Chapter 8

Ultrastructural observations of tegument of *in vitro* treated *Fasciola gigantica* with three selected medicinal plant extracts by transmission electron microscope
Introduction

Fasciolosis is a major parasitic disease of livestock. Fasciolosis, caused by *F. gigantica* is one of the most prevalent helminth infections of the ruminant. Fascioliasis has long been considered an important veterinary problem leading to high annual economic losses of $2-3 Billion. Now it is also recognized as a human disease of major public health importance including high pathogenicity. Animal and human cases are being increasingly reported from Europe, the Americas, Oceania, Africa and Asia. Hence, animal and human fasciolosis are considered now as a zoonosis of major global and regional importance (MasComa, 2005 and Rioux *et al.*, 2007).

Control of fascioliasis is greatly dependent on the use of synthetic drugs; triclabendazole and albendazole have been used to control fasciolosis since 1983 as a veterinary drug due to unavailability of effective vaccines till now. However, continued use of these drugs is under threat due to development of resistance, high cost, development of resistance, chemical residue in milk and meat, toxicity problem and failed snail control measures the majority of world population depend on traditional remedies have prompted the search for new compounds or for better use of existing drug (Fairweather, 2005 and 2009; Githori *et al.*, 2006; Brenann *et al.*, 2007; Wedrychowicz *et al.*, 2007; Halferty *et al.*, 2009; Saowakon *et al.*, 2009 and Massoud *et al.*, 2012).

So, there is a pressing need to find new fasciolicidal drugs that are effective against different parasites stages, for both treatment and control of this trematode infection. Nowadays, researchers are directed towards compounds of plant origin back to nature instead of using synthetic ones, which may be more dangerous than the disease itself. The plant kingdom is known to provide a rich
source of anthelmintic, antibacterial and insecticide. Several anthelminthic plants *Trigonella foenum-graecum*, *Clitorea ternatea*, *Balanites aegyptica*, *L. amplexicaule*, *M. philippinensis*, *W. somnifera*, *A. indica*, *A. juss* and *C. colocynthis* were used to control and treatment of *Pheretima posthuma*, *Fasciola buski*, *Fasciola hepatica*, *Fasciola gigantica*, *Toxocara vitulorum*, *Paramphistomum microbothrium* and *Haemonchus contortus*, *Schistosoma mansoni*, *Schistosoma japonicum*, *Raillietina echinobothrida*, *R. tetragona* and *Ascardia galli*. Parasites (Shuhua et al., 2002; Meaney et al., 2002; Jabbar et al., 2006; Bhalke et al., 2008; McConvile et al., 2008; Diab et al., 2010; Khadse and Kakde, 2010; Nahar et al, 2010; Salhan et al., 2011; Ebeid et al., 2011; Roy et al., 2012a & 2012b; Shalaby et al., 2012; Intisar et al., 2013; Swargiary, 2015; Shalaby et al., 2016; Buchineni & Kondaveti, 2016 and Nandi et al., 2017).

The efficacy of ethnomedicinal plant aqueous extracts such as *Allium sativum*, *Lawsonia inermis*, *Opuntia indica* and *Caparis decidua* stem, *Moringa olifera* leaves *in vitro* in comparison with the chemotherapeutic agent Oxyclozanide and albendazole on *F. gigantica* adult (Jeyathilakan et al., 2012; Sumaia et al., 2012).

Shalaby et al., (2012) investigated the comparative morphological effects of Ivermectin and *Nigella sativa* oil combination and each of them separately against adult helminth parasites includes *Haemonchus contortus*, *Moniezia expansa*, and *F. gigantica*.

