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IN VITRO ANTIHELMINTHIC ACTIVITY OF ETHANOL *ZINGIBER OFFICINALE* EXTRACT ON *FASCIOLA GIGANTICA* IN COMPARISON TO TRICLABENDAZOLE

BY

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Abstract

Fascioliasis, caused by *Fasciola hepatica* and *F. gigantica,* is considered one of the most important helminthes diseases among both humans and animals. The use of triclabendazole (TCBZ) as the only antihelminthic drug against fascioliasis faced recent problems being of many side effects and development of the drug resistance by the parasites. Given the widespread use of ginger (*Zingiber officinale*) in many traditional medicines and the various metabolic properties, this study aims to investigate the *in vitro* antihelminthic activity of *Z. officinale* ethanol extracts on *F. gigantica* in comparison to TCBZ. Fifty-four live adult *F. gigantica* worms were divided into nine groups of six in each, including positive control (G1), negative control (G2), triclabendazole sulfoxide (TCBZSO) of 20μg/ml (G3), ginger extract of 5, 25 and 50 mg/ml (G4, G5 and G6, respectively) and combined TCBZSO with ginger extract of 5, 25 and 50 mg/ml (G7, G8 and G9, respectively). The worm motility scores, survival index and histological examination were utilized to further analyze the effect of treatments on the worms' morphology. Results indicated a marked decrease in worms' motility treated with ginger extracts compared to TCBZSO group. The observed decrease was positively correlated to both time and concentration. Histological examination showed that a higher concentration of ginger extract alone or in combination with TCBZSO caused severe tegumental alterations, more than those observed in TCBZSO treatment alone. In conclusion, the results strongly confirm the plausible development of ginger-based antihelminthic drug against *F. gigantica* infection.

Keywords: *Fasciola gigantica*, *Zingiber officinale*, Antihelminthic activity, *in vitro*

Introduction

Fascioliasis is a major helminthes disease among humans and animals that inflect both serious health problems as well as significant economic losses worldwide (Alajmi, 2019). It is caused by two trematodes of the genus *Fasciola*; *F. hepatica* (temperate liver fluke) and *F. gigantica* (tropical liver fluke). The distribution of both species can overlap clinically in many regions of Africa and Asia (Degheidy and Al-Malki, 2012; Ashrafi *et al,* 2014; Phalee *et al,* 2015).

The fascioliasis effect on livestock is considered a significant problem as the infection usually leads to reduced growth, poor meat and milk production as well as other complications such as reduced fertility, abortion in late stages of pregnancy, anemia and even mortality, resulting in an annual economic loss of US$3 billion (Jaja *et al,* 2017).

The World Health Organization (WHO) classifies fascioliasis as a neglected tropical disease, with an estimated 17 million people infected and 180 million people at risk of infection (Mas-Coma *et al,* 1999). The significant impact on agriculture and human health combined with increased demand for animal-derived food products to support global population growth suggested that fascioliasis is a major health problem (Cwiklinski *et al,* 2016).

The pathogenesis involved different phases of the fluke's life cycle inside the liver, which causes hepatitis, maturity and the establishment of the adult parasite in the bile ducts causing infection in the obstructive

biliary tract. The main pathological changes in fascioliasis occur because of the immature migratory stage of the fluke. This stage of infection can cause extensive hemorrhage and fibrosis in the liver as the young flukes move through the liver. Adults in bile ducts cause inflammation and edema, which in turn stimulate fibrosis in the walls of bile ducts, resulting in atrophy of liver parenchyma, cirrhosis, and impaired liver function. In severe infections, the gall bladder is damaged, and the walls of bile ducts are completely eroded; therefore, the worms re-enter liver, again causing marked eosinophilic and granulomatous reaction (Raina *et al,* 2011; Nassef *et al,* 2014; Jaja *et al,* 2017).

In the last decades, animal fascioliasis outbreaks have spread to different parts of Saudi Arabia (Khanjari *et al,* 2014). *Fasciola* infection in sheep was considered a very serious problem in Riyadh Animal Markets, which forced consumers to shift from domestic to imported sheep (Degheidy and Al-Malki, 2012).

There are no effective commercial vaccines yet; the anthelmintic drugs were mainly used to control the fluke (Massoud *et al,* 2012). Triclabendazole (TCBZ) is the drug of choice used for treating *Fasciola* infection. It can affect both stages of the fluke (juvenile and adult), providing effective control for both acute and chronic stages of fascioliasis. Various studies confirmed that the TCBZ-resistance in livestock worldwide (Alvarez-Sanchez *et al,* 2006; Ortiz *et al,* 2013; Shalaby *et al,* 2016). Such confirmations convey a serious issue, as there are no other effective drugs available (Massoud *et al,* 2008).

With respect to new compounds for use as antihelminthics, there has been increasing interest in natural plant products used as traditional medicines in developing countries (Flynn *et al,* 2010; Nassef et al., 2014).

