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Nematocidal effects of tobacco infusion (*tuibur*) against intestinal helminth parasites of chicken

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Abstract

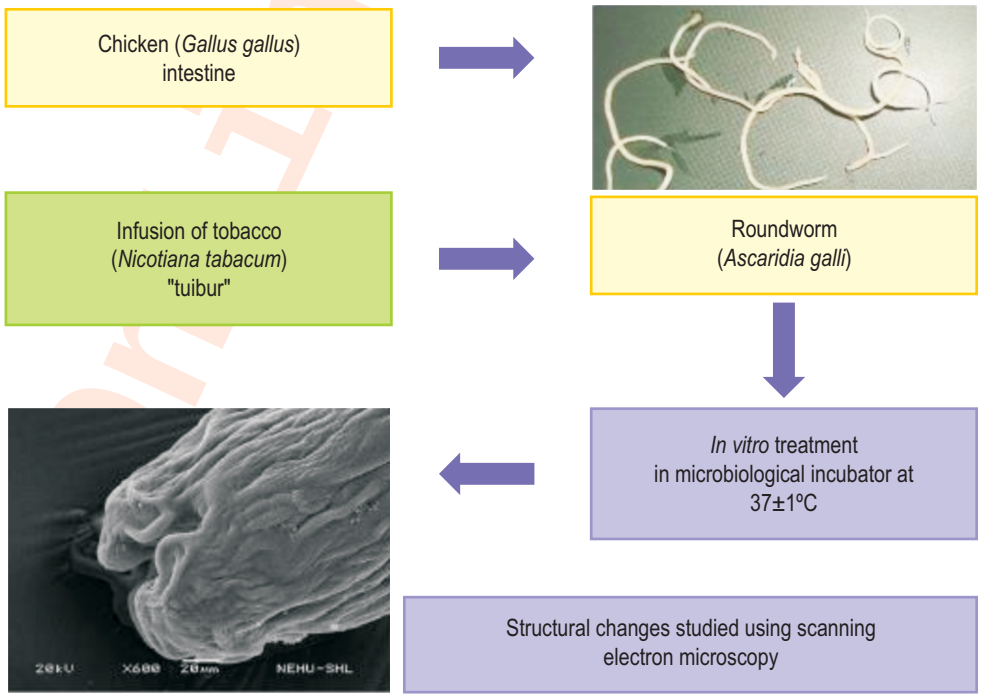
Aim: To study the effect of tobacco infusion, locally known as *tuibur* in Mizoram, as an antiparasitic agent.

Methodology: A traditionally prepared tobacco infusion was tested for its possible effects on the nematode parasite, *Ascaridia galli*. The tobacco infusion was prepared in an exponential increase of concentrations, *viz.* 12.5, 25 and 50% from the original solution. Live and sentient *A. galli* were collected from the intestines of freshly slaughtered chicken, *Gallus gallus domesticus*. They were maintained in culture plates that contained phosphate-buffered saline (PBS) in a biological incubator maintained at 37±1°C.

Results: Both tobacco infusion and standard drug showed a dose-dependent efficacy against the nematodes. Scanning electron microscopy of tobacco infusion-treated nematodes showed extensive structural damage. The sensory amphid was ruptured, and the surrounding lips collapsed. The cuticle was distorted and shrunk all over the body as patchy rows of longitudinal corrugations.

Interpretation: Our data shows evidence that tobacco infusion has anthelmintic activity on parasitic nematodes and may serve as a promising candidate for pharmaceutical development.

Keywords: Anthelmintic, *Ascaridia galli*, Nematode, Tobacco infusion, *Tuibur*



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Introduction

Labelled as neglected tropical disease, helminthiasis is an outstanding factor of morbidity and morbidity-associated mortality in humans and livestock animals. In humans, major morbidity and mortality due to infections are caused by soil-transmitted nematodes (Becker *et al.,* 2018). According to the latest WHO estimate, 1.5 billion people are infected by these nematode parasites (WHO, 2019). Despite global strategies on mass deworming programmes, the situation is aggravated by rapid and widespread evolution of drug resistance in all major parasites. Every anthelmintic drug is at a peril of waned or complete loss of effectiveness (Moser *et al.*, 2019). The threat is particularly alarming in animal industry as massive economic losses are accounted for – this is a serious wakeup call for searching new drugs (Schulz *et al.*, 2018; Kyne *et al.*, 2019).

