Gastrothylax crumenifer: ultrastructure and histopathology study of in vitro trematodiciidal effect of Microlepia speluncae (L.) Moore

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ABSTRACT
An in vitro incubation study was carried out to determine the efficacy of Microlepia speluncae (MS) fronds’ extract on the worm motility and viability, morphology and histopathological changes in/of Gastrothylax crumenifer. The methanolic extract of MS fronds was used for in vitro study at 1, 2, 3, 4 and 5 mg/ml concentration as test extracts. Oxyclozanide (OXY) at 1% (250 mg/25 ml) as standard control and Hedon-Fleig (H−F) salt solution as negative control. The solutions (25 ml), with 25 amphistomes each, distributed to 7 petriplates (90 mm diameter) and placed in an incubator with 5% CO₂ at 37°C. The motility of control and treated flukes was observed at regular time intervals, 0, 10, 15, 30 and 60 min respectively with specific score 3, 2, 1, 0 for motility. Relative Motility assay and LC₅₀ were determined by probit regression analysis. In vitro incubation study revealed death of all trematodes, lethal at 10 min incubation time at 5 mg/ml concentration, indicated RM value is 0, very much effective than Oxyclozanide, RM value was 0.05. The LC₅₀ was determined as 3.666. The worms subjected to in vitro trials were studied under Scanning Electron Microscope and Light Microscope for significant morphological changes.

1. Introduction
Paramphistomosis is one of the major problems affecting the productivity of livestock throughout the world. In context to India, livestock are of great economic importance as they are closely associated with the life activities of resource-poor rural people. They contribute to financial independence for the people by providing milk, meat and skin. Paramphistomosis has a wide geographical distribution in subtropical and tropical areas, where the infection leads to mortality and low productivity (WHO 2002; Sharma & Busang 2013). It is a disease caused by a group of various species of trematode parasite: Paramphistomum cervi, Gastrothylax spp., Cotylophoron spp., Orthoecolium spp., which are found to be predominant in domestic ruminants. In developing countries, they cause severe morbidity and contribute to the prevalence of malnutrition, anaemia, eosinophilia and pneumonia (Scantlebury et al. 2013). Control of helminth infection in domestic animals is widely based on anthelmintic drugs. Its prolonged use has led to the additional problem of emergence of anthelminthic resistant in livestock, which has become a constant concern. Dependence of control programmes on a limited number of compounds makes drug resistance an even greater concern and careful management of those available is imperative (Kundu et al. 2014). According to World Health Organization, medicinal plants would be the best source to obtain a variety of drugs. Therefore, such plants should be investigated to better understand its safety and efficacy (Nascimento et al. 2000). The origin of many effective drugs is found in traditional medicine practices and has made several researchers to undertake studies for evaluating folklore medicinal plants on their proclaimed anthelmintic efficacy (Mehlhorn et al. 2011). Use of medicinal plants and studies on the chemistry and pharmacology of natural products have grown considerably in the second half of twentieth century (Hossain et al. 2012). Therefore, there has always been a need to find new anthelmintic drugs because current drugs do not control all parasitic infections well. Moreover, high treatment frequency, single-drug regimen or frequent use of the same anthelmintic has led to the development of resistance among helminth population (Geerts & Gryseels 2001). Similarly, its negative, undesired effects, residual effects in products as well as limited availability to the rural areas further restricted the effective control of helminthic diseases (Waller 1997), causing new threat to human society. Hence, investigations into the efficacy of new drugs based on traditional knowledge and traditionally used medicinal plants as an alternative remedies (Mali & Mehta 2008; Tandon et al. 2011) is important. Microlepia speluncae (L.) Moore (MS) belongs to ‘Dennstaedtiaceae’, which is commonly known as ‘limp leaf fern’. Most of the species are native to Asia, with many endemic to China, although a few species occur also in Australia, Africa, the West Indies, Latin America and various oceanic islands (Smith et al. 2006). The plant is seen in terrestrial regions and grows in
moist places on slope sides in open or semi-shaded places or on edges of forests at lower elevations. This species is widely distributed in pantropical regions. The medicinal uses of Pteridophytes have been viably reported (Benjamin & Manickam 2007; Benniamin 2011). The anti-trematodal property of flowering plants has been reported worldwide (Hossain et al. 2012; Alvarez-Mercado et al. 2015); Blakemore et al. (1964) and Amritpal Singh (2011) reported on the anthelmintic property of ferns. Kalpana Devi et al. (2016) reported on the anti-trematodal property of Blechnum orientale against Gastrothylax crumenifer. Another fern, Pteridium sp. belonging to the same has been reported to have anthelmintic property (Benjamin & Manickam 2007). MS also belong to the family Dennstaedtiaceae, whose anthelmintic property is ascertained for the first time by this study. The toxic potential of ferns as well as a challenge to promote this baseline study towards in vivo animal model and further assessment of its toxicity is equally important.

