

## Anthelmintic efficacy of extract of *Stephania glabra* and aerial root extract of *Trichosanthes multiloba* in vitro : two indigenous plants in Shillong, India

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In the traditional medicinal practices in Meghalaya (Northeast India) aqueous concoction of the powders of the dried rhizome of *Stephania glabra* and aerial root of *Trichosanthes multiloba* is used as anthelmintic against intestinal worms. To ascertain their efficacy, the alcoholic crude extracts of these materials were tested against the various helminth parasites - nematode: *Heterakis gallinarum*, *Ascaridia galli*, *Ancylostoma ceylanicum* and *Ascaris suum*; cestode: *Railletina echinobothrida* and trematode: *Fasciolopsis buski* in dosages ranging between 25-100 mg/ml in 0.9% phosphate buffered saline (PBS, pH 7.2) at  $38 \pm 1^\circ\text{C}$ . The controls, kept in PBS, survived for average 22 h (trematode), 72 h (cestode) and 55->380 h (nematodes). Treatment with *S. glabra* showed pronounced effect on the cestode and trematode; a dose-dependent gradual decline in physical motility was observed in *R. echinobothrida* and *F. buski*. The isolated active principle (Compound X) of the rhizome pulp also showed good efficacy against *F. buski*. In contrast, much less effect was revealed in respect of nematodes; in treatments with 50 mg/ml all the smaller nematodes attained paralysis within 7-10 h, whereas *A. suum* remained unparalyzed even after 40 h. In treatments with *Trichosanthes* alone the onset of paralysis in all the test worms took much longer than *Stephania*, though in concoction of the two crude extracts the paralytic effect was discernible in less than half the time taken for individual treatments. The phytochemical(s) of *S. glabra* seems to be effective against platyhelminth parasites that have a tegumental interface, but not so against cuticle-covered nematodes.

**Keywords:** Anthelmintic; Cestode; Nematode; Parasites; *Stephania glabra*; *Trichosanthes multiloba*; Trematode

A large number of plants with medicinal properties have been reported from India (Chopra et al., 1956; Dhar et al., 1965, 1968, 1973; Dhawan et al., 1980; Rao and Krishnaiah, 1982; Bhakuni et al., 1988; Shilaskar and Parasher, 1989) and some of them have been documented as curative against worm infections (Perry, 1980; Dharma, 1985; Chhabra et al., 1990). Meghalaya, one of the biodiversity hotspots of Northeast India, has a wide variety of plants used by the natives as curatives against worm infections (Rao, 1981). Besides

*Flemingia vestita* (family Fabaceae) that is used widely as an anthelmintic against gastrointestinal worms (Tandon et al., 1997), two other plants, *Stephania glabra* Miers (family Menispermaceae) and *Trichosanthes multiloba* Clarke (family Cucurbitaceae), are similarly used in the traditional medicinal system. While the rhizome pulp of *S. glabra* is used as anthelmintic locally, the aerial root of *T. multiloba* is used in concoction with *S. glabra* pulp for more effective cure.

With a view to establishing anthelmintic efficacy of the above-mentioned plants and to find out clues to the plausible mode of their action, a thorough study

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seemed desirable. The present study, therefore, aimed at investigating the efficacy of the phytochemicals on the model nematode, cestode and trematode parasites of zoonotic significance. Physical motility and survival time including the onset of the paralytic state and alterations in the surface architecture of the parasites constitute the parameters of activity.

## MATERIALS AND METHODS

**Plant crude extract:** Fresh rhizomes of *S. glabra* and aerial roots of *T. multiloba* were collected in winter from Mawsynram, a small hamlet, about 60 km from Shillong (Meghalaya). After washing thoroughly with water the rhizomes were thinly peeled and the pulp was diced into small pieces; the aerial roots were also chopped into small unpeeled pieces. Each of these materials was soaked separately in rectified alcohol for about a month; in the case of *S. glabra*, the alcoholic contents turned dark brown, whereas the solvent with *T. multiloba* aerial roots attained only a golden tinge. The alcoholic solutions thus obtained were distilled in a reflux condenser for isolating the concentrated crude extract.