Plant-derived compounds are among the most interesting sources of alternative or new drugs. Many bioactive phytocompounds with good anthelmintic properties have been reported, but the crucial experimental validations are still scarce. These compounds fall under flavonoids, phenolic compounds, saponins, alkaloids, glycosides and terpenoids (Patil *et al.*, 2019). Among these compounds artemisinin, dioscin, cryptopine, gingkoneolic acid, narigenin, protopine, osthol, palmitic acid, pharnilatin A, rutin, and santonin are confirmed to have anthelmintic activities *in vitro* and *in vivo* (Romero-Benavides *et al*., 2017). Tobacco (*Nicotiana tabacum* L.) is an interesting plant because of its notoriety as the leading cause of preventable cancer and as a medicinal plant of importance on the other side. The plant is established as a storehouse of more than 70 different carcinogens (Leon *et al.,* 2015), and for this it is infamously attributed as the single largest cause of cancer worldwide (Secretan *et al.,* 2009). But not all its compounds are harmful, as some of them also have therapeutic applications (Budzianowski, 2013; Barreto *et al.,* 2015).

On the contrary, tobacco is used for various ailments in different traditional medicines. It is a good remedy for fever, cold, indigestion, constipation, strangulated hernia, hydrophobia, gout, tetanus, malaria, ringworms and worms. In fact, the scientific name *Nicotiana* is derived from Jean Nicot, a 16th-century French ambassador to Lisbon, who was impressed with the curing effects of this plant on nasal polyps (Charlton, 2004). Tobacco is also used for treating dropsy, epilepsy, insomnia, constipation and even hiccoughs (Perry *et al.,* 1999).

The Mizo people use the tobacco infusion, which they call *tuibûr*, mainly as a recreational purpose by simply keeping it in the mouth. Chemical analysis of the tobacco infusion using Fourier transform-infrared spectroscopy has been reported (Lalmuanpuii and Muthukumaran, 2016). Its cytotoxicity is demonstrated against different cancer cell lines such as HeLa, Dalton's lymphoma ascites, Chinese hamster carcinoma cell (V79), as well as cultured human peripheral blood lymphocytes (Lalruatfela *et al.*, 2017). In Mizo traditional medicine, it is used for the treatment of tetanus and as a potent deworming agent against helminth parasites (Sawmliana, 2013). Therefore, this study aims to establish the scientific rationale for such medicinal claim as an anthelmintic agent.

Materials and Methods

Tobacco infusion: The tobacco infusion (*tuibûr*) was purchased from a local market in Aizawl, Mizoram, India.

Chemicals and drug: All chemicals were standard analytical grades. Osmium tetroxide, sodium cacodylate and tetramethylsilane were supplied from Merck India, Mumbai. Methanol was procured from SD Fine-Chem Ltd., Mumbai. All other chemicals were obtained from HiMedia Laboratories Pvt. Ltd., Mumbai, India. Albendazole (ZENTEL®) was a product of GlaxoSmithKline Pharmaceuticals Ltd., Mumbai, India.

***In-vitro* survival test:** Anthelmintic activity of tobacco infusion with albendazole as reference was tested on the survival of intestinal nematode, *Ascaridia galli* Schrank, 1788. Live nematodes were dissected out and recovered from the intestines of local fowls (*Gallus gallus domesticus* Linnaeus, 1758). They were collected in neutral phosphate-buffered saline (PBS) maintained at 37±0.1 ºC in a microbiological incubator. The original concentration of tobacco infusion was diluted serially, i.e., at concentrations of 50%, 25% and 12.5% using PBS supplemented with 1% dimethylsulfoxide (DMSO) in separate culture plates. A 20 mgml^{-1} of albendazole was prepared by dissolving in PBS + DMSO as a positive control. A set of two nematodes were introduced into each culture media. One set of nematodes was maintained as negative control in a medium that contained only PBS with 1% DMSO. The duration of survival was determined from the onset of complete paralysis, i.e., the nematodes failed to show any sign of movements upon agitation such as dipping in lukewarm PBS (45 °C). Each test was performed in triplicates.

