NEMATOCIDAL EFFECTS OF PIPERAZINE AND THE EXTRACT OF *ACACIA OXYPHYLLA* STEM BARK ON THE POULTRY NEMATODE, *ASCARIDIA GALLI*

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Summary

Acacia oxyphylla Graham ex Bentham (Mimosaceae) is a leguminous plant widely used in the traditional medicines of north-east Indian tribes. Among the Mizo tribes, the stem bark is commonly used as fish stupefying and deworming agents. This present experiment was aimed at evaluating the nematocidal effect, if any, upon *Ascaridia galli* Schrank (Nematoda), the intestinal roundworm of domestic fowl, in comparison to that of piperazine. Different concentrations of the plant extract and piperazine (0.5, 1, 2, 5, 10 and 20 mg/mL) were prepared in 0.9% phosphate buffered saline with 1% dimethylsulfoxide. *In vitro* treatment of the nematodes indicated concentration-dependent efficacy of both piperazine and the plant extract in terms of mortality effect. Piperazine was significantly effective ($P < 0.05$) at all concentrations tested. However, the plant extract showed significant efficacy only at the concentrations of 5, 10, and 20 mg/mL. Photomicrographs of the female nematodes treated with 20 mg/mL of the plant indicated extensive structural alterations involving detachment of the cuticle, disintegration of the muscular layers, rupture of the ovaries and deformity on the egg membranes. The results show that *A. oxyphylla* is an effective nematocide, supporting the feasibility of its usage as traditional anthelmintic.

Keywords: *Acacia oxyphylla*, anthelmintic, *Ascaridia galli*, cuticle, muscle, nematode.

Introduction

Nematodes remain the major gastrointestinal parasites of livestock animals and are responsible for decreased productivities resulting in heavy economic losses in animal-based industries. Despite remarkable achievements in the discovery and improvement of pharmaceutical anthelmintics, diseases due to nematode infections continue to be the greatest constraint in sustainable livestock production worldwide, primarily due to rapid evolution of drug resistance in these parasites to all classes of anthelmintics [1]. In addition, global appreciation and general endorsement of organic farming pose serious restriction to the prophylactic use of synthetic drugs [2].

Ascaridia galli Shrank (Nematoda) is a roundworm parasitizing the small intestine of birds, and is by far the most prevalent of all helminths infecting poultry [3]. *A. galli* infections continue to be the most debilitating factor impeding poultry productivity resulting in retarded growth, weight loss, diarrhoea, poor absorption of nutrients, death and even the spread of fatal bacterial infections [4].

In spite of 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 [5]. 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 evidences have 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 [5,6].

Acacia oxyphylla Graham ex Bentham 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 fish stupefying agent and as remedy to gastrointestinal infections. In view of its traditional usage, the ethanolic extract of the stem bark reportedly exerted significant mortality effects associated with structural damages on the tapeworm, *Raillietina echinobothrida* Megnin [7], and inhibition of vital tegumental enzymes [8]. The present work, therefore, is an attempt to explicate structural changes on the common poultry roundworm, *A. galli*, induced by the plant extract.

Materials and Methods

Plant material and preparation of the extract

Collection, identification and cataloguing of the plant specimen were reported elsewhere [7]. The stem barks of *Acacia oxyphylla* were peeled off, thoroughly washed with deionized water, and dried in a hot air oven at 50ºC. The dried parts were macerated and then refluxed with methanol (100g/L) for 8 h at 60ºC. The solution obtained was filtered and then desiccated to complete dryness at 50ºC. The ethanol extract obtained as a deep brown precipitate was refrigerated at 4ºC until further use. 1 h prior to experimental assay, varying concentrations of the extract, viz 0.5, 1, 2, 5, 10, and 20 mg/mL, were prepared by dissolving in 0.9% phosphate buffered saline (PBS), and supplemented with 1% dimethylsulfoxide (DMSO).

Chemicals and drug

All the chemicals used were of standard analytical grades, obtained either from S.D. Fine-Chemicals Limited, India, except where otherwise stated. Methanol was supplied by Bengal Chemicals, Kolkata, India, and piperazine hydrate is a drug of choice against avian ascariasis and was obtained from GlaxoSmithKline Pharmaceutical Limited, India.

In vitro assessment of nematocidal activity

Live local fowls (*Gallus domesticus* Linnaeus) obtained from the local abattoir in Aizawl, Mizoram, India, were sacrificed and on immediate necropsy, live *A. galli* were recovered from the small intestines. The nematodes were directly introduced to the different concentrations of the plant extract and piperazine in separate Petri dishes maintained at 37 ± 1ºC in an automated glass-chambered incubator. Each incubation medium consisted of 5 replicates. A set of nematodes was maintained as control in only PBS with 1% DMSO. Physical activity of the nematodes was observed and time taken to attain death was recorded. Death was substantiated when complete immobility was noted upon dipping the parasites in tepid PBS (~45ºC) 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. Comparison of the mean values of the treatments against those of the control group was made using unpaired Student's *t*-test, and the level of probability considered significant when $P < 0.05$.

