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Scanning Electron Microscopic Study of *In Vitro* Effects of *Tinospora cordifolia* on *Gastrothylax crumenifer*

Jogpal B., Swarnakar G.*, Sen H. and Chouhan H.S.

Department of Zoology, Government P.G. Meera Girls College, M.L.S. University, Udaipur 313001, India

*Corresponding Author

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Abstract: *Gastrothylax crumenifer* parasites have been found in the rumen of goats. *G. crumenifer* is listed as one of the most pathogenic parasite that causes paramphistomiasis, directly or indirectly negatively affects the dairy and food industries in tropical and sub-tropical region. Live *G. crumenifer* parasites were collected from the infected rumen of freshly slaughtered domestic goats at the local meat market and Zoo abattoir of Southern Rajasthan. The parasites were divided into four groups: The first group of the worms was used for the identification of species of amphistome *G. crumenifer* by whole mount preparation. The second group of *G. crumenifer* was used as a control group. The third group of worms was incubated in different concentrations of the alcoholic extracts of medicinal plant *Tinospora cordifolia*. The fourth group of *G. crumenifer* was treated with synthetic drug albendazole at different concentrations. Parasites were fixed in fixative Karnovsky for scanning electron microscope (SEM). The *T. cordifolia* treated parasite revealed prominent conformational changes. After exposure to *T. cordifolia* plant extracts, the surface of parasites becomes deformed, destructions, swelling and shrinkage occurred through body length. The alcoholic extracts of *T. cordifolia* caused intense distortion, extensive erosion, breakage and severe alterations in different part of *G. crumenifer*. The results indicate the highest toxic effect of alcoholic extracts in comparison to albendazole. Findings may help to initiate pharmacological aspects of the extracts *T. cordifolia* herbal plants to prepare less costly, ecofriendly anthelmintic and veterinary drugs.

Keywords: *Gastrothylax crumenifer*, Goat, *In vitro*, Rumen, Scanning electron microscope, *Tinospora cordifolia*

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Introduction

Gastrothylax crumenifer is listed as one of the most pathogenic parasite that causes paramphistomiasis, directly or indirectly negatively affects the dairy and food industries in tropical and sub-tropical region. The state of Rajasthan is famous

for its cattle wealth. The economy of rural people largely depends on cattles. The paramphistomiasis diseases cause morbidity and mortality in goats affecting socio-economic conditions of people. *G. crumenifer* is also major cause of economic losses

and productivity in Rajasthan (Swarnakar *et al.*, 2014a, 2015, 2021).

Various veterinary drugs have been available to eliminate amphistome parasites from goats but they are unaffordable for poor farmers and these synthetic drugs having major problem of drug resistance in several parasites, hence there is urgent need to develop herbal anthelmintic drug that are able to act on parasites for longer time and easily available to poor farmers.

Several plants that have been reported to possess anthelmintic properties due to the presence of some chemical compounds directly acted on various parasites. Plant derived natural compounds are more stable and provide diversity that can prevent occurrence of resistance. Anthelmintic activity of herbal plants was revealed on the surface topography of amphistome parasites which were observed under scanning electron microscopy (SEM) (Nahla *et al.*, 2012; Shalaby *et al.*, 2012; Swarnakar *et al.*, 2014b; Abdelaal *et al.*, 2016; Lokesh and Veerakumari 2016; Shalaby *et al.*, 2016; Swarnakar and Kumawat 2016; Rajesh *et al.*, 2017; Roat and Swarnakar, 2017; Devi *et al.*, 2018). The observations enlightened the anthelmintic effect of ethanol extract of medicinal plant extracts that could be used as antihelminthic agents against amphistome parasites in ruminants (Minsakorn *et al.*, 2019; Menaria *et al.*, 2020; Minsakorn *et al.*, 2021). The efficacy of alcoholic extract of *Cassia fistula* on the tegument of liver fluke *Fasciola gigantica* in liver of domestic goat was observed by light microscope (Sen *et al.*, 2021).

The medicinal plant *Tinospora cordifolia* is an eco-friendly feed supplement for human, poultry and veterinary nutrition that showed many biological activities such as anti-inflammatory, anti-oxidant, anti-hyperglycemic, antimicrobial, osteoprotective and anti-stress effects. It constitutes many bioactive compounds such as polysaccharides, lactones, alkaloids, steroids etc. It is one of the important herbal plant used in Ayurvedic medicine for the treatment of fever,

cold, rheumatoid arthritis and diabetes (Bharathi *et al.*, 2018; Tiwari *et al.*, 2018; Saeed *et al.*, 2019). The interaction of *Tinospora cordifolia* with helpful interspecific plants triggers the modulation in its secondary metabolic profile. The targeting of COVID-19 (SARS-CoV-2) main protease was investigated through active phytochemicals of ayurvedic medicinal herbs- *Tinospora cordifolia* (Giloy). The effect of *Tinospora cordifolia* and *Azadirachta indica* on physiological parameters of growing lambs has been reported (Kumari *et al.*, 2020; Shree *et al.*, 2020; Balkrishna *et al.*, 2021). The above review of literature observed the importance of medicinal plants in the treatment of lots of diseases of veterinary animals. The present study is very significant from medico-veterinary standpoints and for understanding the physiological aspects of *Gastrothylax crumenifer* parasites.