One of the most famous herbs used as spice and for medicinal purposes is ginger (*Zingiber officinale* Roscoe). It belongs to the tropical and subtropical family Zingiberaceae, originating in Southeast Asia (Gupta and Sharma, 2014). Ginger has been identified as an herbal medicinal product with pharmacological effect (Grzanna *et al,* 2005; Abouel-Nour *et al,* 2015). The ginger extract has been used since old times to treat diseases such as rheumatoid arthritis, sore throats, nausea, constipation and infectious diseases like helminthiasis (Gupta *et al,* 2016).

The use of *Z. officinale* to treat parasitic infections has received considerable attention lately. Several experimental and clinical trials have demonstrated ginger for its range of antihelminthics activity against schistosomiasis (Mostafa *et al,* 2011) and the protoscolices of hydatid cyst (Moazeni and Ahmadi, 2016). Thus, it has an antiprotozoal effect against *Toxoplasma gondii* (Choi *et al,* 2013), *Giardia lamblia* (Mahmoud *et al,* 2014), *Trypanosoma brucei brucei* and *Blastocystis* spp. (El-Sayed and El-Saka, 2015). Besides, Moazeni and Khademolhoseini (2016) showed that *F. hepatica* eggs were susceptible to methanolic extract of ginger with ovicidal effect of 100% after 24 and 48 hours at concentrations of 10 and 5mg/ml, respectively.

The effect of *Z. officinale* on *F. gigantica* has not been studied previously; therefore, the current study aimed to investigate the *in vitro* antihelminthic activity of *Z. officinale* ethanol extract on the *F. gigantica* in comparison to TCBZ.

Materials and Methods

Liver flukes: Fifty-four live *F. gigantica* adult worms were collected from the bile ducts of the naturally infected cattle and sheep slaughtered at Jeddah slaughterhouse. To eliminate all traces of blood and bile, the flukes were washed several times with warm normal saline solution (37ºC) and the healthy flukes with normal histological structure and good motility were selected. Healthy adult flukes were kept in a petri dish containing 20ml of M-199 medium (Sigma-Aldrich, St. Louis, MO, USA) and transferred to the lab of Parasitology Department, Jeddah University, Saudi Arabia, until the

experimental beginning.

Plant extract: *Z. officinale* rhizomes were purchased from local market in Jeddah, Saudi Arabia. The rhizomes were cleaned, scrapped of superficial skin, cut into small pieces, air-dried for a week then ground using a mechanical grinder. The ground product was macerated in absolute ethanol for 24 hours, filtered with a clean white cloth and the filtrate was concentrated using a rotary evaporator in a water bath at 40-50 ºC. The ethanolic extract was refrigerated until used (Moazeni and Khademolhoseini, 2016). For final use, the ethanolic residue was dissolved in M-199 culture medium immediately before use. Three doses of ginger ethanolic extract were investigated, 5, 25 & 50mg/ml (Bazh and El-Bahy, 2013).

Drug and media: Triclabendazole (Sigma-Aldrich) was initially prepared as a stock solution in dimethyl sulfoxide (DMSO) and then added to the culture medium M-199 (Sigma-Aldrich) containing antibiotics (penicillin 50 IU/ml and gentamycin 30 IU/ml) to give a maximum concentration of 0.1% (V/V) (Mestorino *et al*, 2008). A concentration of 20µg/ml triclabendazole sulfoxide (TCBZSO) was used (Sanyal, 1995).

Experimental design: Adults *F. gigantica* were recovered under sterile conditions in a laminar flow cabinet. The worms were kept in petri dish containing 20 ml M-199 medium. They were divided into nine groups containing six flukes each. Group (1) included six non-drug exposed *F. gigantica* worms served as positive control group and were incubated in M-199 medium (Chang and Flors, 2015). Group (2) included six *F. gigantica* worms incubated in M-199 medium containing 0.1 % DMSO served as negative control group (Shalaby *et al*, 2016). Group (3) included six *F. gigantica* worms incubated in M-199 medium containing 20 µg/ml of TCBZSO (Hegazy *et al*, 2007). Groups (4, 5 & 6) each included six *F. gigantica* worms exposed to 5, 25 & 50mg/ml ginger extract, respectively (Bazh and El-Bahy, 2013). Groups (7, 8 & 9) included six *F. gigantica* worms each, exposed to 5, 25 & 50mg/ml ginger extract with the 20µg/ml TCBZSO, respectively.

Parasitic viability: Worms' viability was evaluated in each group using a stereomicroscope (Chang and Flors, 2015). The inhibition of motility and/or mortality of flukes were observed after 3, 6, 12 & 24hrs.

Parasitic survival index: Survival index (SI) was evaluated using stereomicroscope. Percentages of live worms after a given time (3, 6, 12 or 24hrs) were evaluated with scored system (Jiraungkoorskul *et al*, 2005).