Indian traditional spices such as *Allium sativum*, *Ferula asafoetida*, *Ocimum sanctum*, *Syzygium aromaticum*, *Terminalia catappa* and Thymoquinone and Curcumin have several medicinal and pesticidel properties such as antioxidant, antiviral, antibacterial, antifungal, molluscicida, antihelminthic and larvicidal the *in vitro* toxicity of species such as *A. sativum*, *F. asafoetida* and *S. aromaticum* on the liver fluke *F. gigantica* (Kumar et al., 2014; Mahardika et al., 2017 and Ullah et al., 2017).
Very little research work was observed on ultrastructure alteration of the tegument of trematodes parasites, due to in vitro effects of anthelminthic medicinal plants and their research work found good result comparatives to synthetic (Keiser et al., 2006a and b; Keser and Morson, 2008a and b; Osama et al., 2010; Veerakumari et al., 2012 and Devi et al., 2018). On the basis of above review of literature, it was decided to work on ultrastructural observations of tegument of in vitro treated Fasciola gigantica with alcoholic extract of seeds of Centratherum anthelminticum (Kalijiri), fruit of Citrullus colocynthis (Indrayan or Kharatumba) and seeds of Trachyspermum ammi and compared with albendazole by transmission electron microscope (TEM).
Result

Tegument of control *Fasciola gigantica* by Transmissions electron microscope

On the basis of the presence of various organelles and density of the tegumental skeletal of the tegument of *Fasciola gigantica* divided into 4 layers. First and outer most layers are known as syncytium epithelium layer that is thick with the density of the cytoplasmic matrix, secretory granules, and some organelles. The cytoplasm of the first layer consists of strongly packed microtubules of very thin filaments. The outer surface of syncytium layer has many rounded and branched glycocalyx and ridges or microfolds inverted by rectangle pits as visualized. The grooves between major folds may run deep down in the tegument. The second layer was a thin strip of cytoplasmic that contained a high concentration of discoid bodies, endoplasmic reticulum, mitochondria, lysosomes and spherical bodies. The third layer was the widest zone of the tegument cytoplasm that contained a high concentration of mitochondria, secretory granules and dense scaffold of the cytoskeleton network and lysosomes are present. It contained evenly distributed discoid as well as spherical bodies, but with much lower concentration than in the first two layers. The fourth layer was the basal zone where there were in the folding of the basal plasma membrane. The basal plasma membrane rested on the thick basal lamina which was coupled to the former by series of hemidesmosomes.

Tegument of *F. gigantica* with first syncytium epithelium layer, second basal lamina layer, third musculature and fourth layer contains many different types tegumental cell with many cell organelles namely endoplasmic reticulum, mitochondria, Golgi complex, and ribosomes are present. Numerous parenchymal cells observed between the tegumental cells. Tegument of *F. gigantica* also exhibited tegumental outer parts of ridge and pits in the tegument layer first syncytium zone and high concentration of glycogen granules and lysosomes in the tegument layer second basal lamina (Plate 1, Fig. 1A and 1B). Numerous pointed and comb-like spines are present in the tegument. All spines are embedded in the
whole thickness of the tegument whereas, tip portion projected outside of surface syncytium, is covered by tegumental thin layer. Spins are originated from basal or muscular part of tegument from the muscular (Plate 1, Fig. 1C, 1D, 1E and 1F & Diagram 2).

**Tegument of in vitro treated Fasciola gigantica with alcoholic seeds extract of Centratherum anthelminticum, fruits of Citrullus colocynthis and seeds of Trachyspermum ammi by Transmissions electron microscope (TEM)**

*Fasciola gigantica* parasites are treated with alcoholic extracts of seeds of *Centratherum anthelminticum*, fruits of *Citrullus colocynthis* and seeds of *Trachyspermum ammi*. Treated parasites became clumped, paralyzed and died after 15 hours exposure time at a concentration of 50mg/ml alcoholic extracts of these three of plants. The present investigation revealed that the alcoholic extract of medicinal plants caused highly destructive alternation and distortion in the tegumental architecture of treated *Fasciola gigantica* parasites.

1. **Tegumental alterations in Fasciola gigantica treated with seeds extract of Centratherum anthelminticum by Transmission electron microscope (TEM)**

Anterior region tegument of treated *F. gigantica* showing damaged tegument, removed outer syncytium epithelium, vacuolization and damaged are showing in basal lamina and muscle fibers. Damages, vacuolization, and breakage are also observed in longitudinal muscles and circular muscles of tegument (Plate 2, Fig. 2A and 2B). Tegument of the middle region of treated *F. gigantica* exhibited removed tegumental layer first and damaged basal lamina and tegumental circular muscles with vacuolization exist between the calls in the underlying tissue (Plate 2, Fig. 2C and 2D). Posterior region of treated *F. gigantica* also showing removing tegument outer layer and damaged and removed internal tissue of tegument and muscles fibers. Highly breakage in basal lamina and vacuolization in the tegument due to removed all internal tegumental muscles (Plate 2, Fig. 2E and 2F).
2. **Tegumental alterations in *Fasciola gigantica* treated with alcoholic fruits extract of *Citrullus colocynthis* by Transmissions electron microscope (TEM)**

