent efficacy of albendazole and the ethanol extract of *A. oxyphylla* stem bark on the survival of *A. galli*.

Incubation medium	Dose (mg/mL)	Time (h) taken for complete paralysis or death	Student's *t*-test
Control	0	84.83 ± 0.89	
Albendazole	0.5	55.17 ± 1.04	$P < 0.05$
	1	38.31 ± 1.08	$P < 0.05$
	2	20.28 ± 0.89	$P < 0.05$
	5	10.67 ± 1.12	$P < 0.05$
	10	07.25 ± 1.10	$P < 0.05$
	20	05.31 ± 0.77	$P < 0.05$
A. oxyphylla	0.5	83.98 ± 0.74	NS
	1	83.75 ± 0.83	NS
	2	79.84 ± 0.94	$P < 0.05$
	5	62.58 ± 0.81	$P < 0.05$
	10	44.45 ± 0.92	$P < 0.05$
	20	32.78 ± 1.14	$P < 0.05$

Values are expressed as mean ± SE ($n = 5$); P value significant at < 0.05 for comparison of treated against control groups; NS, not significant (i.e. $P \geq 0.05$).

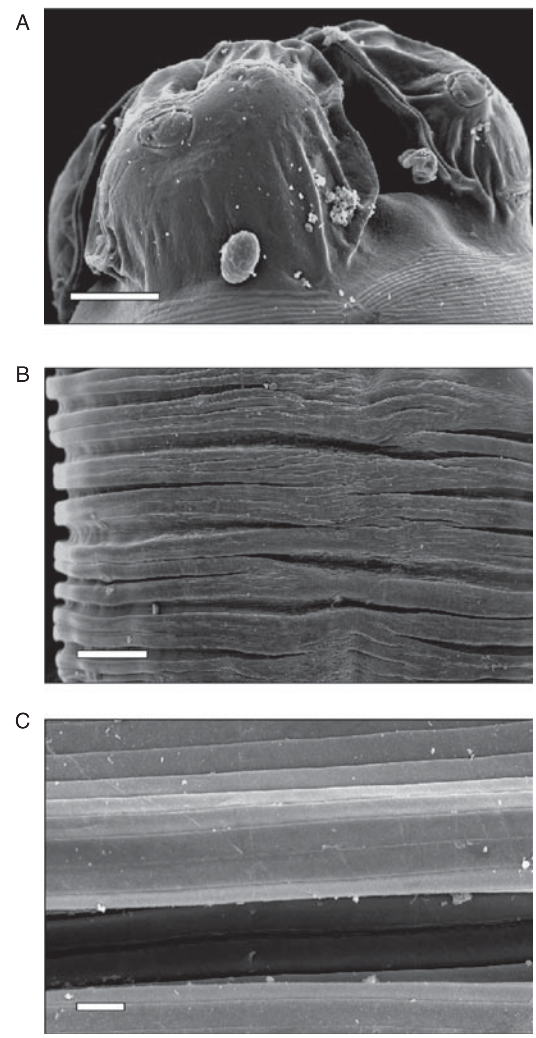

Figure 1. Scanning electron micrographs of untreated control nematode *A. galli.* A) Anterior end showing three denticulate lips surrounding a central mouth, a latero-ventral lip facing at the center of which is a sensory papilla, × 200 (scale bar, 100 μm). B) The cuticle with distinct ridges and furrows throughout the body, × 270 (scale bar, 200 μm). C) Enlarged portion showing a series of lighter transverse striations and intercalated darker annulations, × 1,000 (scale bar, 50 μm).

A

B

C

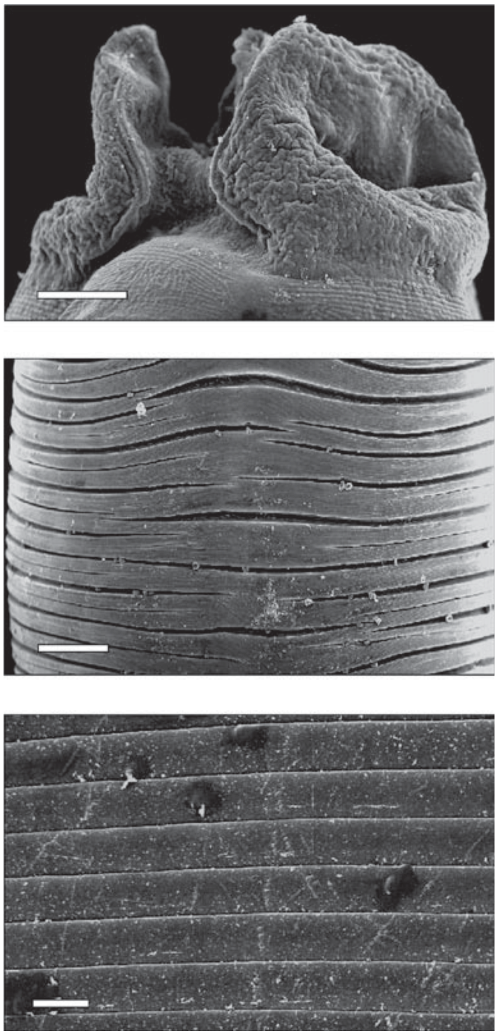

Figure 2. Scanning electron micrographs of *A. galli* treated with 20 mg/mL of the ethanol extract of *A. oxyphylla* stem bark. A) Anterior end with severe deformity, lips collapsed, papillae destroyed and shrunk, and the cuticle wrinkled, ×200 (scale bar, 100 μm). B) Cuticular surface showing irregular rings bearing a number of unusual dark spots, ×270 (scale bar, 200 μm). C) Transverse rings developing external blebs as conspicuous dark blisters, and a ruptured bleb at the left bottom, ×1,000 (scale bar, 50 μm).