Morphological identification of adult *Fasciola*: One fluke from each group was fixed in 10% buffered formalin, dehydrated, cleared and embedded in paraffin. Serial 5µm sections were stained with hematoxylin and eosin (H&E) after Bancroft and Gamble (2008) for routine histological study. The layers of the tegument were examined under light microscope to check for abnormalities and get photographed (Hanna *et al*, 2013).

Statistical analysis Correlation data between TCBZ treatment and ginger treatment were entered into Microsoft excel sheet and were analyzed using IBM Corp. IBM SPSS Statistics for Windows, Version 22.0. (Armonk, NY, United States). Further comparison using Chi-square was applied and significance appointed at P value of <0.5.

Results

The motility/mortality scores of all treated groups were observed under a stereomicroscope with scores ranging between (score 3) for normal whole-body movement to (score 0) for dead. All treated groups were observed after 3, 6 or 12hrs (Tab. 1). The positive control (G1 group) and negative control (G2 group) showed no change in worms' motility (score 3) after all incubation times points. The group treated with 20µg/ml of TCBZSO (G3) showed reduced movement (score 2) for most of the flukes after 3hrs of incubation and the motility score decreased after 6 hours to include three dead (score 0), two immobilized and alive (score 1) and one reduced movement (score 2). After 12hrs of

incubation, most of them were dead (score 0).

But, the ginger treated groups (G4, G5 & G6) with increased ginger extract dose (5, 25, 50mg/ml, respectively) showed concentration- and time-dependent changes. Group 4 showed only two flukes with reduced movement (score 2) after 3hrs of incubation while after 6hrs, there was one dead (score 0), one immobilized and alive (score 1) and two with reduced movement. After 12hrs, most of the flukes were dead (score 0) with one immobilized and alive (score 1). The effect of increased dose in G5 was markedly observed after 3 hours of incubation as there were two dead flukes (score 0), three immobilized and alive (score 1) and one with reduced movement, while after 6hrs the number increased to four dead (score 0) and two immobilized (score 1). All flukes were dead after 12hrs of incubation. Similar correlations were observed in G6 as most of the flukes died (score 0) after 3hrs of incubation and all flukes were dead after 6 and 12hrs.

The combined ginger and TCBZSO treated groups (G7, G8 & G9) with 20µg/ml of TCBZSO and increased ginger extract dose (5, 25, 50mg/ml, respectively) had the most intense effect on flukes regardless of the incubation time. Three hours incubation revealed two dead (score 0) in G7, five in G8 and all six in G9, while after 6hrs the dead flukes increased to four in G7 and all six in G8 and G9. The 12hrs incubation showed all six flukes dead in G7, G8 & G9.

Statistical analyses were applied on data and showed Chi-square value of 221.833 with significant P values (<0.05).

Survival index scores (in percentage) of *F. gigantica* after 3, 6, 12 & 24 hours were recorded under stereomicroscope. The control groups (G1and G2) exhibited active movement and maximum survival index (SI = 100%) after all incubation times. The TCBZSO treated group (G3) showed declining SI (from 100% to 16.6%) with increasing incubation time. Similar decline was observed in both the ginger treated groups (G4, G5 & G6) and combined ginger and the TCBZSO treated groups (G7, G8 & G9) in accordance with time and concentration increase (Tab. 2, Fig. 1).

Control groups (G1 & G2) showed no abnormalities and normal histological composition of the tegument, which was lodged with spins enclosed with the muscle layer and parenchyma. The outer rim of the tegument and tips of the spines were deeply stained. The tegument rested on basement membrane underlying muscle layers (longitudinal and diagonal) surrounding the mesenchyma (Fig. 2).

Group 3, treated with TCBZSO, showed mild separation of tegument between the spines from underlying tissue with dislodged spines (Fig. 3).

The sever histological changes were observed in the ginger extract treated groups (G4, G5 & G6) as well as a positive correlation with extract concentration. Group 4 showed swelling intact tegument, embedded spines and distracted muscle layers (Fig. 4A) while group 5 showed severe swelling of the tegument, partial separation between the tegument and the distracted muscle layers underneath with the spines either surrounded by the tegument or completely dislodged (Figure 4B). Moreover, group 6 showed separated tegument and marked multiple dislodged spines with furrows embedded into the muscle layer (Fig. 4C).

The combined ginger and TCBZSO treated groups (G7, G8 & G9) had the most sever observations compared to all other groups. Group 7 showed swelled and detached tegument, dislodged spines with empty spine sockets and extensive cracking of the muscle layer (Fig. 5A). In addition to G7 observations, G8 also showed clearly surrounded spines by tegument with some embedded into the muscle layer as well as the appearance of many vacuoles (Fig, 5B). Group 9 showed completely detached tegument with appearance of multiple empty spine sockets and sever destruction of the underlying muscle layer (Fig.5C).