Histological processing for light microscopy

Female nematodes were selected from the control and 20 mg/mL extract media, and were fixed in Bouin's fluid overnight. Females were selected because of their elaborate structures. They were

completely dehydrated through a series of graded alcohols. After clearing in xylene and clove oil, they were embedded in paraffin. Sections were cut at 7-9 µm thickness using Erma Japan type Rotary microtome. The sections were then deparaffinized followed by another round of complete dehydration, double staining with eosin and haematoxylin, and finally mounted with DPX. Photomicrographs of the sections were taken with Zeiss image analyzer HBO 50.

Results

A. galli in the control group effectively survived for 84.83 ± 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 piperazine. Piperazine was found to be a highly effective nematocide exhibiting profound dose-dependent activity at all concentrations tested. Significant mortality effects were observed at 55.17 ± 1.04 h in the lowest concentration (0.5 mg/mL), and at 5.31 ± 0.77 h in the highest concentration (20 mg/mL).

The extract of *A. oxyphylla* stem bark also indicated concentration-dependent efficacy on the nematode, but only at the concentrations of 5, 10 and 20 mg/mL, in which the extract indicated significant nematocidal efficacy at 78.86 ± 0.79, 76.47 ± 0.82, and 71.28 ± 0.89 h, respectively.. However, at lower concentrations, i.e. 0.5, 1 and 2 mg/mL, mortality was not significantly different to those in the control.

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

Incubation medium	Concentration (mg/mL)	Time (h) of survival	Student's *t*-test
Control	0	84.83 ± 0.89	
Piperazine	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	83.72 ± 1.00	N.S.
	1	83.83 ± 0.92	N.S.
	2	83.83 ± 1.26	N.S.
	5	78.86 ± 0.79	$P < 0.05$
	10	76.47 ± 0.82	$P < 0.05$
	20	71.28 ± 0.89	$P < 0.05$

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

Histology of a normal *A. galli* revealed that the cuticle is composed of several concentric proteinaceous layers running continuous around the body (Figure 1). Below the cuticle is a syncytial epidermis, giving rise to a complex layer of longitudinal muscles towards the outside and a meshwork of connective tissues towards the inner side. The muscular layer comprises two distinct portions: a fibrillar, contractile muscular portion, which runs lengthwise against the

epidermis, and a granular non-contractile protoplasmic portion projecting towards the centre of the body. The middle portion of the body is a large cavity called pseudocoel. A large gut lies centrally in the cavity, and runs throughout the length of the body. Near the gut, two ovaries are present in females that run into extended oviducts and tubular uterus. Egg cells or oogonia are essentially formed in the ovaries, and the mature polyhedral eggs are transported and housed in the uterine chambers, which are fully lined with tough muscle layer.

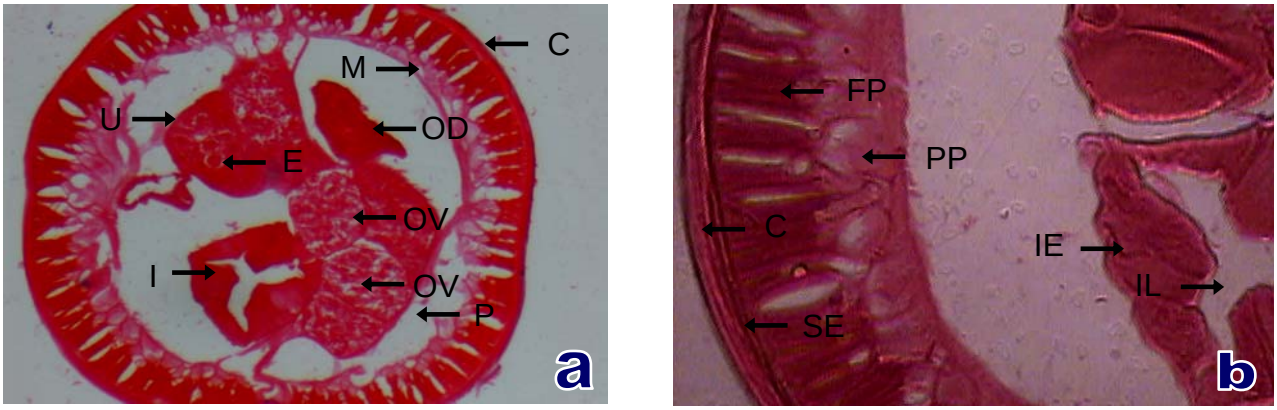

Figure 1. Photomicrographs of histological sections of control female *A. galli*. a) Middle part of a body showing the cuticle (C) with the underlying layer of longitudinal muscle (M), that encloses pseudocoel (P), where ovary (OV) containing numerous oogonia, oviduct (OD), uterus (U) with maturing eggs (E), and an intestine (I) are situated. x 50. b) A portion magnified showing a thick cuticle formed of concentric layers of syncytial epidermis (SE), associated with fibrillar portion (FP) and protoplasmic portion (PP) of the muscular layer. The intestine consists of epithelial layer (IE) and a central lumen (IL). x 200.