   Anterior region tegument of treated *F. gigantica* showing damaged and removed tegumental syncytium epithelium, glycocalyx, and damaging basal lamina and longitudinal muscles (Plate 3, Fig. 3A and 3B). Middle region tegument of treated *F. gigantica* also observing removed spines and tegumental outer layer extract drinking by pinocytes and brakes in basal lamina (Plate 3, Fig. 3C and 3D). Posterior region tegument of treated *F. gigantica* exhibited removed spines and tegumental outer layer granules and lysosomes, damages in the tegumental tissue and vacuolization, breakage with a detachment in basal lamina and muscles fiber (Plate 3, Fig. 3E and 3F).

3. **Tegumental alteration in *Fasciola gigantica* treated with seeds extract of *Trachyspermum ammi* by Transmissions electron microscope (TEM)**

   Anterior region tegument of treated *F. gigantica* showing large space created by removed spines and damaged tegument glycocalyx, granules and lysosomes, damaged and vacuolization are also observing in basal lamina (Plate 4, Fig. A and B). Middle region tegument exhibited large space formed by removed spines and damaged tegument with vacuolization are present in the tegument and muscles fiber due to highly damaged and removed internal organ of tegument (Plate 4, Fig. 4C and 4D). Posterior region tegument of treated *F. gigantica* also exhibited removed all spines and removed the outer layer of tegument, glycocalyx, granules and lysosomes are damaged with shrinkage in basal lamina (Plate 4, Fig. 4E and 4F).

4. **Tegumental alteration in *Fasciola gigantica* treated with synthetic drug albendazole by Transmissions electron microscope (TEM)**

   Tegument of treated *F. gigantica* showing few damaged spine with few vacuolization present in the tegumental basal lamina. Swelling in muscles fibers
observing moderate effective (Plate 5, Fig. 5A and 5B). Tegument of treated *F. gigantica* showing internal material, small spaces exist between the calls in the underlying tissue, basal lamina, few vacuolization and damages in muscles fibers. Few vacuolization are also observing in syncytium epithelium and the pinocytes developed in basal lamina (Plate 5, Fig. 5C and 5D).
Discussion

The present study has shown ultrastructural observations of tegument of *in vitro* treated *Fasciola gigantica* with an alcoholic extract of seeds of *Centratherum anthelminticum* (Kalijiri), fruit of *Citrullus colocynthis* (Indrayan or Kharatumba) and seeds of *Trachyspermum ammi* and compared with albendazole by transmission electron microscope.

Transmission electron microscopy of tegument of trematode parasites revealed that internal cellular structure changes as the location and possible variation of any disruption due to drug treatment. Several alterations were observed during present study like swelling, blebbing and dislodgement of spines with disruption in the covering tegument.

Becker *et al.*, (1980) revealed disruption and scattering in apical tegumental layer, degeneration of sensory structure, damages in parenchymal tissue and musculature, shrinkage of granular endoplasmic reticulum and vacuolization in the tegument of *Schistosoma mansoni* and *Dicrocoelium dendriticum* after treatment with praziquantel and artemether.

*Fasciola hepatica* revealed disruption in the tegument, extensive loss of syncytium layer, autophagy vacuoles appear, Golgi complex becomes smaller in size, swelling in mitochondria, granular endoplasmic reticulum and secretory bodies and lipid droplet appeared after treatment with the flukicidal drug clorsulon and compound alpha. Vacuolization in apical cytoplasm, T1 bodies become depleted in cell bodies, cytoplasmic processes, and disruption in musculature bundles; circular and longitudinal muscle was observed. Tegumental changes were considered to be an indicator of stress reactions by the fluke in an attempt to replace damaged surface (Stitt and Fairweather, 1993; Meaney *et al.*, 2002; Keiser and Morson 2008b and McConvile *et al.*, 2008).