The present study aimed at investigating the in vitro efficacy of M. speluncae against trematode model G. crumenifer and its effect on the morphology and histopathology of the parasites.

2. Materials and methods

2.1. In vitro trematodcidal activity

2.1.1. Collection and preparation of fern extracts

Microlepia speluncae (Figure 1(a)) (Synonym: Polypodium speluncae L. Sp.), plants belonging to the family Dennstaedtiaceae (Figure 1(a)), samples were collected from lower Kodayar (Latitude: 8°31′24.42″, Longitude: 77°21′11.40″, Altitude: 1250 M), a southernmost region of Western Ghats, Kanyakumari district, Tamil Nadu, India. They were identified (Sujana 2013) and authenticated at Scott Christian College, Nagercoil, India and a voucher specimen (Figure 1(b)) (SPCH 1007) was deposited at A. Veeraiya Vandayar Memorial Sri Pushpam College, Thanjavur district, India. The ferns were collected during June, 2015, shade dried, pulverized and extract was prepared as per method of Samundeeswari et al. (2013). Fronds portion of the fern was taken for the study. Briefly, 1 g of dried powder was extracted with 100 ml methanol (Merck, extra pure) for 1 min using an Ultra Turax mixer (13,000 rpm) and soaked overnight at room temperature and filtered through Whatman No. 1 paper and evaporated at 40°C to a constant weight and stored at 18°C and used for in vitro incubation study, performed during July 2015.

2.1.2. Collection and processing of trematodes

Live and healthy adult amphistomes were collected from the rumen of sheep slaughtered at Orathanadu abattoir, Thanjavur District, Tamil Nadu, during July 2015. Random collections were performed early morning, in varied number of sheeps slaughtered on the same day and an in vitro incubation study was conducted within 1 h from time of collection. The distance covered from collection site and laboratory was almost 5 km. The collected worms were washed thoroughly in physiological saline and maintained in Hedon–Fleig (H–F) solution (pH 7.0) for in vitro maintenance of amphistomes (Veerakumari 1996). H–F salt solution (Hajare et al. 2012) contain NaCl 119.82 mM, KCl 4.01 mM, MgSO4 0.29 mM, CaCl2 0.40 mM, NaHCO3 17.8 mM, glucose 22.3 mM, streptomycin sulphate 6900 unit 10 mg/l and benzyl pencillin 9900 units/l at 38 ± 1°C in a BOD incubator. Collected samples contain admixture of trematodes, including G. crumenifer, Fischoederius elongatus and other trematodes. From these, G. crumenifer (Figure 4) alone were identified based on morphology (Soulsby 1982), segregated and subjected to in vitro incubation study as stated. G. crumenifer is mostly found in enormous number (large sample size) during varied sample collection subjected to the study.