Values of the survival times recorded in hours were normalised against those of the negative control and were presented as mean ± SD. Anthelmintic efficacy was assessed by Student's *t*-test, and significant difference was taken at $p < 0.05$.

Scanning Electron Microscopy: *A. galli* in control medium and those treated with 50% tobacco infusion were studied for scanning electron microscopy. After complete paralysis in the treatment media, they were washed with PBS and fixed in 10% formaldehyde (buffered with 0.1 M sodium cacodylate) at 4 °C for 4 hr. Secondary fixation was done with 1% buffered osmium tetroxide (OsO_4) at 4 °C for 1 hr. The fixed specimens were dehydrated through increasing concentrations of acetone and finally in pure acetone. They were then treated with

tetramethylsilane, Si $(CH_3)_4$ for 15 min and left to dry in air-drying chamber at 25 °C. Different parts were carefully selected and mounted on metal stubs, and then sputter coated with gold in ion-sputtering chamber (JFC-1100, JEOL Ltd., Tokyo, Japan). Finally, the images were taken with a Scanning Electron Microscope (JSM-6360, JEOL Ltd., Tokyo, Japan) at an electron accelerating voltage of 20 kV.

Results and Discussion

Analysis of the *in-vitro* survival test of *A. galli* after treatment with tobacco infusion and albendazole is presented in Table 1. Nematodes in the control media thrived well for 187.01 hours. Survival times normalised against the control showed that 5, 10 and 20 $mgml^{-1}$ of albendazole was able to kill all the nematodes in 15.93, 9.63, and 2.15 hrs, whereas tobacco infusion at 12.5., 25, and 50% concentrations took 27.42, 24.42, and 17.10 hrs to kill the nematodes.

Scanning electron microscopy of normal untreated nematode is shown in Fig. 1. The body is cylidrical in shape and the surface called the cuticle has fine transverse rings, called annulations. At the posterior end there are three bulbuous lips surrounding a mouth. On the lips are small blebs called amphids, which are the sensory organs. The marginal rim of each lip is thickened and is called denticle. The tobacco infusion caused distinctive anthelmintic effects of the body surface of *A. galli.* In Fig. 2, general shrinkage of the cuticle is visible and is marked by numerous longitudinal folds. Lips surrounding the mouth (Fig. 3) display severe collapse with total abrogation of the smooth surface. The denticles are invisible which suggest their complete removal. Minute eye-like spot, called amphid, is seen towards the bottom-left of the lip. This sensory organ is splintered with a tiny hole at its centre. The shrinkage extends all over the body including the main body as shown in (Fig. 4).

Longitudinal corrugations are present everywhere. The tail end is particularly deflated (Fig. 5). Cuticle in nematode is a unique structural and functional component. It not only serves as a protective layer between the internal organs and the external environment, but also acts as a durable exoskeleton for maintaining the rigidity but somewhat flexible contour of the body (Wright, 1987). It is, therefore, highly resistant to external factors such as chemicals and digestive enzymes of the host. In contrast to other helminth parasites such as tapeworms (cestodes) and flukes (trematodes), nematodes are bestowed with proper mouth and digestive system so that they are capable of ingesting food materials. Yet, anthelmintic drugs act on them principally though transcuticular diffusion as in other helminths (Lanusse *et al.*, 2016).