Finally 200 g dry crude extract was obtained from 4 kg pulp, and 40 g from 2.5 kg peel of *S. glabra*. From about 5 kg aerial roots of *T. multiloba*, 50 g crude extract was retrieved.

**Compound X:** Purified active component from the crude extract of *S. glabra* rhizome pulp and peel was isolated following the method of Rao and Reddy (1991). The alcoholic crude extract of the plant material was mixed well with hexane and the supernatant solution was decanted. This process was repeated 20 times. The extract was reduced in volume through distillation and passed through silica gel column using hexane, benzene and ethyl acetate as solvents. The column was prepared using hexane and benzene mixture (6:4) and silica gel 100-200 mesh (about 350 g). The sample (10 g) was mixed with silica gel 60-120 mesh and loaded on top of the column. Elution was carried out with hexane: benzene mixture successively at the ratio of 6:4, 5:5, 4:6 and so forth to pure benzene and thereafter with benzene: ethyl acetate (9:1, 8:2, 7:3, 6:4, 5:5 till pure ethyl acetate)

using about 300 ml of mixed solvent at each ratio.

From the rhizome pulp and peel only one major isoflavone could be isolated, the chemical constitution of which is yet to be ascertained; hereafter this is referred to as Compound X. For *T. multiloba*, due to very less quantity of the crude extract recovered from the available material, isolation of active components could not be pursued.

**Parasites:** Parasites (nematodes: *Heterakis gallinarum* and *Ascaridia galli* from domestic fowl and *Ascaris suum* from pigs; cestode: *Raillietina echinobothrida* from domestic fowl; trematode: *Fasciolopsis buski* from pigs) were collected in phosphate buffered saline (PBS; pH 7.2) from freshly slaughtered hosts at the local abattoirs. *Ancylostoma ceylanicum* was obtained from infected hamsters maintained in the laboratory. The parasites were exposed to different treatments within 1 h of collection.

**Treatments:** The test parasites were incubated at  $38 \pm 1^\circ\text{C}$  in 0.9% phosphate buffered saline (PBS, pH 7.2) containing crude extract in concentrations of 25, 50 and 100 mg/ml supplemented with 1 % DMSO. While *F. buski* and *A. suum* were kept singly for each test, *H. gallinarum* and *A. ceylanicum* were incubated in batches of 5 worms, each of approximately same size. For *R. echinobothrida*, on the other hand, 5 strobila, each with scolex and of approximately equal length, were used. For each experiment three replicates were used. The reference drugs praziquantel (PZQ, cestocide), oxiclozanide (flukicide) and mebendazole (nematocide) were used in various concentrations with 1 % DMSO, as established in earlier studies (Visen et al., 1987; Roy and Tandon, 1996; Tandon et al., 1997). Controls were maintained in 0.9 % PBS with 1% DMSO at  $38 \pm 1^\circ\text{C}$ . The parasites, both in control and treated, were brought back to the slightly warm PBS to examine their motility at every 15 min gap and the time taken for the reversible loss of motility (paralysis) and death (no motility) was recorded as per the procedure followed in earlier similar study (Tandon et al., 1997).

**Scanning electron microscopy:** Soon after they attained a paralytic state, the test materials (*R.*

*echeinobothrida*, *F. buski* and *A. ceylanicum*), treated with various dosages of the rhizome-pulp extract of *S. glabra*, were fixed along with their respective controls in 10% neutral buffered formalin at 4°C for 24 h, washed in PBS and dehydrated with ascending grades of acetone to pure dried acetone. The specimens were then critical-point dried using liquid carbon dioxide as the transitional fluid or treated with tetramethylsilane following Roy and Tandon (1991). The gold-coated specimens were observed using LEO 435 VP SEM or JEOL at electron-accelerating voltages ranging between 10-20 kV.

**Statistical analysis:** Data were statistically analyzed and presented as mean  $\pm$  SEM ( $n = 3$ ). Comparisons of the paired mean values between the experimental and respective reference were calculated using Student's t-test (Croxtton et al., 1982) and differences with  $P < 0.05$  were regarded as statistically significant.

