Anthelmintic Efficacy of Medicinal Plants from Northeast India against Hookworms: an *In Vitro* Study on *Ancylostoma Ceylanicum*

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Summary

The root tuber peel of *Flemingia vestita* (Fabaceae) and a concoction of rhizome pulp of *Stephania glabra* (Menispermaceae) with aerial roots of *Trichosanthes multiloba* (Cucurbitaceae) are used against intestinal helminths by the natives in Meghalaya (Northeast India). In order to find out the nematocidal efficacy of these medicinal plants, the crude extracts of their usable plant parts were tested on the experimental hookworm model, *Ancylostoma ceylanicum*. Adult parasites collected from the intestine of golden hamsters (*Mesocricetus auratus*) were exposed to various concentrations of crude extracts and compared with a reference nematocidal drug, mebendazole. Physical activity, motility, survival and alterations in the surface topography of the test parasites were chosen as the parameters to assess the effect of the plant-derived components. The control parasites in PBS showed motility for a considerable long period (56.5 ± 0.05h); whereas, the *in vitro* treated parasites showed a dose-dependent onset of paralytic state. At a concentration of 100 mg/ml of rhizome pulp of *S. glabra* and root extract of *T. multiloba* and a concoction (1:1) of these phytochemicals took lesser time, 5.26 ± 0.07h, 4.20 ± 0.04h and 2.71 ± 0.06h, respectively, for onset of paralysis in the parasite *A. ceylanicum* in comparison to the lower concentrations. On exposure of the parasites to root peel extract (100mg/ml) of *F. vestita*, the time taken for paralysis in the parasites was 6.18 ± 0.04h. Genistein (10mg/ml), the major active principle of *F. vestita*, caused paralysis in the nematode in about 0.93 ± 0.03h, which was much faster than the effect of mebendazole (2.5 ± 0.12h) at same concentration. Scanning electron microscopic observations revealed no seemingly visible changes in the surface cuticlular features of the worms treated with various test materials as compared to controls. The phytochemicals of the test plants thus suggestedly may have a vermifugal role with regard to nematode parasites, though less effective than in soft-bodied flukes and tapeworm parasites.

Keywords: *Ancylostoma ceylanicum*; *Flemingia vestita*; *Stephania glabra*; *Trichosanthes multiloba*; Genistein; Mebendazole.

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Introduction

The use of traditional medicine in getting rid of worm infections is wide spread across the world. It is an undeniable fact that in today's world, herbal medicine plays a vital role in health care of large sections of the population, particularly in developing countries, where they often bridge the gap between the availability and demand for modern medicines [1]. In many parts of India, especially those inhabited by tribal populations, there persists a rich folklore regarding the vermicidal and vermifugal properties of many plants [2,3]. In Meghalaya (Northeast India) various medicinal plants are used by the natives as curatives against worm infections [4]. Of these, *Stephania glabra* Miers (Menispermaceae) and *Trichosanthes multiloba* Clarke (Cucurbitaceae) are used in the traditional medicinal system besides *Flemingia vestita* (Fabaceae) as cure against gastrointestinal worms and have been shown to cause flaccid paralysis as well as various detrimental effects in these parasites [5]. The activity of the enzymes associated with the co-ordination system of the parasite, nitric oxide synthase in particular, have been shown to be altered by the phytochemicals from *F. vestita*, which cause many other alterations in the metabolism in cestode and trematode parasites that in general have a syncytial tegument forming their outermost body surface [6-11]. A concoction of the crude rhizome pulp extract of *S. glabra* and aerial root of *T. multiloba* was also observed to have a definite effect on the cestode and trematode worms [12].

The body surface of the parasite, the interface between the parasite and its microenvironment inside the host, seems to be the foremost potential target for action of any anthelmintic. *In vitro* treatments of the parasites with different plant extracts have been shown alteration in the structure and composition in their tegumental architecture [5,13-17].