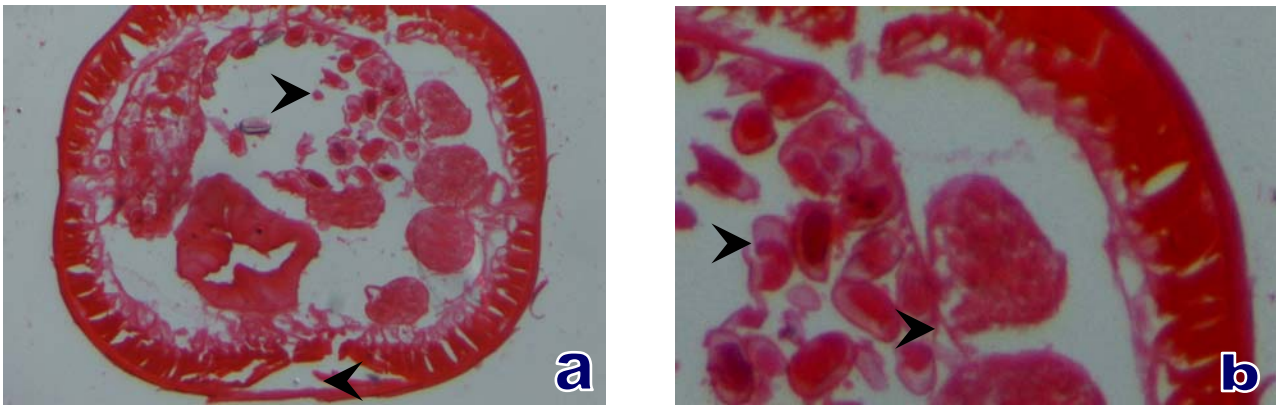

Figure 2. Photomicrographs of histological sections of female *A. galli* treated with 20 mg/mL of *A. oxyphylla* extract. a) Rupture of one uterus releasing the eggs (upper arrowhead) and disintegration of the muscle layer (lower arrowhead). x 50. b) A portion magnified to show disrupted egg (upper arrowhead) and a portion of ovarian wall collapsed (lower arrowhead). x 200.

Figure 2 shows *A. galli* treated with the extract of *A. oxyphylla* stem bark indicating disintegration of the cuticle with evident disruptions at the underlying syncytial epidermis and removal of the associated muscle layer. The longitudinal muscle layer was intensely damaged with the muscle fibres disengaged from one another. The intestinal epithelial cells appeared diffused and contracted. The two uteri were entirely disrupted due to collapse of the muscular wall,

resulting in the scattering of eggs into the pseudocoel. The ovaries appeared completely ruptured. The egg membranes were distinctively distorted throughout.

Discussion

A good number of plants have been experimentally evaluated to show significant nematocidal acticites of varying degrees. A closely related species of *Acacia* used in the present study, *A. auriculiformis* extract reportedly caused significant activity against the nematode *Dirofilaria immitis* of dogs [9], the bovine nematode *Setaria cervi* [10], and a cestode *Hymenolepis diminuta* [11]. Feeding of *A. karoo* leaves also caused significant decrease in the faecal egg count of the nematode *Haemonchus contortus* in goats [12].

A number of plants including *Melia azedarach* [13], *Caesalpinia crista* [14], *Piliostigma thonningii* [15], *Embelia ribes* [16], *Cleome viscosa* [17], *Mimusop elengi* [18], *Carica papaya* [19] and *Ocimum sanctum* [20] were demonstrated to have efficacy against *A. galli*. The present investigation also evidently showed that extract of *A. oxyphylla* stem bark is an effective nematocide upon *A. galli*. Tandon *et al.* [21] had also reported similar results using *Flemingia vestita* on nematodes such as *Ascaris suum*, *A. lumbricoides*, *A. galli* and *Heterakis gallinarum*.

The structural alterations in *A. galli* revealed by photomicrography were clearly evidences of the nematocidal effects of the plant extract. *Onchocerca volvulus* microfilariae treated with amocarzine and milbemycin resulted in disintegrations of the cytoplasm, myofilaments and mitochondria of the muscle cells, associated with progressive separation of the cuticle from the hypodermis [22]. Tribendimidine also caused severe disruption of the cuticle and intestinal epithelium in *Necator americanus* [23]. Cyclosporin A was shown to cause disorganization of the cuticle in *Trichinella spiralis*, especially between the hypodermal pores which appeared somewhat thickened and irregular, with obliteration of the grooves between the cuticular ridges [24].

PPZ in combination a depsipeptide BAY 44-4400 caused vacuolization in the hypodermal layer, degeneration of the muscle fibres, distension of the nerve fibre axons, and rupture of the epithelial cells of *Heterakis spumosa* [25]. Extensive comparative studies of Aukštikalnienė *et al.* (2000, 2005) had demonstrated that the intestinal epithelial cells of *Toxocara canis* filled with vacuoles and granules, associated with degeneration of the microvilli and swelling upon treatment with albendazole, levamisole, pyrantel pamoate and nitroscanate.

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