## RESULTS.

**Efficacy:** The observations of the efficacy tests are summarized in Table. The control parasites, in conformity with earlier studies (Roy and Tandon, 1996; Tandon et al., 1997), showed active physical activity for a considerably long period than in all treatments. The time taken for paralysis showed an orderly decline with increase in concentration of the crude-pulp extract of *S. glabra*. The results indicate that the crude-pulp extract of *S. glabra* showed greater effect on the trematode and cestode worms than on nematode parasites. The plant-derived components showed more efficacy at a concentration of 100 mg/ml compared to the other dosages; paralysis occurred at  $1.44 \pm 0.11$  h and  $0.91 \pm 0.21$  in case of *R. echinobothrida* and *F. buski*, respectively, whereas in nematodes it took a longer time. The crude-peel extract of *S. glabra* showed less effect in comparison to its pulp extract at the same concentration in *R. echinobothrida*, but it took almost similar time for paralysis in *A. ceylanicum*; at the concentration of 100 mg/ml, the paralysis occurred at  $3.01 \pm 0.05$  h in the former and at  $3.18 \pm 0.037$  h in the latter case. Compound X, tested only against *F. buski*, showed considerably

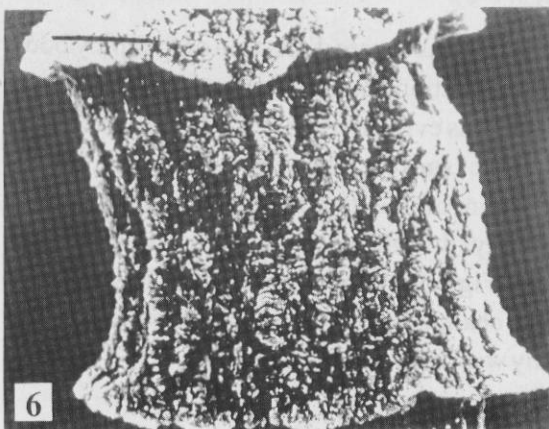
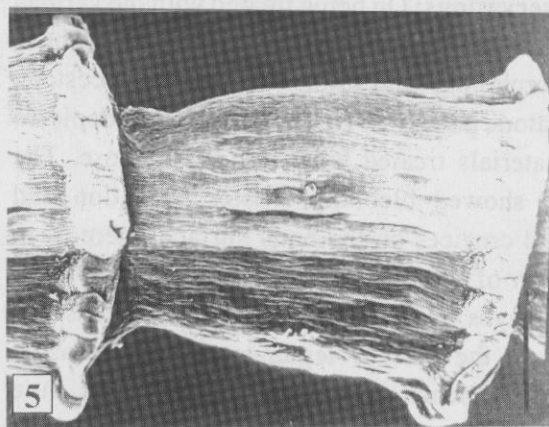
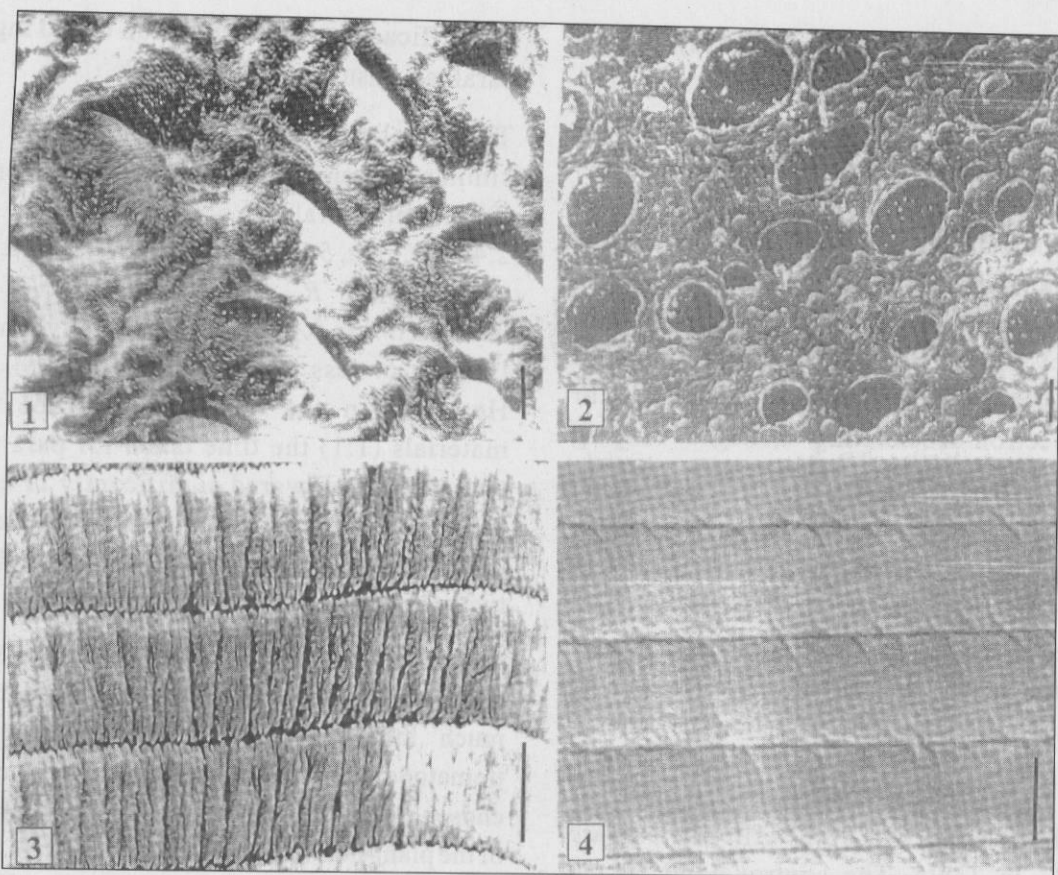
high efficacy at a concentration of 1.0 mg/ml, with paralysis ensuing in less than 1 h.