In order to authenticate the use of traditional medicinal plants by the Meghalayan natives and practitioners through this study, we aimed at investigating the efficacy of the phytochemicals on the model nematode *Ancylostoma ceylanicum*, which is a parasite of great importance in human and veterinary medicine. Onset of the paralysis in *A. ceylanicum* with treatments of different dosages of test materials and alterations in the surface architecture of the parasite form the parameters for this study.

Materials & Methods

Chemicals Genistein (G 6649) and Osmium tetraoxide (OsO_4) were obtained from Sigma Chemicals (St. Louis, USA) whereas mebendazole was from Concept Pharmaceuticals, India. Other chemicals used in this study were from local sources and of analytical grade.

Preparation of plant extracts Rhizomes of *S. glabra*, aerial roots of *T. multiloba* and tuberous roots of *F. vestita* were collected from nature in neighbouring forested areas of Shillong (Meghalaya) and thoroughly washed. The rhizome and tuberous roots of *S. glabra* and *F. vestita*, respectively, were peeled off and only the pulp of the former and the peel of the latter were used in the experiment; the whole aerial root of *T. multiloba* was used, cut

into pieces and left for air drying. A crude extract was prepared from *S. glabra* (rhizome pulp), *T. multiloba* (aerial roots) and *F. vestita* (root peel) by soaking the plant materials in methanol for two to three weeks and followed by extraction in a Soxhlet apparatus as described earlier [5]. After 24h of extraction, the solvent was changed, filtered through Whatman filter paper No 1 and concentrated by rotary vacuum-evaporation. The crude extracts were kept at 4 ^{0}C for further use.

In vivo parasite culture Adult Syrian golden hamsters (*Mesocricetus auratus*) of both sexes of about 4-5 weeks age and weighing approximately 40-50 g were obtained from the animal house of National Institute of Nutrition, Hyderabad, India, and maintained in a colony. Faecal pellets of *A. ceylanicum*-infected hamsters were collected from Parasitology lab, Indian Drugs Pharmaceutical Limited, Hyderabad, and cultured in activated granular charcoal at 27ºC in Biological Oxygen Demand incubator [18], the infective third stage larvae (L_3) were retrieved from the culture [19]. About 60 numbers of L_3 larvae of *A. ceylanicum* in 0.5 ml of distilled water were administered orally using a blunt 18 gauge feeding needle. After infection, the hamsters were maintained under proper diet and environmental conditions and were properly labeled according to the nature of the experiment. Faecal pellets of individual hamsters were collected on day 15 post infection and faecal smears examined for *A. ceylanicum* ova to know whether the infection was established or not. Adult *A. ceylanicum* were dissected out from the small intestine of infected hamsters and collected in 0.9 % (w/v) phosphate buffered saline (PBS, pH 7.2).

In vitro treatments The parasites collected from the intestine of infected hamsters were incubated *in vitro* in 0.9% (w/v) PBS (pH 7.2) at 37 ± 1^{0}C containing 5, 10, 25, 50 and 100 mg/ml of the crude plant extracts and a concoction of rhizome pulp of *S. glabra* with aerial root of *T. multiloba* (1:1) dissolved in 1% (v/v) of dimethylsulphoxide (DMSO). The parasites were also tested with genistein and mebendazole at various concentrations (0.1, 0.5, 1.0, 5.0 and 10 mg/ml). Control parasites were maintained in 0.9% (w/v) PBS (pH 7.2) containing 1% (v/v) DMSO only. Each concentration was tested against batches of 5 worms each weigh approximately 5mg. Three replicates for each experiment were used and the time required for onset of paralysis and death in the parasite was recorded. Flaccid paralysis in the treated parasites was monitored by bringing them back at every 15 min gap to the warm (45 ± 1 °C) 0.9% (w/v) PBS (pH 7.2) to examine their motility as described earlier [5].