2.1.3. In vitro study

The concentration of elute, extracted solvent of M. speluncae (L.) Moore was used for the in vitro incubation study of G. crumenifer trematodes. Twenty-five millilitres each of H–F salt solution containing various concentrations (1, 2, 3, 4 and 5 mg/ml) of fern extracts respectively were poured in five petri plates (90 mm, diameter) as test extracts, Positive control constituted of oxyclozanide (Oxy, I.P. (vet) 3.4% w/v Pack from Vetco Pharma, Gujarat) at 1% (250 mg/25 ml) was poured in the sixth petri plate and

Figure 1. Microlepia speluncae (L.) Moore.
negative control with only H–F salt solution in the seventh petri plate. Twenty-five adult parasites were randomly added to each of the seven petri plates and placed in an incubator with 5% CO₂ at 37°C. A total of three replicates of in vitro incubation study were performed. The flukes were assessed for motility and viability at (0, 10, 15, 30 and 60) minutes of incubation time. The motilities of the worms were scored using the criteria proposed by Kiuchi et al. (1987). The flukes with no motility were stained with the vital dye (1% methylene blue-diluted in 0.85% NaCl (w/v)) for 2 min and the excess dye was washed out with 0.85% NaCl, to test whether live or dead. Motility score were assigned using the following criteria:

3 = movement of the whole body
2 = movement of only part of the body
1 = immobile but not dead (unstained with 1% methylene blue)
0 = immobile and dead (stained with 1% methylene blue)

The efficacies of the drugs were based on calculated Relative Motility (RM) values using the formula below (Saowakon et al. 2009; Tansatit et al. 2012; Saowakon et al. 2013):

$$RM = \frac{MI_{test} \times 100}{MI_{control}}.$$  

Motility Index (MI) = \sum \frac{nN}{N}

n = motility score, N = number of flukes with the score of n.

Lethal Concentration 50 (LC₅₀), a concentration at which the drug was able to kill half of the population, was determined by assessing the motility of the flukes using probit regression analysis (Tayo et al. 2014). A smaller RM value indicates higher mortality rate.

2.2. Gross morphology of amphistomes under carmine staining

The morphological and histopathological variations of the dead worms were studied under light and scanning electron microscope for correlation and comparison.

Amphistomes were subjected for carmine staining for gross morphological study. The amphistomes were washed thoroughly with 0.1 M phosphate buffer saline, pH 7.4 and pressed in between two slides, tied both slides with rubber band and submerged in formalin for at least 12 h for fixing, then they were washed in running tap water overnight. The washed amphistomes were dehydrated with 70% alcohol three times and stained with acetic carmine dye overnight. The amphistomes were destained with 1% acid alcohol, washed in ammonia water, dehydrated with graded series of ethanol and cleared in xylene and permanently mounted with DPX (Soulsby 1982; Jeyathilakan et al. 2012). The stained specimens were examined for abnormalities under stereo-zoom and light microscope and photographed.

2.3. Haematoxylin and eosin staining for histopathology study

Flukes from the groups with lowest (1 mg/ml), highest concentrations (5 mg/ml) of the fern extract, positive control and negative control were stained using haematoxylin and eosin, for observing histopathological changes. The worms were fixed in 10% formaldehyde for 24 h, dehydrated with ascending series of ethanol and cleared with xylene. They were then embedded in paraffin, sectioned longitudinally and transversely at thickness of 5 µm, stained with haematoxylin and eosin (Jeyathilakan et al. 2012) and examined for abnormalities under light microscope and photographed.

2.4. Scanning electron microscope study of amphistomes

Scanning electron microscope (SEM) was used for ultrastructural changes of G. crumenifer subjected to in vitro incubation study. After incubation, worms from the groups as above were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer solution (PBS), pH 7.4 for 12 h at 4°C. Samples were washed in phosphate buffer three times, and post fixed in 1% osmium tetroxide (OsO₄) in 0.1 M PBS, pH 7.4 at 4°C for 2 h (Saowakon et al. 2013). They were washed repeatedly in cold double distilled water three times, dehydrated through ascending concentrations of ethanol, dried in a Hitachi HCP-2 critical point drying machine using liquid CO₂, mounted on aluminium stubs, coated with gold-palladium in a JEOL JSM- 5610LV INCA EDS ion-sputtering apparatus set at 10 mA for 9 min. Specimens were observed and photographed at various magnifications using JEOL JSM- 5610LV INCA EDS SEM operating at 0.5–30 kV.