The aerial root extract of *T. multiloba* showed a similar pattern of efficacy; at the concentration of 100 mg/ml, paralysis occurred at  $2.19 \pm 0.001$  h,  $2.41 \pm 0.52$  h,  $5.26 \pm 0.072$  h and  $11.02 \pm 0.003$  h in the case of *R. echinobothrida*, *F. buski*, *A. ceylanicum* and *A. galli*, respectively. This efficacy is more or less comparable to that of *S. glabra*. However, in the concoction of the two plant materials (1:1) the time taken for paralysis in the test parasites lowered significantly in comparison to the treatment with the crude-pulp extract of *S. glabra* alone, indicating a synergistic effect of the two at a dosage of 50 or 100 mg/ml.

All the nematode species showed a negligible effect as they survived the various treatments for comparatively much longer periods than the cestode and the trematode. The effect with the reference drugs at chosen concentrations was more or less similar to that of the plant-derived components.

**SEM observations:** On being treated with the various plant-derived components there were major changes in the morphology and ultrastructure of the cestode and trematode parasites. This effect was best depicted in the materials treated with 100 mg/ml dose. The tegument showed blebbing, blister formation and cracks and crevices on the body surface. Stereoscan observation of the treated parasites further revealed major damages in the form of wrinkles, lesions and ruptures on the surface tegument of the parasite. However, the nematode cuticle showed no or little effect (Figs. 1-6).

In *F. buski* there was severe distortion of the spiny body surface; pit formation and sloughing off of spines from the tegument was evident (Figs. 1, 2). In the cestode the surface of the suckers showed cracks and severe disorganization of the tegumental architecture. All over the body the microtriches depicted a clumpy appearance (Figs. 5, 6). In *A. ceylanicum*, however, no appreciable change and distortion of the cuticular fine topography was discernible (Figs. 3, 4).



**Figs. 1-6.** Surface architecture of the parasites following treatment with *S. glabra* crude rhizome pulp extract (100 mg/ml):

**1, 2. *Fasciolopsis buski*- ventral tegument**

1. Control, showing the spination of the normal surface. (Scale bar = 10 $\mu$ m).
2. Treated fluke; distortion of the spines, sloughing and pits are noticeable. (Scale bar = 30 $\mu$ m).