Scanning electron microscopy The paralysed nematodes and their respective controls were processed for scanning electron microscopy (SEM) following standard protocol. In brief, the parasites were fixed in 10% (v/v) neutral buffered formalin at 4^{0}C for 24 h, washed repeatedly in 0.1 M PBS (pH 7.2), post fixed in 1% (v/v) OsO_4 for 2 h at 4^{0}C, dehydrated through gradient acetone, critical-point-dried using liquid CO_2 in Polaron Jumbo Critical Point Dryer, mounted on aluminum stubs with adhesive tape and metal coated with gold palladium in an ion sputter coater (Argon P7340) and viewed in a JEOL-JSM-35 CF SEM with an electron accelerating voltage of 10-15 KV.

Statistical analysis Data were presented as mean ± SEM. Statistical differences between means were determined using student *t*-test and a *P* value less than 0.05 was considered statistically significant.

Results

1. Control parasites

The body surface is covered with cuticle that shows striations (Fig. 1a). A buccal cavity with conspicuous cutting plates (Fig. 1b), a bursa in the male (Fig. 1c) and tail end in female (Fig. 1d) are typical of the genus Ancylostoma.

Fig. 1.

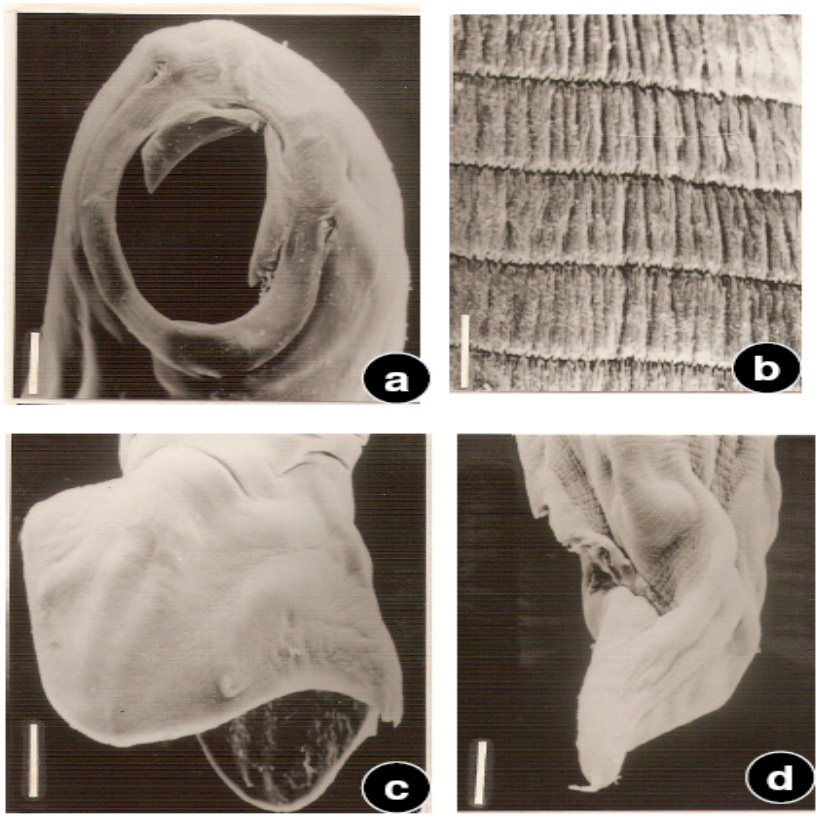

Fig 1 Scanning electron micrographs of *Ancylostoma ceylanicum* showing; a) the buccal cavity (scale bar = 0.06 μm), b) Cuticular striations on the body surface (scale bar = 0.25 μm), c) Bursal end of male (scale bar = 0.03 μm) and d) Tail end of female (scale bar = 0.06 μm).

2. Effect of test materials on *A. ceylanicum*

The test materials were tested on the parasites to investigate the effects in *A. ceylanicum*. Table 1 depicts the paralysis and death time in *A. ceylanicum* treated with various test materials at varying dosages. A dose-dependent effect was evident in all treatments since the time for onset of paralysis and death of the parasite (Table 1). Control parasites showed physical activity for about 56.5 ± 0.5h.