3. Results

3.1. In vitro incubation study

The results of in vitro incubation study (depicted in Table 1) suggested the RM values decrease when exposed to increased duration of incubation time, for standard control and all varied concentrations (1–5 mg/ml) of fern extracts used. The higher concentration (5 mg/ml) kills all the worms (100%) at 10 min incubation time more potent than the standard drug action of Oxyclozanide (95%). However lower concentration of plant extracts (viz, 1, 2, 3 and 4 mg/ml) took more incubation time rather than killing at 10 min incubation time (Table 1). The LC₅₀ was graphically determined from the probit regression analysis (Figure 2). Using probit regression analysis in determining the LC₅₀, 10 min time was the most suitable one. The results of the entire study could be presented as such, based on which 10 min timing could be expressed as the best one for estimation of LC₅₀.  

Table 2; Figure 2 stated that probit regression was significant. Chi-square test for heterogeneity indicated adequacy of probit model to the data. The concentration for 50% death (LC₅₀) was 3.666 with a 95% confidence interval (CI) of 1.508–4.046 (Table 3).

3.2. Morphology and histopathology studies

Flukes from the control group did not show any morphological changes with characteristic features: Body elongated, length: 10.84–18.39 mm, width: 5.01–7.32 mm, S-shaped uterus, ventral pouch present, reaching to posterior level of posterior
testis, Acetabulum terminal (diameter: 1.62–4.52 mm), Oesophagus (length: 0.45–0.72 mm), testes deeply lobed, side by side, between caecal ends and acetabulum, intestinal caeca long, wavy, reaching up to anterior level of testes (Figure 3). Standard control showed degenerative changes towards Oral sucker, acetabulum, teguments and testes (Figure 4). Flukes from lower concentration drug showed least changes when compared to high concentrated drug. Lower extract concentration (1 mg/ml) showed deformity, vacuolization and degenerative changes of oral sucker, teguments, acetabulum and testes, whereas higher extract concentration showed degenerative changes in Oral sucker, detached teguments around acetabulum, degeneration of testes and Vitelline gland (Figure 4). No changes were notified in other internal organs like intestines of both test and standard control group.

Histopathology of normal, standard and test groups' amphis- tomes was compared under light microscope. Flukes from negative control showed normal features, including anterior sucker, sub-syncytium, testes and Vitelline gland. Standard control showed mild vacoular degeneration of anterior sucker, sub-syncytium and mild degeneration of testes and Vitelline gland. Test groups showed severe degenerative changes towards anterior sucker, sub-syncytium, testes and Vitelline gland (Figure 5).

In SEM study, flukes of the control group revealed a pouched body with anterior end bounded by thick muscular rim. The tegument was composed of transverse major folds alternating with major grooves. The oral sucker was positioned at the anterior tip, and the genital pore located ventrally at the middle of the anterior third of the body. The acetabulum (caudal sucker) was positioned close to posterior tip. No tegumental changes were observed in control flukes. In OXY-treated flukes, general features appeared similar to control flukes at 1 h post incubation. However when observed at higher magnification, tegumental surface around oral sucker exhibited blebs on the papillae. There was wide and deep furrows between transverse major folds. The degree of tegumental damages was more on ventral surface. Drug-treated

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### Table 1. Motility index and RM values of *Gastrothylax crumenifer* on various time mode of exposure to *Microlepia speluncae* (L.) Moore extract.