**3, 4. *Ancylostoma ceylanicum*- cuticle, mid body**

3. Control, showing striated surface pattern. (Scale bar = 2.5 $\mu$ m).
4. Treated; not much distortion of the cuticular architecture is discernible even at higher magnification. (Scale bar = 5 $\mu$ m).

**5, 6. *Raillietina echinobothrida*- tegumental surface of the gravid proglottid**

5. Control, showing normal surface contour. (Scale bar =100  $\mu$ m).
6. Treated worm; cracks and wrinkles in the tegumental surface, with clumpy microtriches are evident. (Scale bar =100  $\mu$ m).

Table : Efficacy of the rhizome pulp and peel extracts of *Stephania glabra* and aerial root extract of *Trichosanthes multiloba* against various helminth parasites\*

Treatment (mg/ml)	Time (h) taken for complete loss of movement					
	<i>Raillietina echinobothrida</i>	<i>Fasciolopsis buski</i>	<i>Heterakis gallinarum</i>	<i>Ancylostoma ceylanicum</i>	<i>Ascaridia galli</i>	<i>Ascaris suum</i>
<b><i>S. glabra</i></b>						
Crude rhizome pulp extract						
25	7.4±0.05	5.04±0.02	-	11.38±0.12	-	-
50	2.24±0.17 <sup>a</sup>	2.34±0.46 <sup>a</sup>	10.19±0.16	7.31±0.03 <sup>b</sup>	10.2±0.07	55.04±0.03
100	1.44±0.11 <sup>a</sup>	0.91±0.21 <sup>a</sup>	7.19±0.09 <sup>b</sup>	4.20±0.05 <sup>a</sup>	6.9±0.04 <sup>b</sup>	43.54±0.01 <sup>a</sup>
Crude rhizome peel extract						
25	-	-	-	11.01±0.02	-	-
50	5.05±0.13	-	-	6.80±0.07 <sup>b</sup>	-	-
100	3.01±0.05 <sup>b</sup>	-	-	3.18±0.04 <sup>a</sup>	-	-
Compound X						
0.5	-	1.37±0.01	-	-	-	-
1.0	-	0.74±0.02 <sup>b</sup>	-	-	-	-
<b><i>T. multiloba</i></b>						
Crude aerial root extract						
25	3.76±0.01	-	-	12.6±0.1	15.58±0.01	-
50	3.39±0.02 <sup>a</sup>	4.07±0.71	-	8.1±0.02 <sup>b</sup>	13.7±0.01 <sup>a</sup>	-
100	2.19±0.01 <sup>b</sup>	2.41±0.52 <sup>b</sup>	-	5.26±0.07 <sup>a</sup>	11.02±0.01 <sup>b</sup>	-
<b><i>S. glabra</i> (pulp) : <i>T. multiloba</i> (1:1)</b>						
50	-	1.18±0.03	-	3.21±0.04	-	-
100	-	0.32±0.05 <sup>a</sup>	-	2.71±0.04 <sup>b</sup>	-	-
Praziquantel						
0.001	2.9±0.05	-	-	-	-	-
0.005	0.89±0.04 <sup>a</sup>	-	-	-	-	-
0.01	0.47±0.07 <sup>b</sup>	-	-	-	-	-
Oxyclozanide						
5	-	1.97±0.01	-	-	-	-
10	-	1.12±0.02 <sup>b</sup>	-	-	-	-
20	-	0.68±0.03 <sup>a</sup>	-	-	-	-
Mebendazole						
2.5	-	-	-	5.08±0.03	-	-
5	-	-	-	3.78±0.02 <sup>b</sup>	-	-
10	-	-	-	2.51±0.02 <sup>a</sup>	-	-

\* The parasites survived in control (0.1% DMSO in PBS, pH 7.2) for considerably longer periods than in any treatments : *R. echinobothria*, 72±0.05 h; *F. buski*, 20±2 h; *H. gallinarum*, 120.04±0.04 h; *A. ceylanicum*, 56.5±0.05 h; *A. galli*, 168±0.08 h and *A. suum*, 384±0.1 h. Values are expressed as mean ± S.E.M. (n=3).