Table 1: Motility scores count of *F. gigantica* worms' number after 3, 6 & 12hrs incubation (each n=6 worms).

Variables		Motility Scores			
Group	Time (hrs)	Dead (0)	Immobile & alive (1)	Reduced movement (2)	Normal movement (3)
G 1	3	0	0	0	6
	6	0	0	0	6
	12	0	0	0	6
G 2	3	0	0	0	6
	6	0	0	0	6
	12	0	0	0	6
G 3	3	0	0	5	1
	6	3	2	1	0
	12	5	1	0	0
G 4	3	0	0	2	4
	6	1	1	2	2
	12	4	1	1	0
G 5	3	2	3	1	0
	6	4	2	0	0
	12	6	0	0	0
G 6	3	5	1	0	0
	6	6	0	0	0
	12	6	0	0	0
G 7	3	2	0	2	2
	6	4	1	1	0
	12	6	0	0	0
G 8	3	5	1	0	0
	6	6	0	0	0
	12	6	0	0	0
G 9	3	6	0	0	0
	6	6	0	0	0
	12	6	0	0	0

Table 2: Survival index (SI) values (%) of *F. gigantica* worms after different incubation times.

Variables	Incubation Time		
Group	3 hours	6 hours	12 hours
G 1	100	100	100
G 2	100	100	100
G 3	100	50	16.6
G 4	100	83.3	33.3
G 5	66.6	33.3	0
G6	16.6	0	0
G 7	66.6	33.3	0
G 8	16.6	0	0
G 9	0	0	0

Discussion

Fascioliasis is a foodborne zoonotic disease caused by the two parasite species *Fasciola hepatica* and *Fasciola gigantica* (Grabner *et al*, 2014). The effectiveness of using natural plant extracts as alternative drugs has been heavily investigated in recent years (Anthony *et al*, 2012; Nassef *et al*, 2014); especially in cases were the drug can cause deleterious side effects and/or have acquired resistance (Hanna *et al*, 2015; Kelley *et al*, 2016), as antihelminthics drugs controlling fascioliasis (Geary *et al*, 2012; Hossain *et al*, 2012).

Ginger has been reported in many studies to cause marked the significant nematocidal, cestocidal, trematocidal, antiprotozoal, insecticidal, molluscicidal and anti-leech effects (Al-Sharkawi *et al*, 2007; Choi *et al*, 2013; Lin *et al*, 2014; El-Sayed and El-Saka, 2015; Abouel-Nour *et al*, 2016; Dyab *et al*, 2016, Fakhrieh-Kashan *et al*, 2018). Moreover,

Iqbal *et al.* (2006) reported that the ginger anthelmintic activity against gastrointestinal nematodes in sheep. Also, Abdel-Hafeez *et al.* (2015) proved the in vivo antiprotozoan effects of the *Zingiber officinale* extracts on experimentally infected mice with *Blastocystis hominis*.

For such reasons, this study pioneered the *in vitro* antihelminthic activity of *Z. officinale* (ginger) ethanol extracts on *F. gigantica* in comparison to the commonly used TCBZ drug.

In the present study, ginger ethanolic extract have demonstrated strong fasciolicidal activity independently and in combination with TCBZSO drug with the fluke's tegument being a major target. The tegument isolates the fluke from the surrounding environment, maintains its homeostasis, absorbs nutrients, and defends direct contact with drugs, in addition to synthesis and secretions of antigens (Anuracpreeda *et al,* 2017). So, damage of tegument cause vital changes that can affect many downstream internal processes.

The effect on fluke's tegument was markedly observed both in motility/mortality scores as well as in histological examination of the worm, which is an important parameter for estimating the effectiveness of any anthelmintic drugs (Shalaby *et al,* 2009). All treated groups were observed after 3, 6 or 12hrs and the results were positively correlated with both time and concentration. The positive and negative control groups (G1 & G2, respectively) showed normal composition of the tegument, spines, muscles and basement membrane after all tested incubation times. Similar results were confirmed with others (Anuracpreeda *et al,* 2017). These flukes groups showed normal active movement (score 3) and maximum SI (100%). These results agreed with Jeyathilakan *et al.* (2010) and Ullah *et al.* (2017) who reported that all control liver flukes remain active and motile after different time of incubation.

The group treated with 20µg/mL TCBZSO (G3) showed reversed correlation between motility and time with higher mortality scores after 6 hours of incubation. This could be attributed to the inhibited secretion of proteolytic enzyme and disrupted microtubule-based secretory processes that responded to the worm mobility (Massoud *et al,* 2012). Histological examination showed mild separation between the spines from underlying tissue with dislodged spines, which concur with previously published findings of disrupted tegument, spine loss and decreased worms' motility after 24 hours incubation with 15µg/mL TCBZSO (Massoud *et al,* 2012). Besides, Shalaby *et al.* (2009) and McConville *et al.* (2009) reported that disruption and spine loss were characteristic of a stressed fluke.