| Concentration (mg/L) | 0 min | 10 min | 15 min | 30 min | 1 h | Replicates | Average RM | Replicates | Average RM | Replicates | Average RM | Replicates | Average RM | Replicates | Average RM |
|----------------------|-------|--------|--------|--------|-----|-----------|------------|-----------|------------|-----------|------------|-----------|------------|-----------|------------|
| 0 (NC)               |       |        |        |        |     |           |            |           |            |           |            |           |            |           |            |
| 3.00                 | 1.00  | 1.00 ± 0.00 | 1.00  | 1.00 ± 0.00 | 1.00  | 1.00 ± 0.00 | 1.00  | 1.00 ± 0.00 | 1.00  | 1.00 ± 0.00 | 1.00  | 1.00 ± 0.00 | 1.00  | 1.00 ± 0.00 | 1.00  | 1.00 ± 0.00 |
| 1                    |       |        |        |        |     |           |            |           |            |           |            |           |            |           |            |
| 3.00                 | 1.00  | 1.00 ± 0.00 | 2.28  | 0.76 ± 0.021 | 2.00  | 0.67 ± 0.021 | 1.40  | 0.47 ± 0.020 | 0.00  | 0.00 ± 0.00 | 0.00  | 0.00 ± 0.00 | 0.00  | 0.00 ± 0.00 | 0.00  | 0.00 ± 0.00 |
|                     |       |        |        |        |     |           |            |           |            |           |            |           |            |           |            |
| 2                    |       |        |        |        |     |           |            |           |            |           |            |           |            |           |            |
| 3.00                 | 1.00  | 1.00 ± 0.00 | 1.92  | 0.64 ± 0.015 | 1.44  | 0.48 ± 0.009 | 0.92  | 0.31 ± 0.000 | 0.00  | 0.00 ± 0.00 | 0.00  | 0.00 ± 0.00 | 0.00  | 0.00 ± 0.00 | 0.00  | 0.00 ± 0.00 |
|                     |       |        |        |        |     |           |            |           |            |           |            |           |            |           |            |
| 3                    |       |        |        |        |     |           |            |           |            |           |            |           |            |           |            |
| 3.00                 | 1.00  | 1.00 ± 0.00 | 1.80  | 0.61 ± 0.212 | 1.32  | 0.44 ± 0.011 | 0.91  | 0.31 ± 0.000 | 0.00  | 0.00 ± 0.00 | 0.00  | 0.00 ± 0.00 | 0.00  | 0.00 ± 0.00 | 0.00  | 0.00 ± 0.00 |
|                     |       |        |        |        |     |           |            |           |            |           |            |           |            |           |            |
| 4                    |       |        |        |        |     |           |            |           |            |           |            |           |            |           |            |
| 3.00                 | 1.00  | 1.00 ± 0.00 | 0.52  | 0.17 ± 0.003 | 0.00  | 0.00 ± 0.00 | 0.00  | 0.00 ± 0.00 | 0.00  | 0.00 ± 0.00 | 0.00  | 0.00 ± 0.00 | 0.00  | 0.00 ± 0.00 | 0.00  | 0.00 ± 0.00 |
|                     |       |        |        |        |     |           |            |           |            |           |            |           |            |           |            |
| 5                    |       |        |        |        |     |           |            |           |            |           |            |           |            |           |            |
| 3.00                 | 1.00  | 1.00 ± 0.00 | 0.00  | 0.00 ± 0.00 | 0.00  | 0.00 ± 0.00 | 0.00  | 0.00 ± 0.00 | 0.00  | 0.00 ± 0.00 | 0.00  | 0.00 ± 0.00 | 0.00  | 0.00 ± 0.00 | 0.00  | 0.00 ± 0.00 |
|                     |       |        |        |        |     |           |            |           |            |           |            |           |            |           |            |
| OXY (PC)             |       |        |        |        |     |           |            |           |            |           |            |           |            |           |            |
| 3.00                 | 1.00  | 1.00 ± 0.00 | 0.20  | 0.07 ± 0.012 | 0.00  | 0.00 ± 0.00 | 0.00  | 0.00 ± 0.00 | 0.00  | 0.00 ± 0.00 | 0.00  | 0.00 ± 0.00 | 0.00  | 0.00 ± 0.00 | 0.00  | 0.00 ± 0.00 |
|                     |       |        |        |        |     |           |            |           |            |           |            |           |            |           |            |
|                     |       |        |        |        |     |           |            |           |            |           |            |           |            |           |            |

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### Table 2. Parameter estimate.