Anthelmintic drugs target the cuticular proteins as a primary route of action (Page *et al*., 2014). But different drugs exhibit different degrees of diffusion to penetrate the cuticle; hence, different efficacy. For instance, albendazole as a broad-

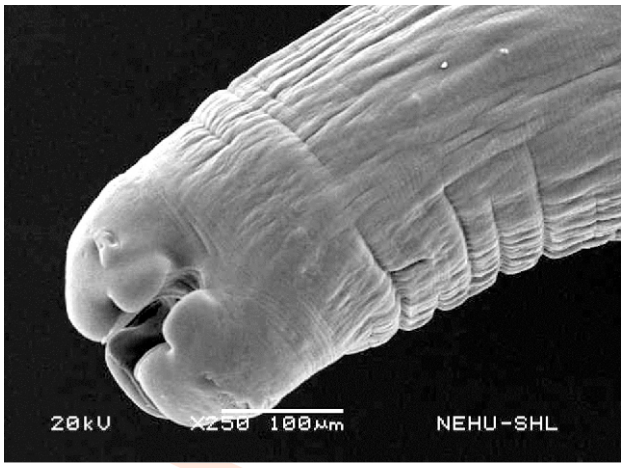

Fig. 1 : Scanning electron microscopy of the anterior part of the body of normal *A. galli*. Three bulbuos terminals are the lips, the central hollow space is the mouth, and scar-like blebs on the lips are sensory amphids.

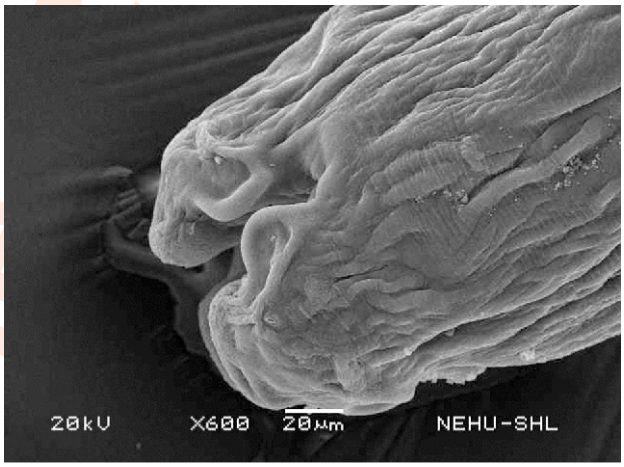

Fig. 2 : Scanning electron microscopy of the anterior part of the body of *A. galli* treated with tobacco infusion (50% concentration). Shrinkage of cuticle is evident.

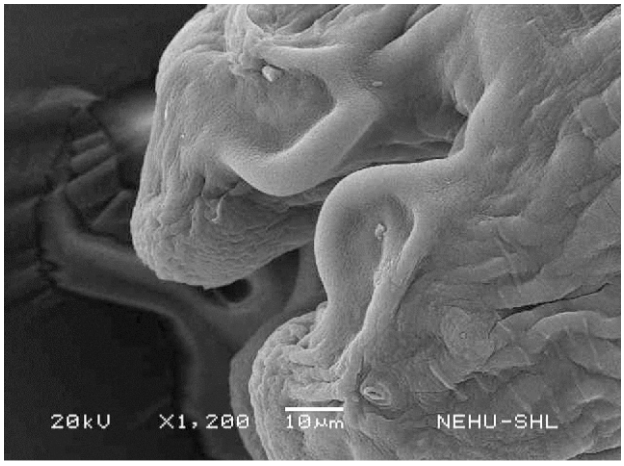

Fig. 3 : Scanning electron microscopy of the lips of *A. galli* treated with tobacco infusion. The lips are collapsed and deflated. A small amphid ruptured on the bottom lip.

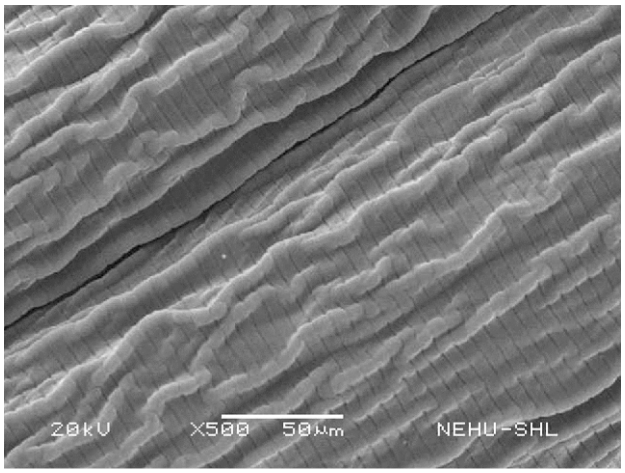

Fig. 4 : Scanning electron microscopy of the main body of *A. galli* treated with tobacco infusion. Longitudinal folds are seen all over.