In the present study, the groups treated with different concentrations of ginger extract (5, 25 & 50mg/ml; G4, G5 &G6, respectively) revealed sever and significant decrease in the worms motility as well as increased mortality scores in positive correlation with both time and concentration. Histological examination showed disrupted tegument, in the form of mild swelling with partial separation in low ginger concentration (G4) and marked swelling with multiple sloughed spines and embedded furrows in the muscle layer in high ginger concentrations (G5 & G6). These tegumental changes immobilized the flukes then died. Similar observations were confirmed by Jeyathilakan *et al.* (2010); Shalaby *et al.* (2016) and Anuracpreeda *et al.* (2017) who demonstrated that the tegumental swelling is the first sign of change and characterized by osmotic disturbance due to the destruction of the tegument membrane and plasma membranes' ion pumps. This swelling of tegument is the main target of many flukicides pharmacological action (Devine *et al,* 2012).

In the present study, TCBZSO group (G3) and ginger extract groups (G4, G5 & G6) indicated that higher ginger dosage afflict more sever observations than the drug. Such observation confirmed the effectiveness of

the ginger extracts as an alternative drug to treat fascioliasis infection.

The combination of the 20µg/ml TCBZSO and different ginger extract concentrations (5, 25 & 50mg/ml; G7, G8 & G9, respectively) had a foreseeable sever and stringent effect in as early as post-3 hours of incubation. Low motility and high mortality scores were markedly observed as well as very low SI scores. These results confirm previous reports that *Z. officinale* extract markedly reduced the motility and caused death of flukes (score 0) after 3 hours (Jeyathilakan *et al*, 2010). In addition, histological examination showed severe dislodgment of many spines from their sockets, the muscle layer appeared markedly cracking with many vacuoles and the tegument had been completely dislocated in some specimen. Shalaby *et al.* (2016) reported that the tegument once detached, the drug would be able to penetrate deeper into the internal tissues of the fluke leading to a more widespread destruction. Such tegumental damage may trigger the release of the surface antigen that interacts with specific antibodies in the blood, promoting a rapid and severe immunopathological response to destruct and kill the fluke.

Conclusion

The outcome results strongly support a plausible development of ginger-based antihelminthics drug against *F. gigantica* infection. The combination of ginger extract (5, 25 & 50mg/ml) and 20µg/ml the TCBZSO showed fasciolicidal properties of the *Z. officinale*.

These compounds inhibited the worms' motility and severely disrupt the tegumental surface. Furthermore, molecular, ultrastructural, and physiological studies are recommended to evaluate ginger extract effect.