| Parameter | Estimate | Std error | Z     | Sig. | 95% Confidence interval |
|-----------|----------|-----------|-------|------|-------------------------|
| PROBITa con | 14.078  | 6.205 | 2.269 | .023 | 1.917 | 26.240 |
| Intercept | −7.942  | 3.873 | −2.051 | .040 | −11.816 | −4.069 |

*PROBIT model: PROBIT(p) = Intercept + BX (Covariates X are transformed using the base 10,000 logarithm).*
flukes showed swelling followed by blebbing, resulted in formation of bulbous structure. Blebs were disrupted and eroded and sloughed off exposing basal lamina, devoid of syncytium.

The severity of erosion in the flukes from the test groups might be attributed due to penetration of plant secondary metabolites (PSMs) and was dose dependant and highest in 5 mg/ml than 1 mg/ml and positive control (Figure 6).

4. Discussion

Various studies on in vitro and in vivo anti-trematodal activity of medicinal plants viz., alcoholic extract of *Allium sativum* and *Piper longum* (Singh et al. 2015), *Balantia aegyptica* alcoholic extract (Swarnakar et al. 2015), bark of *Prosopis cineraria* (Manigandan & Veerakumari 2015), Plumbagin on newly excysted and 4-weeks-old juvenile parasites of *Fasciola gigantica* (Natcha et al. 2014), and Plumbagin on motility, survival and tegument structure of *Paramphistomum cervi* (Saowakon et al. 2013) have been carried out. Similarly, an in vivo study in natural fascioliosis-infected cattle (Shokier et al. 2013) has been reported. The present study is the first to report on *Pteriodophytic* plants having anti-trematodal efficacy.

The effect of *M. speluncae* extracts against rumen amphistomes, *G. crumenifer* was tested and it was found that complete paralysis and death of the worm occurred at higher concentration with least incubation time. Gross microscopical changes under light microscope showed drug-treated worms became small with shrunken tegument. Control worms showed smooth tegument followed by surface syncytium, sub-syncytial zone, longitudinal and circular muscles, similar to the study of *Swarnakar et al. (2014)*.

The results were in accordance with many other similar works on amphistomes (Jeyathilakan et al. 2010, 2012; Veerakumari et al. 2013) and demonstrated that depending on dose, *M. speluncae* can reduce motility and cause death of adult *G. crumenifer* on varying time mode as evaluated by RM values. The major target organ that was highly affected is the tegument, whose damages were observed by LM and SEM. The effects of MS extracts (at 5 mg/ml) were more severe than OXY. It is possible that OXY takes longer time and higher dose to immobilize and kill the trematodes (Hossain et al. 2012). When observed by SEM, the sequences of tegumental surface changes were similar for all doses of MS as well as OXY, and consisted of swelling, fibrous network formation between major and minor folds, blebbing, which was later ruptured, leading to erosion and desquamation of the tegument, resulting in the lesion, and finally the exposure and disruption of basal

**Figure 3.** General morphology of *Gastrothylax crumenifer* (Negative control). Oral sucker (Os), Pharynx (Ph), Oesophagus (Oe), Ventral pouch (Vp), Caecum (C), Uterus (U), Vitelline follicle (Vf), Vitelline gland (Vg), Testes (T), Ovary (O) and Acetabulum (Ac).
lamina. The tegument is an important structure of parasite because it provides covering and protection of the parasite’s body, and supports internal organs. It also controls the secretion, synthesis, perception of sensory stimuli and osmoregulation. It was demonstrated here that tegument is a major target of MS, which was probably absorbed by the tegument. The initial tegument swelling was believed to be part of the general response of the fluke to a stress situation, representing an attempt by the fluke to replace damaged surface membrane (Stitt & Fairweather 1993), caused by osmotic imbalance, due to the disruption of ion pumps present on the apical plasma membrane (Skuce et al. 1987). This was followed later by swelling, blebbing, disruption, erosion and lesion. Once the surface layer is totally destroyed, the drug could penetrate deeper into the muscular layer and caused motility reduction and cessation that lead finally to death. Regional difference of responses to the MS were also observed, with the ventral side being more severely affected than the dorsal surface, and the anterior and middle third regions as well as the lateral margins of the flukes were generally more affected than the posterior region. The early changes were found at the oral sucker and the genital pore, which exhibited swollen appearance with scattered blebs along their rims. The acetabulum also was distorted. Surface changes observed in the present study resemble that demonstrated on *F. gigantica* treated with aqueous extract of *Artocarpus lakoocha* (Saowakon et al. 1997).