For all the plant crude extracts tested herein, it was noticed that the parasites initially became paralysed followed by death. The time taken for paralysis in the parasite showed an orderly decline with increasing concentration of the test material; at a dosage of 100mg/ml of crude peel extract of *F. vestita* the time taken for paralysis was about 6.18 ± 0.03h and death occurred in about 8.51 ± 0.04h, whereas it was 20.53 ± 0.15h and 23.71 ± 0.06, respectively, at 5mg/ml. With crude rhizome pulp extract of *S. glabra* and crude aerial root extract of *T. multiloba* at 100mg/ml concentration, paralysis in the parasite took around approximately 4.20 ± 0.05h and 5.26 ± 0.07h, respectively, while death occurred at 4.91 ± 0.07h and 7.76 ± 0.04h, respectively, at post incubation. However, a concoction of 100mg/ml of rhizome pulp crude extract *S. glabra* and aerial root crude extract of *T. multiloba* (1:1 ratio) showed onset of paralysis in *A. ceylanicum* in about 2.71 ± 0.04h and death occurred in about 4.05 ± 0.02h. Time taken for paralysis in *A. ceylanicum* treated with the concoction of *S. glabra* rhizome pulp extract and *T. multiloba* aerial root extract was significantly ($P<0.05$) lower than that observed in medium containing their individual extracts.

The time taken for onset of paralysis in the case of the mebendazole-treated parasites was 3.78 ± 0.02h and 2.51 ± 0.02h for 5mg/ml and 10mg/ml, respectively, which is comparable to the paralytic time for the parasites treated with the concoction of 50mg/ml and 100mg/ml of *S. glabra* and *T. multiloba*, respectively. In genistein-treated parasites, paralysis occurred at 0.93 ± 0.06 and death at 1.78 ± 0.04h at the concentration of 10mg/ml, which is significantly ($P<0.01$) lower than that in the case of mebendazole-treated parasites at same concentration. However, the paralysis time (6.18 ± 0.03h) occurred in the case of *F. vestita* crude peel extract (100mg/ml) treated parasites can be compared with the genistein-treated (1mg/ml) ones.

3. Surface topography

In the control parasites, the cuticular surface shows prominent transverse striations and longitudinal wrinkles (Fig 2a). However, the SEM observations of the parasites treated with various crude plant extracts revealed no seemingly visible changes in the surface cuticular features as compared to the control (Figs. 2b-e). The genistein- and mebendazole-treated parasites at 10mg/ml also showed no variation in the cuticular structures as compared to the control (Fig 2f-g).

Table 1.
Effects of various test materials on paralysis and death time in *Ancylostoma ceylanicum in vitro*. Data represent as mean ± SEM (n = 3). Control parasites in 0.9% (w/v) PBS survived for 56.5 h ± 0.05.

Test materials	Concentration (mg/ml)	Time taken (h) for paralysis (P) and Death (D) of *A. ceylanicum*	
		P ± SE	D ± SE
Stephania glabra (rhizome pulp)	5	18.55±0.12	19.03±0.12
	10	15.13±0.03	16.30±0.03
	25	11.38±0.12	12.78±0.01
	50	7.31 ±0.03	8.18±0.04
	100	4.20±0.05	4.91±0.07
Trichosanthes multiloba (aerial root)	5	19.01±0.02	24.01±0.02
	10	16.18±0.04	19.21±0.03
	25	12.60±0.10	14.71±0.04
	50	8.10±0.02	10.33±0.08
	100	5.26±0.07	7.76±0.04
S. glabra (rhizome pulp) and *T. multiloba* (aerial root) (1:1)	5	13.55±0.06	15.10±0.02
	10	9.21±0.04	11.25±0.10
	25	6.26±0.03	8.06±0.04
	50	3.21±0.04	6.01±0.03
	100	2.71±0.04*	4.05±0.02
Flemingia vestita (root tuber)	5	20.53±0.15	23.71±0.06
	10	17.51±0.08	20.30±0.06
	25	13.30±0.09	17.71±0.04
	50	9.03±0.14	11.06±0.09
	100	6.18±0.03	8.51±0.04
Mebendazole	0.1	15.58±0.06	27.05±0.05
	0.5	10.21±0.06	19.38±0.04
	1	8.18±0.02	16.08±0.03
	5	3.78±0.02	7.35±0.07
	10	2.51±0.02	5.25±0.03
Genistein	0.1	13.25±0.08	19.08±0.06
	0.5	8.00±0.04	12.01±0.05
	1	6.00±0.01	9.13±0.05
	5	1.05±0.02	4.08±0.02
	10	0.93±0.06**	1.78±0.04