- not tested.

<sup>a,b</sup>: p-value significant at <0.01 and <0.05 level, respectively in comparison to lower dosages.

<sup>a</sup> : Not significant.

## DISCUSSION

The present study demonstrates that the test plant materials have a deleterious effect on *R. echinobothrida* and *F. buski*. In ultrastructural studies, disorientation of the microtriches, spines and scales was observed along with severe distortion and deformity of the tegument, breakage and sloughing off of the tegumental surface structures. Cestodes and trematodes are soft-bodied parasites and the tegument is their only or major interface, through which various basic physiological functions such as digestion and absorption take place. The plant-derived components seem to affect this normal physiological functioning causing paralysis and subsequent mortality of the parasite, as has been described before in case of *R. echinobothrida* and *F. buski* on being treated with *F. vestita* and its active component genistein (Roy and Tandon, 1996; Tandon et al., 1997; Pal and Tandon, 1998).

Tegumental distortion and severe vacuolization on exposure to flukicidal drugs have been observed in several species of trematodes (Jiang and Xia, 1992; Schmahl, 1993; Stitt and Fairweather, 1993; Xu-Lin et al., 1994; Anderson, and Fairweather, 1995); the extent of damage induced was reported to increase with the exposure time. Similar changes were noticed in case of cestode parasites (Delabre-Defayolle et al., 1989; Perez et al., 1994; Pal and Tandon, 1998). In *Fasciola hepatica*, treatment with diamphenetide caused blebbing of the tegument and the blebs increased in size with an increase in the dosage; surface pitting was also observed and the mid body region of the tegument was stripped off to expose the basal lamina beneath (Fairweather et al., 1987). The ultrastructural changes in the tegument are linked to a possible mode of action of the drug as an inhibitor of protein synthesis (Anderson and Fairweather, 1995). Vacuolization and contraction in the parasite body surface are closely related to the levels of  $Ca^{2+}$  concentration of the media used (Bricker et al., 1982; Xiao et al., 1984). Disturbances in ion flux across the membrane, leading to changes in the tegumental integrity in different trematodes on

treatment with PZQ, are well established (Mehlhorn et al., 1983; Schmahl and Mehlhorn, 1985). Tegumental bubbles of different sizes appeared on the surface of *Opisthorchis viverrini* treated with PZQ *in vitro* and *in vivo*. Thus these ultrastructural changes may represent a generalized response of the tegumental surface to an obnoxious agent (Sirisinha et al., 1984). The same fluke when treated with amoscanate exhibited severe swelling and pit formation, leading to total disruption of the surface tegument and suggested that the drug may have caused an imbalance in osmosis, resulting in impaired ion transfer (Sobhon et al., 1986). The changes in the tegumental architecture on treatment with various test materials of plant origin suggest that the plant products bring about permeability changes in the tegument of the worm.

Disruptions of the cuticular interface and/or intestinal epithelium and degenerative changes even in the subcuticular region have been reported in several nematode species exposed to anthelmintics *in vitro* (An, 1990; Storte et al., 1990; Mackenstedt et al., 1993; Rothwell and Sangster, 1996). Though there was also an orderly decrease in the time taken for paralysis with increasing concentration of the plant-derived components in the nematode species in the present study, the onset of paralysis in them took much longer time than the platyhelminth parasites. The synergistic effect of *S. glabra* and *T. multiloba* concoction (1:1) at 50 or 100 mg/ml showed more efficacious results compared to the effect in treatments with individual plant materials.

It can be hypothesized that the paralysis of the flat-bodied helminth parasites on exposure to the plant-derived components is attributable to the severe distortion, degeneration and disruption of the tegumental architecture of the parasite. The plant-derived components do not seem to be much effective against the cuticle-covered nematodes. It may further be assumed that these phytochemicals have a vermifugal action in that the paralyzed parasites are removed from the host by peristaltic movements of the intestine.

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