**Figure 4.** Gross morphological changes of Standard and treated G. crumenifer. Stereo-Zoom Microscope study of G. crumenifer for morphological changes under incubation for 1 h: (A–D: Standard Control) (A) Whole view of G. crumenifer showing shrinkage of teguments, degenerative changes of Oral sucker (Os). (B) Disruption of Oral sucker (Os). (C) Degenerative changes of Oral sucker (Os). (D) Degenerative changes of Testes (T), Oral sucker (Os) and Acetabulum (Ac). (E–H: Test Group) (E) Degeneration of Oral sucker (Os) and Acetabulum (Ac) at 1 mg/ml conc. (F) Deformity of Oral sucker (Os), acetabulum and degenerative changes in testes (T) at 1 mg/ml conc. (G) Whole view of G. crumenifer showing severe degenerative changes of Oral sucker (Os), Testes (T) and Vitelline gland (Vg) at 5 mg/ml conc. (H) Severe degenerative changes in Right Testes (RT) at 5 mg/ml conc.

**Figure 5.** Histopathology study of G. crumenifer. Histopathology showing (A–D) Control treated section of worms showing normal Anterior Sucker 10× (As) (A), Sub-Syncytium 10× (Ss) (B), Testes 10× (T) (C) and Vitelline Gland 10× (Vg) (D). (E–H) 1 mg Concentration test group of worms showing As mild vacuolar degeneration 4× (E), mild sub-syncytium surface disruption 10× (F), normal mild degeneration of sub-syncytium 10× (G), mild degeneration of testes 4× (H). (I–L) 5 mg Concentration test group of worms showing As severe sucker cell vacuolization 20× (I), Degeneration of Posterior sucker 10× (Ps) (J), Testes 10× (Td) (K), Vitelline gland 20× (Vgd) (L).
2009), and on *Paramphistomum microbothrium* treated with artemether (Shalaby et al. 2010).

The tegument of trematode comprises an outer surface syncytium underlined by a thick subsyncytial zone and musculature (Sharma & Hanna 1988). MS might be exerted its effect on the tegument first then permeated through the underlying muscle, which exhibited drastically decreased motility. Gross disruption of tegument clearly visible to the naked eye was observed in all specimens at the higher doses. The flukes' surface appeared dark with tegumental desquamation. On the other hand, the flukes might also ingest MS through the oral sucker because numerous blebs were usually found at the anterior part of worm, especially around the oral sucker. Dome-shaped papillae are commonly present on trematodes' tegument surfaces, and they are believed to have a sensory function (Tandon & Maitra 1982) related to feeding at the oral aperture, pressure detection on the general body surface and sexual reception around the genital pore (Bennett 1975). The papillae were also damaged by MS, which could cause the loss of sensory functions. Besides, the damage of the acetabulum might affect the holding onto the host tissues (Shalaby et al. 2010).

The in vitro incubation study and RM value suggest that this fern extract had reliable source of anti-trematodal property. Hence it is concluded that *M. speluncae* can be used as a potent anti-trematodal drug, cost effective and to overcome anthelmintic resistance against Oxyclozanide and other trematodicidal drugs. This preliminary study could be an outcome initiation for further detailed study as in vivo animal models to ascertain toxicity. Thus knowledge and understanding gained in vitro trematode model could promote in vivo controlled studies in animal model, and an array of bioactive molecules could be discovered for further clinical applications in human and veterinary parasitology.

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**Disclosure statement**

No potential conflict of interest was reported by the authors.

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