All cellular alterations are undoubtedly disrupted many of the physiological processes associated with the tegument including osmoregulation, nutrient uptake secretion, synthesis and immunoprotection (Haltom, 2004). Presence of spines on the surface of trematodes were function as an abrasive apparatus to host epithelium producing debris for feeding and reasonably
suggested to play significant roles in the benefit of the worm; they serve as
attachment function, helping the parasite to anchor to the host surface to maintain
good contact in his surrounding environment which in turn protects it from being
washed out with the host secretions (Srisawangwonk, 1989 and Hong et al.,

Sensory papillae were reported to play several roles in the trematode; they
act as rheo-receptors, sensing the currents of fluids in their environment, also they
are tango receptors, sensing contact with the surface epithelium. Destruction of
spines and sensory papillae may stop the vital functions as chemoreceptor
function of sensory papillae of parasites (Woo et al., 1998). Tegumental changes
observed during the present work similar Meaney et al., (2002) investigations,
who studied the effects of the active sulphoxide metabolite of triclabendazole
Fasciolicide of choice on the surface morphology of F. gigantica in vitro. He
observed that changes became progressively severe with higher concentrations
and longer durations of exposure. Moreover, several studies have documented
extensive tegumental damage in triclabendazole-susceptible and Synthetic drugs
on Fasciola gigantica and Fasciola hepatica, whereas Synthetic drug albendazole
and triclabendazole resistant ones showed only localized and relatively minor
disruption of the tegument (Meaney et al., 2002; Robinson et al., 2002;
Keiser et al., 2006a and b; McConile et al., 2006; Halferty et al., 2009 and
Soliman and Taha, 2011).

Results of the present work are in agreement with those of several studies
evaluated in vitro effects of plants extracts on adult Fasciola gigantica tegument
ultrastructure; Keiser et al., (2006) and Shalaby et al., (2009). Swelling of
tegument with slight loss of spines reported with lower concentrations, while
severe tegumental ulcerations and sloughing, many furrows, extreme swelling and
deformity, swollen suckers and severe loss of spines leaving empty holes occurred
(Meshnick, 2002). Other suggested the mechanisms were, interference with the
parasite sarcoplasmic/endoplasmic reticulum Ca+ ATPase, disruptions of the
parasite mitochondrial function and inhibition of angiogenesis (Golenser et al.,
2006). Tegumental swelling and blebbing to the osmotic imbalance, secondary to
the affection of Na⁺-K⁺ pump, leading to an influx of Na⁺ and water with

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consequent swelling of the syncytium (Saowakon et al., 2009). The tegument of *Fasciola gigantica* has numerous mitochondria especially in close association with the basal membrane infoldings (Sobhon et al., 2000) and these mitochondria might be closely involved in providing the energy for ion transports, Swelling in mitochondria affected oxidative phosphorylation in mitochondria and the ATP production could decrease and disturbed ion transports (Fairweather et al., 1984). Several scientists elucidated osmotic imbalance by damage and disruption of the surface membrane of the tegument. Alterations and damage in the tegument of parasites could be referred to disruption of the microtubule-mediated process and block the transportation of secretory granules across the tegument (Stitt and Fairweather, 1993).

Medicinal plants such as *C. molmol*, *Flemingia vestita*, and *Meryta denhamii* were examined for their *in vitro* schistosomicidal and fasciolicide activity against *Schistosoma mansoni* and adult *Fasciola gigantica* using electron microscopy (Badria et al., 2001; Hassan et al., 2003; Massoud et al., 2004; Fathy et al., 2005 and Toner et al., 2008). Schistosomicidal effects were found on disruption of tegument with the collapse of tubercles, sensory papillae, and disruption of spines, loss of spines covering tegument tubercles, erosion of the tegument. Current investigations are similar the previous scientific reports because of several tegumental changes due to anthelminthic effects of medicinal plants.