There exists no study regarding anthelmintic activity of *Tinospora cordifolia* against *Gastrothylax crumenifer* (Trematoda: Digenea) parasites of goats by scanning electron microscopy. Therefore, we have performed Scanning Electron Microscopic study of *in vitro* effects of *Tinospora cordifolia* on *Gastrothylax crumenifer*.

Materials and Methods

Collection of parasites:

Live amphistome *Gastrothylax crumenifer* were collected from the rumen of the freshly slaughtered goats at local meat market of Chittorgarh, India. These worms were kept in 0.9 % physiological saline for investigations.

Preparation of extracts:

Fresh *Tinospora cordifolia* stem were collected from Udaipur, Rajasthan, India. The plant was identified, authenticated and allotted accession number BT/2021-22/B 123-1 by Dr. Asha Arora, Associate Professor, Department of Botany, B. N. University, Udaipur (Rajasthan, India). *Tinospora cordifolia* stem were dried, grind into fine powder

and alcoholic extract were prepared.

Experimental design:

Anthelmintic activity was studied by *in vitro* petri dish method as described by Githiori *et al.* (2006). Treated parasites were fixed for surface topography studies by scanning electron microscope.

Anthelmintic activity of alcoholic T. cordifolia stems extract and albendazole:

The experiment was performed in three replicates at the optimal temperature (37°C) and pH -7.4. The motility and mortality of flukes were examined after 1, 2, 3, 4, 5 h. The dead flukes were examined visually and mechanically stimulated using a dissecting needle. Motility was scored using the following criteria: Score 3 - Movement of the whole body; Score 2 - Movement of only parts of the body; Score 1 - Immobile but not dead and Score 0 - Dead.

Ultrastructural study by scanning electronic microscope (SEM):

Untreated and *in vitro* treated *Gastrothylax crumenifer* with alcoholic extracts of *Tinospora cordifolia* (Neem giloy) stem were fixed in Karnovsky fixative at 4^o C for ultrastructural study by scanning electron microscope (SEM).

Results

Gastrothylax crumenifer control or untreated and treated with alcoholic extracts of *Tinospora cordifolia* (Neem giloy) stem and compared with synthetic drug albendazole were observed by scanning electron microscopy (SEM).

Scanning electron microscopic study of control G. crumenifer:

In SEM study, parasite of the control group showed elongated and pouched body with anterior end bounded by thick muscular rim with papillae. *G. crumenifer* has two suckers located at terminal end on both side and opening of ventral pouch near to oral sucker. Opening of ventral pouch is found near the oral sucker. Compact tegumental folds with dome and button shaped papillae are arranged concentrically around oral

sucker and opening of ventral pouch. Posterior sucker has large muscular cuticular rim with tegumental major folds which contains deep grooves. Long, sensory papillae around the acetabulum was also found. The tegument of parasite covered from tegumental folds with grooves and ridges. The sensory pitted papillae also found on tegument of whole body surface of control parasites (Figs. 1a, b, c).

SEM study of G. crumenifer in vitro treated with alcoholic extract of T. cordifolia:

In vitro anthelmintic activity of alcoholic extract of *T. cordifolia* stem caused large breakages, shrinkage and distorted tegumental surface of *G. crumenifer*. Tegumental folds of oral sucker became swollen with breakages and detachment due to *in vitro* anthelmintic activity. Sensory papillae around oral sucker were distorted. The papillae surrounding the acetabulum disappeared in some region, large breakage and swelling were also observed around tegumental surface and posterior sucker with flattened and smooth tegumental folds, grooves and minute papillae (Figs. 1d, e, f).

SEM study of G. crumenifer in vitro treated with synthetic drug albendazole:

G. crumenifer treated with albendazole showed some superficial distortion and breakage on the body surface. Minor shrinkage with smooth flattened tegumental folds with grooves and small papillae were observed on tegumental surface of parasite. Minor swelling, breakage, distortion and damage were observed on tegumental folds on whole body of parasite. The long sensory papillae surrounding the posterior sucker were found in normal structure with minor damages around acetabulum (Figs. 1g, h). The synthetic drug albendazole is less effective as compared to alcoholic extracts of *T. cordifolia* that have been used as anthelmintics.