A. galli treated with 20 mg/mL of the ethanol extract of *A. oxyphylla* stem bark indicated pronounced deformities on the fine topographical structure. At the anterior end, all the three lips showed signs of loosening and collapse, with conspicuous irregular wrinkles all over the surface. The labial papillae were lost from view due to severe shrinkage of the lip. On the general body surface, the cuticle showed considerable disorganization and aberration in the otherwise regular striations (Figure 2B). At higher magnification of the cuticle, the annular rings displayed minute dark spots in the form of blebs all over the surface (Figure 2C). It is obvious that blebbings could result in focal erosion of the cuticle as evidently shown by a ruptured bleb.

Discussion

A primary procedure often employed for evaluating anthelmintic efficacy is direct observation on the viability of the helminthic worms upon exposure to the anthelmintic agent. Following this basic technique, several medicinal plants such as *Albizzia lebbek* Bentham (Mimosaceae), *Allium sativum* Linnaeus (Liliaceae), *A. santonica* Linnaeus (Liliaceae), *Cardiospermum halicacabum* Linnaeus (Sapindaceae), *Coriandrum sativum* Linnaeus, *Cucurbita mexicana* Damm (Cucurbitaceae), *Neurolaena lobata* Linnaeus (Asteraceae), *Perilla frutescens* Linnaeus (Lamiaceae), *Spigelia anthelmia* Linnaeus (Loganiaceae), *Spondias mombin* Linnaeus (Anacardiaceae), *Polyalthia suaveolens* Engler & Diels (Annonaceae), *Vernonia amygdalina* Linnaeus (Asteraceae), and *Zingiber officinale* Roscoe (Zingiberaceae) have already been documented to show anthelmintic activity against different nematodes of veterinary importance (Akhtar et al., 2000; El Garhy & Mahmoud, 2002; Iqbal et al., 2003; Boonmars et al., 2005; Fujimaki et al., 2005; Ademola et al., 2005, 2007; Eguale et al., 2007; Adedapo et al., 2007). A closely related species of *Acacia* used in the present study, *A. auriculiformis* Cunningham extract, reportedly caused significant activity against the nematode *Dirofilaria immitis* Leidy of dogs (Chakraborty et al., 1995), the bovine nematode *Setaria cervi* Kunth (Ghosh et al., 1993) and a cestode *Hymenolepis diminuta* Sturdevant (Ghosh et al., 1996). Feeding of *A. karoo* Hayne (Leguminosae) leaves also caused significant decrease in the fecal egg count of the nematode *Haemonchus contortus* Rudolphi in goats (Kahiya et al., 2003).

A number of plants, including *Bassia latifolia* Roxburgh (Sapotaceae) (Asadullah & Sabir, 1980), *Melia azedarach* Linnaeus (Meliaceae) (Akhtar & Riffat, 1985), *Caesalpinia crista* Linnaeus (Leguminosae) (Javed et al., 1994), *Piliostigma thonningii* Schum (Leguminosae) (Asuzu & Onu, 1994), *Embelia ribes* Burman (Myrsinaceae) (Dama & Kirdak**,** 2002**),** *Cleome viscosa* Linnaeus (Capparaceae) (Mali et al., 2007a), *Mimusop elengi* Linnaeus (Sapotaceae) (Mali et al., 2007b), *Carica papaya* Linnaeus (Caricaceae) (Singh & Nagaich, 1999) and *Ocimum sanctum* Linnaeus (Lamiaceae) (Singh & Nagaich, 2002), were demonstrated to have efficacy against *A. galli*. The present investigation also evidently showed that extract of *A. oxyphylla* stem bark is an effective anthelmintic agent upon *A. galli*, comparable to the efficacy of *M. elengi* and *C. papaya*, but less effective than other reported plants. Tandon et al. (1997) had also reported similar observations using the root tuber extract of *Flemingia vestita* Bentham & Hooker (Fabaceae) on different nematodes such as *Ascaris suum* Goeze, *A. lumbricoides* Linnaeus, *A. galli* and *Heterakis gallinarum* Schrank.

The cuticle of nematodes is metabolically active and morphologically specialized to perform selective absorption of nutrients, secretion of glycoproteins for immunoprotection, osmoregulation and (insofar as it supports sense organs) sensory reception. Consequently, passive diffusion through the cuticle is the principal mechanism by which anthelmintic compounds enter the nematode body (Alvarez et al., 2007). Apparently, it has been firmly documented that one of the hallmark effects of any anthelmintic is the direct destruction of the worm's surface (Tippawangkosol et al., 2004; Schmahl et al., 2007).

Albendazole reportedly caused structural damage on the cuticle of *Trichinella spiralis* Owen upon *in vivo* treatment (Hrćkova et al., 1993). Surface distortion and loss of regular cuticular annulations were also observed on *Brugia malayi* Brug (Tippawangkosol et al., 2004). Adult *Wuchereria bancrofti* Wucherer & Bancroft subjected to albendazole and diethylcarbamazine combination therapy exhibited swollen cuticle, formation of spherical and spike-like projections at the anterior region, and leaf-like expansion on the general cuticle (Oliveira-Menezes et al., 2007).

Angiostrongylus cantonensis Chen also developed severe shrinkage and formation of rounded leaf-like expansions on the cuticle throughout the body after *in vivo* treatment with imidacloprid and moxidectin combination (Schmahl et al., 2007). Cysteine proteinases isolated from different fruits were shown to cause wrinkles and folds of the cuticle, often followed by blistering and gradual digestion of the cuticle on different nematodes (Stepek et al., 2005, 2006, 2007). Thus, structural alterations observed on *A. galli* cuticle in the present study were definitely due to the anthelmintic activity of the extract of *A. oxyphylla* stem bark.

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