The results of the present investigation revealed *Fasciola gigantica* exposed to an alcoholic extract of seeds of *Centratherum anthelminticum* (Kalijiri), the fruit of *Citrullus colocynthis* (Indrayan or Kharatumba) and seeds of *Trachyspermum ammi* were documented to have strong *in vitro* anthelminthic and antiflukcidial activities against *Fasciola gigantica*. Also shows dose-dependent mortality and motility found significant destruction in the tegumental structure revealed the anthelminthic activity of plant than albendazole. These plants have phytochemical constituents which are causes anthelminthic activity and control pathogenic helminth parasites caused dose-dependent activity (Khadse & Kakde, 2010 and Nahar et al, 2010). Severe tegumental changes were characterized by swelling, loss of spines, cytoplasm vacillations, the disappearance of secretory
granules, degeneration of nuclear materials and sunken sensory papillae in the swollen tegument. Cytoplasmic and cell organelles variations in the tegument induced by medicinal plants seeds of Kalijiri, Kharatumba, and seeds of Ajvyan extract could use a profound effect upon the parasites metabolic activities due to alteration effect upon the parasites surface rich in glycoproteins. Present research work is important and significant for treatment of veterinary parasites and also found similar observations on other parasites by many researchers (Mohamed et al., 2005; El-Shenawy et al., 2008; Soliman, 2011; Shalaby et al., 2012a; & 2013 and 2016).

Several disruption and vacuolization, erosion in basal lamina, depletion of parenchymal cell, stripping of tegument, clumping of chromatin in nucleus and fuzzy form mitochondria are common destructions in the tegumental cytoplasm were observed due to Alcoholic extract of seeds of Centratherum anthelminticum (Kalijiri), fruit of Citrullus colocynthis (Indrayan or Kharatumba) and seeds of Trachyspermum ammi effects on Fasciola gigantica. Whereas, the extent of damages was also observed in Brachylaima Species treated with Praziquantel (Gallego and Gracenea, 2016), Raillietina tetragona treated with Clerodendrum viscosum (Nandi et al., 2017) and in Cotylophoron cotylophorum in vitro treated with Acacia concinna (Priya and Veerakumari, 2017).

The present study has confirmed that alcoholic extract of seeds of Centratherum anthelminticum (Kalijiri), the fruit of Citrullus colocynthis (Indrayan or Kharatumba) and seeds of Trachyspermum ammi shows encouraging fasciolicidal effects. They caused not only significant changes of the Fasciola gigantica tegument. These findings give hope for new lines towards control and treatment of fascioliasis, using new eco-friendly, less costly, natural and safe and easily available herbal anthelminthic drug.
Diagram 2. Tegument of *Fasciola gigantica*
Explanations of Photographic Plate 1

Ultraphotomicrographs of control *Fasciola gigantica* by transmissions electron microscope (TEM)

Fig. 1 A. Tegument of *F. gigantica* showing outer layer glycocalyx (GL) with different layer first syncytium (SY) epithelium, layer second basal lamina (BL) and layer third musculature (ML) X 2µm.

Fig. 1 B. High magnification of tegument of *F. gigantica* showing outer parts glycocalyx (GL) of ridge (RI) and pits (PI) in tegument layer first syncytium (SY) zone and high concentration lysosomes (L) in the tegument layer second basal lamina X 1µm.

Fig. 1 C. Full single developing spine (S) in the tegument (T) of *F. gigantica* X 10µm.

Fig. 1 D. High magnification of developing spine (S) tip covered with tegument (T) layer of *F. gigantica* X 2µm.

Fig. 1 E. High magnification of basal region of spine (S) in the tegument muscles of *F. gigantica* X 2µm.

Fig. 1 F. High magnification of posterior region spine (S) covered with tegumental (T) layer of *F. gigantica* X 1µm.
Explanations of Photographic Plate 2

Ultraphotomicrograph of in vitro treated Fasciola gigantica with seeds extract of Centratherum anthelminticum by transmission electron microscope (TEM)

Fig. 2 A. Anterior region tegument of treated F. gigantica showing damaged (DA) tegument, removed outer syncytium epithelium, vacuolization (V) and damaged are showing in basal lamina (BL) and muscle fibers (MF) X 1µm.

Fig. 2 B. Tegument of treated F. gigantica showing damages (DA), vacuolization (V) and breakage (BR) in longitudinal muscles, circular muscles and basal lamina X 1µm.

Fig. 2 C. Tegument of treated F. gigantica showing removed tegumental layer first and damaged (DA) basal lamina (BL), vacuolization (V) exist between the calls in the underlying tissue X 1µm.