Discussion

G. crumenifer parasite has soft body with hard tegument that is metabolically active and morphologically specialized to perform various

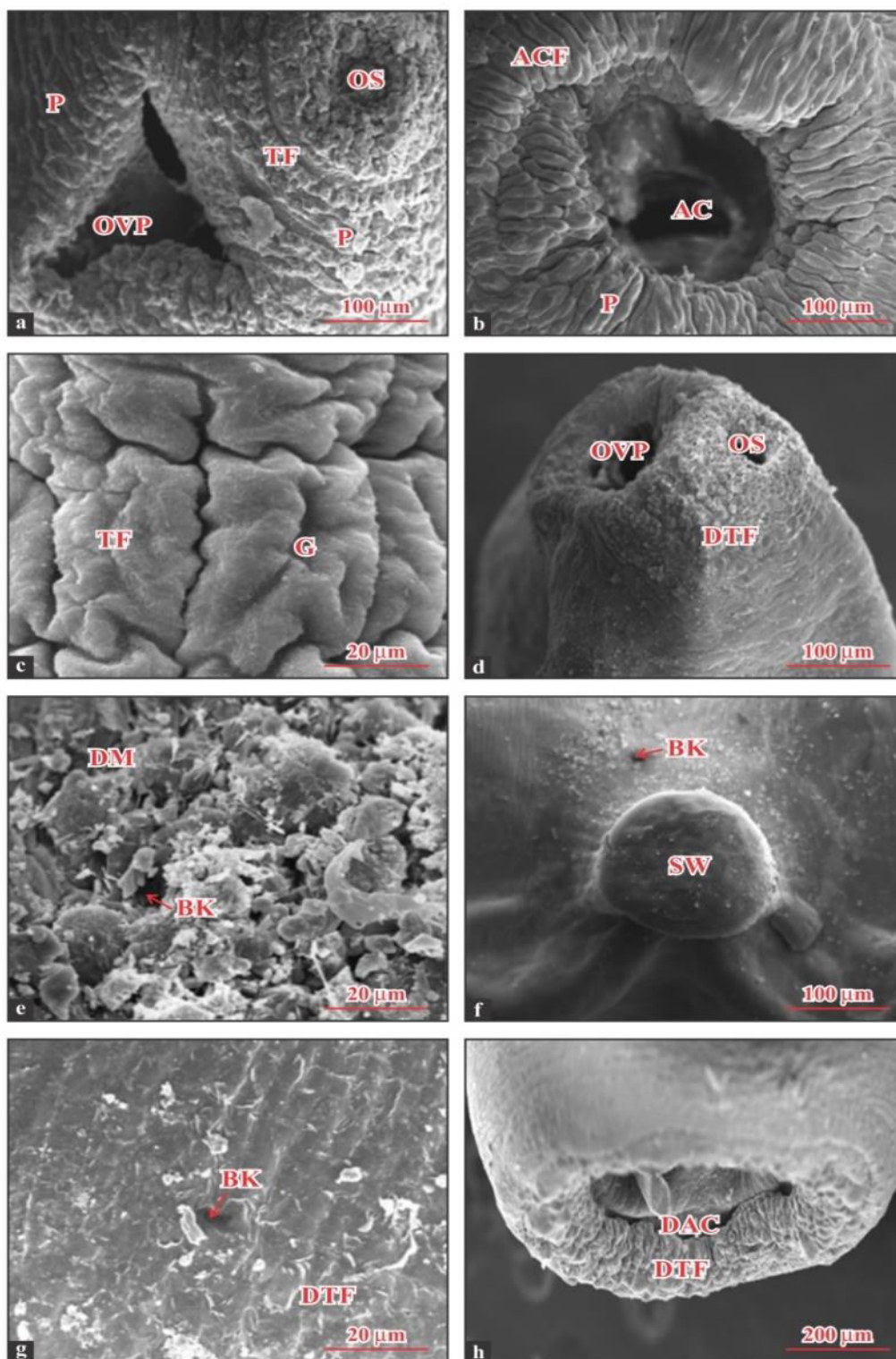


Fig. 1: Surface topography of *G. crumenifer* control (Figs. 1a, b, c) and treated with the stem of *T. cordifolia* alcoholic extract (Figs. 1d, e, f) and albendazole (Figs. 1g, h) by scanning electron microscopy (SEM). AC: Acetabulum, ACF: Acetabulum fold, BK: Breakage, DAC: Damage in acetabulum, DM: Damage, DTF: Damage in tegumental fold, G: Groove, OS: Oral sucker, OVP: Opening of ventral pouch, P: Papillae, SW: Swelling, TF: Tegumental fold, VP: Ventral pouch.

physiological functions like protection, digestion, nutrition, osmoregulation and absorption. Traditional medicinal plants contain the phytochemical components which are effective on the tegument of parasites and make the parasite functionless or dead.