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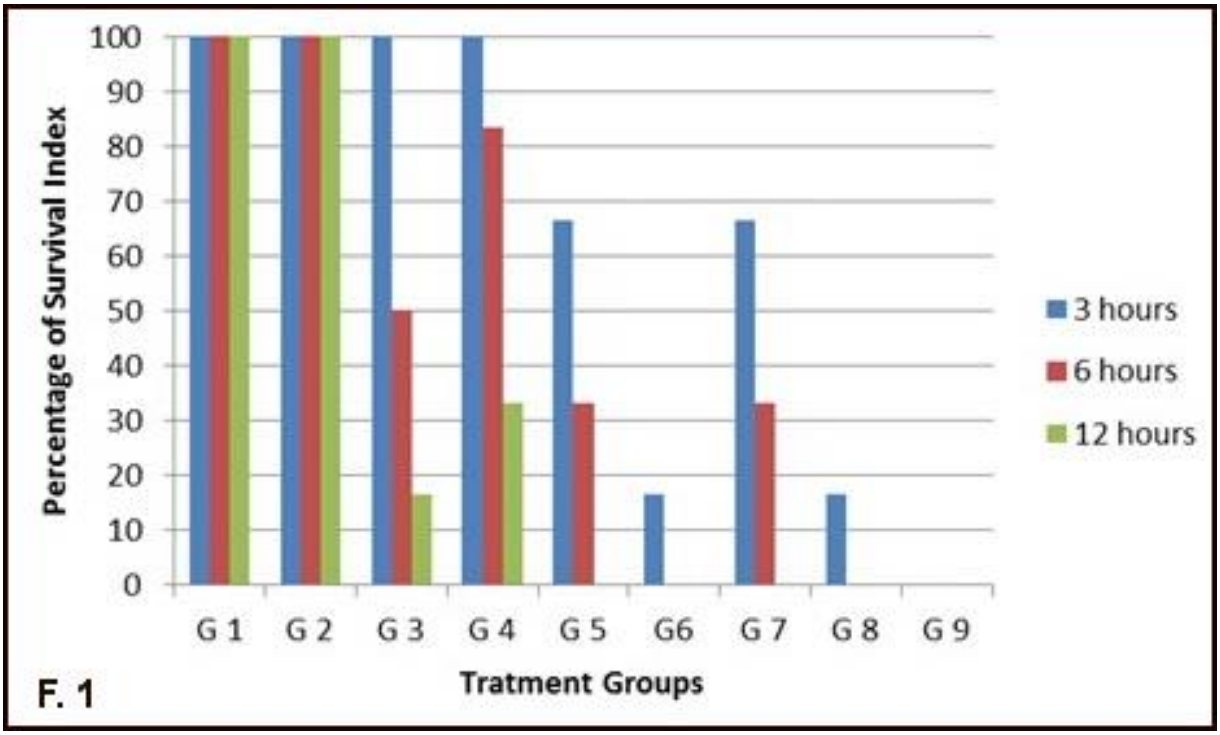
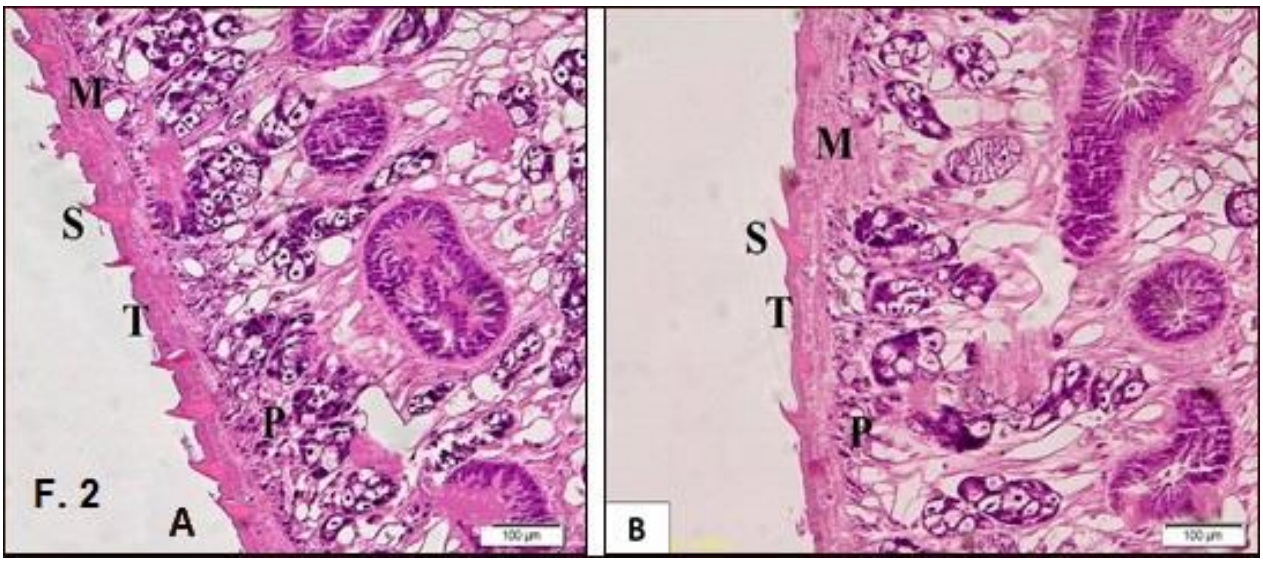
Explanation of figures

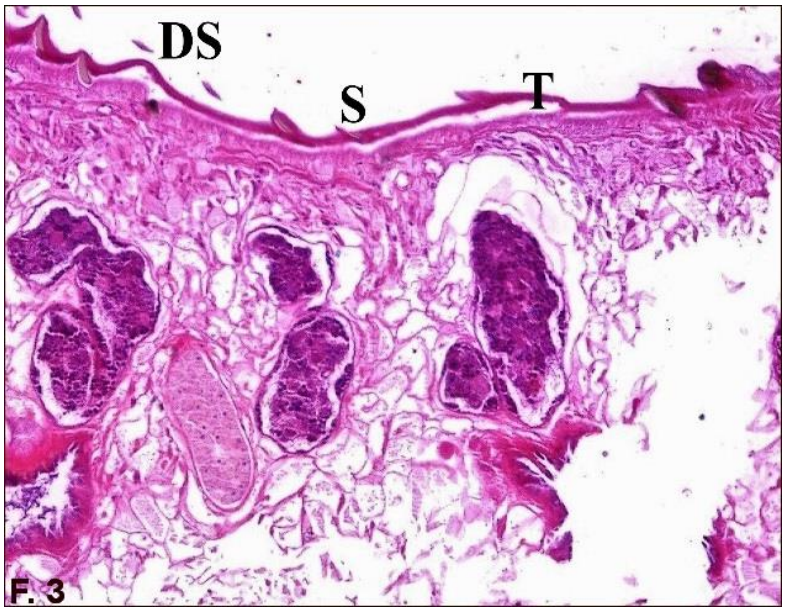
Fig. 1: Bar diagram indicating survival index (SI) percentage of *Fasciola gigantica* worms after different incubation times.