Fig. 2 D. Middle region tegument of treated F. gigantica showing damaged (DA) tegumental circular muscles (CM), vacuolization (V) by removing tegumental tissues. X 1µm.

Fig. 2 E. Posterior region of treated F. gigantica showing removed tegument outer layer, vacuolization (V), damaged (DA) and removed internal tissue of tegument and muscles fibers (MF) X 1µm.

Fig. 2 F. Posterior region tegument of treated F. gigantica showing highly damaged (DA) and breakage (BR) in basal lamina (BL), removed outer layer of tegument and vacuolization (V) by removed all internal tegumental muscles (M) X 1µm.
Explanations of Phtographic Plate 3

Ultraphotomicrograph of in vitro treated *Fasciola gigantica* with fruits extract of *Citrullus colocynthis* by transmission electron microscope (TEM)

Fig. 3 A. Anterior region tegument of treated *F. gigantica* showing damaged (DA) tegument removed syncytium epithelium, glycocalyx, and damaging basal lamina (BL) and longitudinal muscles X 2µm.

Fig. 3 B. High magnification of highly damaged (DA) basal lamina (BL) and removing tegumental outer layer of treated *F. gigantica* X 1µm.

Fig. 3 C. Tegument of treated *F. gigantica* showing removed spines and tegumental outer layer, vacuolization (V) and brakes (BR) basal lamina X 1µm.

Fig. 3 D. High magnification of basal lamina (BL) showed pinocytes (PI) with granules of middle region of treated *F. gigantica* X 0.5µm.

Fig. 3 E. Posterior region of treated *F. gigantica* showing removed spines and tegumental outer layer granules and lysosomes, damaged (DA) tegumental tissue and vacuolization (V) and detachment (DE) in basal lamina X 2µm.

Fig. 3 F. High magnification of posterior region of treated *F. gigantica* showing removed spines and removed tegumental outer layer, breakage (BR) and vacuolization (V) in basal lamina (BL) and muscles fiber X 1µm.
Explanations of Photographic Plate 4

Ultraphotomicrograph of in vitro treated Fasciola gigantica with seeds extract of Trachyspermum ammi by transmission electron microscope (TEM)

Fig. 4 A. Tegument of treated F. gigantica showing large vacuole (V) created by removed spines and damaged (DA) tegument X 2µm.

Fig. 4 B. High magnification of treated F. gigantica showing removed spines, glycocalyx, granules and lysosomes, damaged (DA) and vacuolization (V) in basal lamina (BL) of X 1µm.

Fig. 4 C. Tegument of treated F. gigantica showing large space formed by removed spines, damaged (DA) tegument and muscles fibers (MF) with vacuolization (V) X 1µm.s

Fig. 4 D. High magnification of highly damages (DA) in tegument and vacuolization (V) due to removed internal organ with swelling (SW) in muscles fiber (MF) of treated F. gigantica X 0.5 µm.

Fig. 4 E. Posterior region tegument of treated F. gigantica showing removed all spines, glycocalyx, granules and lysosomes, damaged (DA), shrinkage (SH) and vacuolization (V) in basal lamina (BL) X 1µm.

Fig. 4 F. High magnification of highly damaged (DA), shrinkage (SH) and vacuolization (V) basal lamina and removed outer layer of tegument of treated F. gigantica X0.5µm.
Explanations of Photographic Plate 5

Ultraphotomicrograph of *in vitro* treated *Fasciola gigantica* with synthetic drug albendazole by transmission electron microscope (TEM)

Fig. 5 A. Tegument of treated *F. gigantica* showing few damaged spine (S), and few vacuolization (V) in tegument (T) and basal lamina (BL) X 2µm

Fig. 5 B. High magnification of tegumental basal lamina (BL) showing few swelling (SW), vacuolization (V) in muscles fibers (MF) showing moderate effective X 1µm.

Fig. 5 C. Tegument of treated *F. gigantica* showing internal material, small spaces exist between the calls in the underlying tissue, basal lamina (BL), few vacuolization (V) and damages in muscles fibers X 1µm.

Fig. 5 D. High magnification of tegument of treated *F. gigantica* showing few vacuolization (V) in syncytium epithelium and the pinocytes (PI) developed in basal lamina (BL) X 0.5µm.