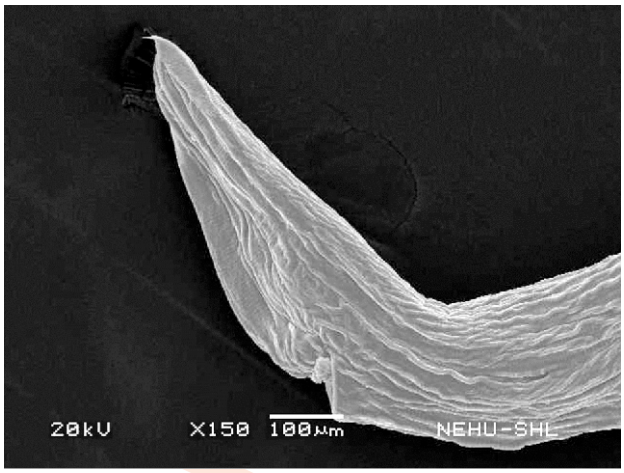

Fig. 5 : Scanning electron microscopy of the tail end of *A. galli* treated with tobacco infusion. General shrinkage and deflation are distinct.

Table 1 : Anthelmintic activity of tobacco infusion against the nematode *A. galli*

Treatment	Concentration	Survival time (hr)[a]	*t*-value	*t*-critical value
Albendazole	5 mg ml^{-1}	15.93 ± 0.69*	56.21	2.57
	10 mg ml^{-1}	09.63 ± 1.46*	56.69	2.36
	20 mg ml^{-1}	02.15 ± 0.53*	65.50	2.57
Tobacco infusion	12.5%	27.42 ± 0.80*	47.93	2.57
	25%	24.42 ± 0.68*	50.25	2.57
	50%	17.10 ± 0.68*	55.13	2.57

[a]Normalised values against those of the negative control; *Significantly different at $p < 0.05$; $n = 6$.

spectrum drug has higher lipophilicity and is more diffusible through the cuticles of *Haemonchus contortus* and *Ascaris suum* when compared to its derivative ABZ sulfoxide (Alvarez *et al.,* 2007). Ivermectin also diffuses through the cuticle in *Caenorhaditis elegans*, *H. contortus* and *Oncocerca ochengi* to act on muscle layers and distort their contraction (Yates *et al.,* 2003). In this study, it was observed that tobacco infusion is a strong anthelmintic agent against *A. galli.* Hallmark anthelmintic effects were clearly discernible. The overall shrinkage of the cuticle indicated detrimental effects on the cuticular proteins. Cyclotides, a family of peptides from different medicinal plants, exert anthelmintic activity by directly destroying the cuticles of *H. contortus* and *Trichostrongylus* colubriformis (Colgrave *et al.,* 2010). The bark of *Acacia oxyphylla* caused cuticular breakdown by damaging the cuticular and muscle layers in *A. galli* (Lalchhandama *et al*., 2009). Isolated from a number of fruits and vegetables, natural cysteine proteinases also damage cuticle of roundworms (Luoga *et al.,* 2015; Phiri *et al.,* 2017).

The cuticle of *H. contortus* was severely deformed following treatment with *Acacia mearnsii* extract (Yoshihara *et al.,* 2015). *Millettia pachycarpa* root bark extract also caused damaging effects on the cuticle of *A. galli*, including shrinkage and collapse of the lips (Lalchhandama, 2019). These evidences indicate that the tobacco infusion contains compounds that are useful for the development of anthelmintic drugs.

An *in-vitro* test of tobacco infusion called *tuibûr* showed that it was effective against an intestinal nematode, *Ascaridia galli*. Scanning electron microscopy revealed that it caused severe damages on the body surface of the nematode.

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