* $P<0.05$ vs. *S. glabra* rhizome pulp extract and *T. multiloba* aerial root extract group individually.
** $P<0.01$ vs. mebendazole-treated

Fig. 2.

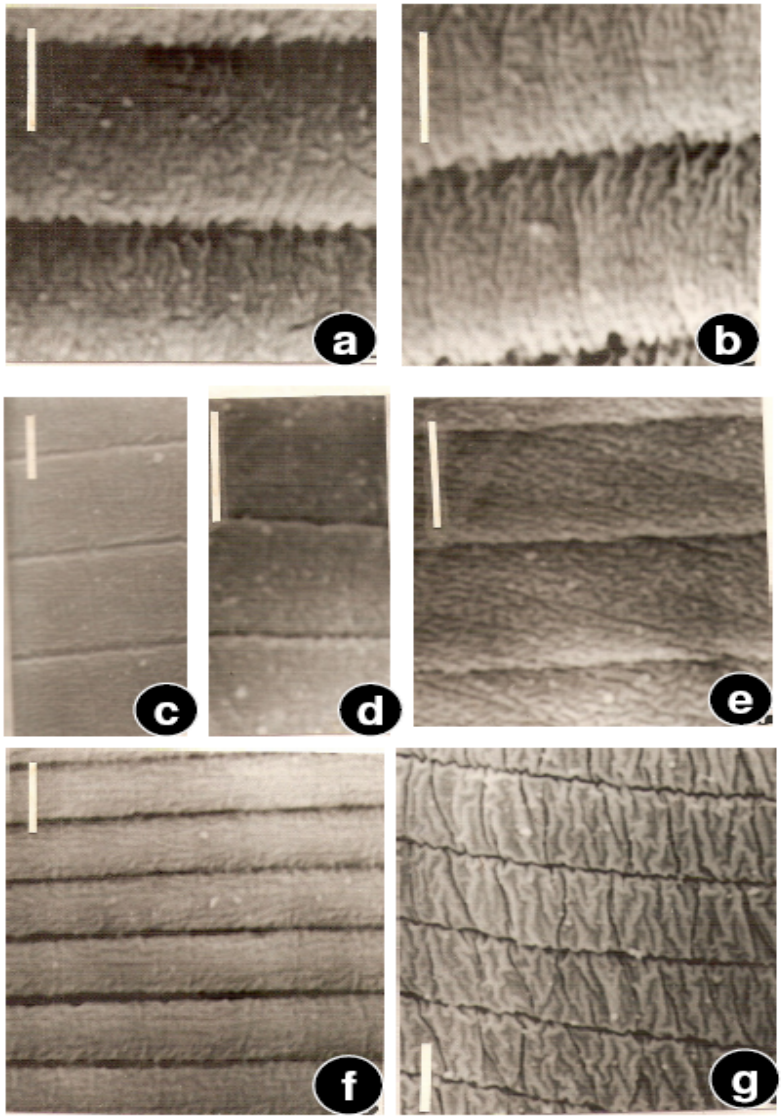

Fig 2 Effects of plant crude extracts on cuticular arrangements of *A. ceylanicum*; a) Control (scale bar = 1μm), b) Crude root peel extract of *Flemingia vestita* (100mg/ml) (scale bar = 1μm), c) Crude rhizome pulp extract of *Stephania glabra* (100mg/ml) (scale bar = 0.5 μm), d) Crude root extract of *Trichosanthes multiloba* (100mg/ml) (scale bar = 1μm), e) A concoction (100mg/ml) of crude rhizome pulp extract of *S. glabra* and root extract of *T. multiloba* (1:1) (scale bar = 1 μm), f) Genistein (10mg/ml) (scale bar = 0.5μm) and g) Mebendazole (10mg/ml) (scale bar = 0.5μm).

Discussion

The SEM microphotographs show that the anterior end of *A. ceylanicum* contains a buccal capsule with large ventral teeth and small inner teeth, while the posterior extremities of the body reveal the sexual dimorphism typically bursate in the male and simple tapering in the female, in conformity with the light microscopic observations [20].

In the present study, the results showed a dose-dependent effect. Many plant derived compounds have been tested *in vitro* against various helminth parasites and a dose-dependent anthelmintic efficacy has been reported [5, 21-25]. A recent study showed that the presence of *Spigelia anthelmia* extracts in the cultures decreased the survival of L_3 larvae of different ovine gastrointestinal nematodes [26]. The inhibitory role of the water extract of the stem bark of *Sacoglottis gabonensis* on hatching of strongyline nematode eggs is comparable to levamisole and albendazole [27]. In the present study, a dose-dependent paralysis was observed in the adult parasite *A. ceylanicum*, when the plant crude extracts were tested with various dosages on the parasite *in vitro*. The control parasites survived for about 56.5 ± 0.5h in the incubation media. Exposure of the nematode *A. ceylanicum* to various concentrations of the crude extract did not cause any portability for sometime to follow but once paralysed it took very short time for death to follow. It may be suggested that even though the plant crude extracts may not have a nematocidal effect, they may be vermifugal in nature