Fig. 2: Light micrographs of adult *F. gigantica* worm stained with H&E with normal histology. A: positive control (G1) showing tegument (T) with normal appearances, i.e., spines (S) embedded in tegument and muscle (M) lying underneath parenchyma (P). B: negative control (G2) in M-199 medium containing 0.1% DMSO showed intact tegument (T) with lodged spines (S) enclosed with muscle layer (M) and parenchyma (P).

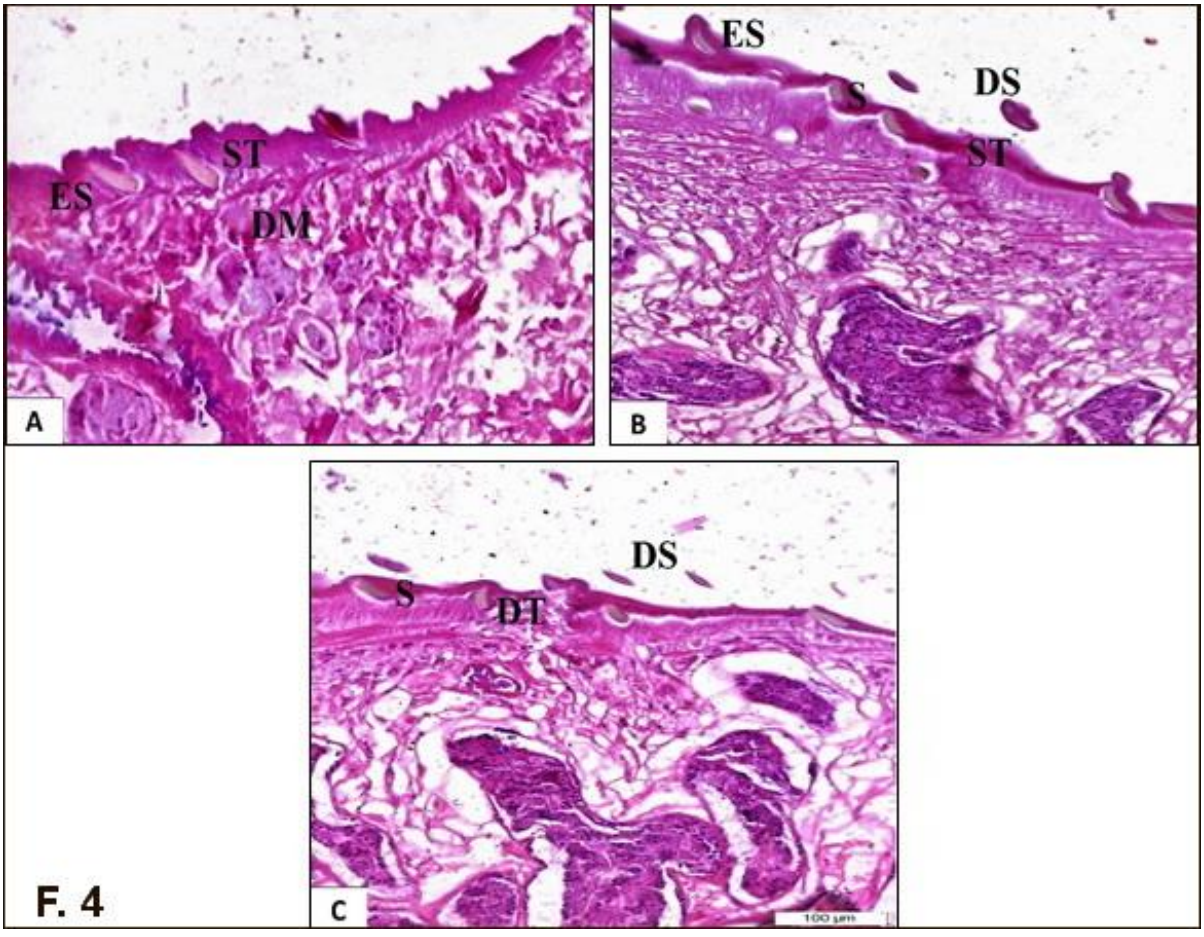
Fig. 3: Light micrographs of adult *F. gigantica* worm stained with H&E and treated with 20µg/ml TCBZSO (G3) showed mild separation of tegument between the spines (S) and underlying tissue (T) and dislodged spines (DS).
Fig. 4: Light micrographs of adult *F. gigantica* worm stained with H&E and treated with 5, 25 or 50 mg/ml ginger ethanolic extract (G4, G5 & G6, respectively). A: G4 showed swelled intact tegument (ST), embedded spines (ES) and distracted muscle layer (DM). B: G5 showed swelling and partial separation of tegument (ST) from a distracted muscle layer and spines (S) surrounded by tegument (ES) while others dislodged (DS). C, G6 showed marked multiple dislodged spines (DS), tegumental swelling with embedded spines (S) and detached with furrows through muscle layer (DT).
Fig. 5 Light micrographs of adult *F. gigantica* worm stained with H&E and treated with 20µg/ml TCBZSO and 5, 25 or 50 mg/ml ginger ethanolic extract (G7, G8 & G9, respectively). A, G7 showed swelled detached tegument (ST), dislodged spines (DS), empty spine sockets (arrow) and extensive cracking of the muscle layer (M). B, G8 showed gross tegumental swelling (ST), spines surrounded by tegument and embedded in the muscle layer (EM) with appearance of many vacuoles (*). C, G9 showed complete detached tegument (DT) with multiple empty spine sockets (arrows).