Some researcher investigated topographic study of amphistome parasites such as tegumental folds with major grooves found all over the body in *Orthocoelium dewasi*, *Orthocoelium scoliocoelium* (Tondon and Maitra, 1987); and concentrically arranged tegumental folds with visible furrows contain various tubercles reported in parasite *Gastrodiscoides hominis*. Finger like projection on tegumental folds were reported in *Cotylophoron cotylophorum* which has tegument covering and dense sensory papillae, this parasite has pear shaped structure (Veerakumari *et al.*, 2012). *Paramphistomum microbothrium* has oral sucker positioned terminal and acetabulum is located sub-terminal region, this parasite has pear shaped body with concentrically arranged tegumental folds and has dome shaped papillae (Shalaby *et al.*, 2016). *Orthocoelium scoliocoelium* has slightly curved body which contains radial tegumental folds with smooth and clearly visible knob shaped papillae (Swarnakar and Kumawat, 2016). *Gastrothylax crumenifer* has pouched body (ventral pouch) showed tegumental folds with major grooves contains papillae and nipple like tip (Rajesh *et al.*, 2017). Similar structures were also observed in present study.

Saowakon *et al.* (2013) reported that *Paramphistomum cervi* have two types of sensory papillae on the surface of tegument, bulbous and nipple like end portion and second is ciliated or sometimes non-ciliated. Both suckers are covered with thick tegumental folds having papillae. Present study revealed that *Gastrothylax crumenifer* having two types of papillae on tegumental surface, first one is smooth and dome shaped and second is sensory wrinkled pitted papillae. The tegument of *G. crumenifer* is morphologically specialized for diffusion of the

anthelmintics would probably be responsible for the degenerative changes and deformation of the surface topography of amphistome. In amphistomes, suckers are important structure for attachment whereas the surface papilla acts as sensory organs.

The extract of *Citrullus colocynthis* and *Trigonella foenum-graecum* were caused distortion in both suckers, deformity in tegument and several damages and disturbance observed in amphistome parasites; *Paramphistomum cervi* and *Orthocoelium scoliocoelium* by scanning electron microscopy (Swarnakar and Kumawat, 2016; Roat *et al.*, 2017). Whereas in present study *G. crumenifer* treated with *Tinospora cordifolia* showed similar structure such as elongated, shrunken, deformed surface architecture and ruptured tegumental folds on whole body. Damages and breakages were present on oral sucker and posterior sucker that are surrounded by tegumental folds.

In vitro treated *Cotylophoron cotylophorum* with *Allium sativum* showed damages, deformed and distorted oral sucker and acetabulum with continuous tegumental folds, complete removal of papillae and few blebs were seen on tegument (Nahla *et al.*, 2012). Present study recorded damages in suckers with unorganized and disappearance of the papillae around acetabulum, on dorsal surface of tegument and oral sucker in the *G. crumenifer* treated with extract of *Tinospora cordifolia* stem. Present study is in agreement with Devi *et al.* (2018) who have demonstrated that *Gastrothylax crumenifer* treated with *Microlepis speluncae* (L.) shows tegumental surface around oral sucker exhibiting blebs, wide and deep furrows between transverse major folds and damage on tegument on ventral surface.

Ultrastructure study of treated *Gastrothylax crumenifer*, *Fasciola hepatica*, *Cotylophoron cotylophorum* and *Fasciola gigantica* shows alterations on worm architecture after treatment with some medicinal plants whereas treatment with synthetic drug indicates less effective as compare to medicinal plants (Abdelaal *et al.*, 2016;

Rajesh *et al.*, 2016; Priya and Veerakumari, 2017; Rajesh *et al.*, 2017; Ullah *et al.*, 2017). Present findings derive support from these studies as tegumental changes were seen in both suckers, distorted genital pore with disorganized radial tegumental folds and blebs and swelling appeared on mid region of body of parasite.

In the present investigation, the scanning electron microscopic study of *in vitro* treatment of alcoholic extracts of *Tinospora cordifolia* on *Gastrothylax crumenifer* showed extensive alterations in the tegumental surface and whole body of parasite. Surface tegument was found to be main target area after treatment with medicinal plants and synthetic drug. Alcoholic extract of plants are cheaper, easily available and more suitable than most of synthetic drugs. The results of present study would be supportive in preparing ecofriendly, less costly, anthelmintic veterinary drug, improve the socio-economic condition of cattle farmers.

Conclusion

The present study suggests that the alcoholic extract of *T. cordifolia* could offer a suitable and cheaper alternative anthelmintic in comparison to synthetic drugs. Consequently, it might help to reduce the occurrence of parasites in the goats after treatments and decrease in motility or death of goats.

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