and the inactiveness caused would last long enough for the parasites to be swept out of the host's body [28]. At 100mg/ml concentration, the time taken for paralysis in the parasite treated with *S. glabra* rhizome pulp extract and *T. multiloba* aerial root extract individually was not significantly ($P>0.05$) different and varied between 4.20 ± 0.05h to 5.26 ± 0.07h, whereas on exposure to a concoction (1:1 ratio) of the two at the same concentration, the onset of paralysis was significantly ($P<0.05$) faster, thus indicating their synergistic action. Genistein, the major isoflavone present in the root peel extract of *F. vestita* [29], was found to be significantly efficacious at lower concentration in comparison to the plant crude extract. However, at corresponding concentrations, the paralyzing effect of mebendazole was slower than that of genistein. Genistein also exhibits significant metacestodicidal activity *in vitro* [30].

In our study, there were no conspicuous changes noticed in the cuticular architecture in *A. ceylanicim* when treated with various test materials despite their vermifugal efficacy against the parasites as shown by their effect on the motility of *A. ceylanicum*. However, earlier studies showed severe tegumental deformity and disruption of tegumental architectures in the case of soft-bodied cestodes and trematodes when exposed to crude peel extract of *F. vestita* [5, 13]. Thus, it may be suggested that the test plants components do not work through a transcuticular route. The loss of motility and paralysis might be reflecting an effect of the phytochemicals on the neuromuscular system of the worm [31,32,33].

Conclusion

Thus, from the present study, we hypothesize that the plant extracts of *S. glabra, T. multiloba* in concoction (1:1) and crude peel extract of *F. vestita* possess a vermifugal potential against the model parasite *A. ceylanicum*. Recently, a compound tetrahydropalmatine has been isolated from *S. glabra* (unpublished data). Thus, this study supports the traditional use of these plants as putative anthelmintic, however, the nature of the active principles from these plants and their precise mode of action need to be investigated in details for delineation of their therapeutic efficacy.

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