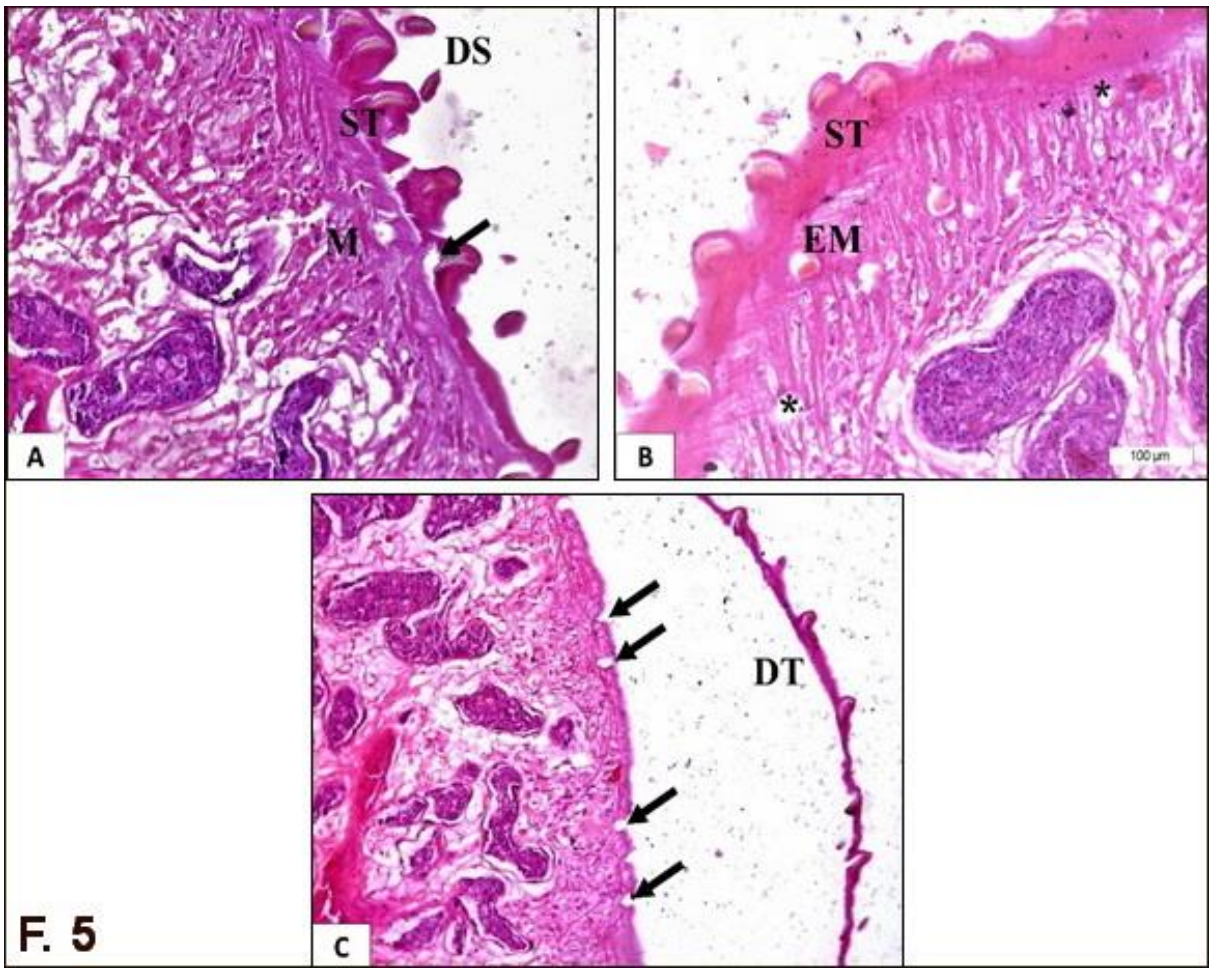




F. 3



F. 4



F. 5