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THERAPEUTIC EFFECT OF *NIGELLA SATIVA* AND IVERMECTIN VERSUS ALBENDAZOLE ON EXPERIMENTAL TRICHINELLOSIS IN MICE

By

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Abstract

Anthelminthics are used for trichinellosis elimination in intestine and encysted larvae in muscles. This study evaluated the effects of *Nigella sativa*, ivermectin versus albendazole on experimental trichinellosis. One hundred and twenty mice were orally infected with 200 larvae of *T. spiralis/* mouse. Drugs were tested against mature worms in the small intestine at 7 days post infection (dpi) and encysted larvae at 35 dpi. Parasitological assessment by counting of adult worms and encysted larvae was done as well as histopathologically. Biochemical measuring of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein, albumin, globulin, and urea, creatinine and creatinine phosphokinase (CPK) values was done. High significant reductions in mean adult count was detected in *N. sativa* prophylactic, ivermectin and albendazole treated mice with efficacies of 45.9%, 97.4% & 83.6% respectively with mild significant reduction in *N. sativa* treated mice with percent reduction 26.5%. At 35 dpi, there was high significant reduction in mean larval count as compared to the control infected mice with reduction of 43.7%, 65.3%, 99.4% & 80% in *N. sativa* prophylactic, *N. sativa* treated, ivermectin and albendazole respectively. Reduction of AST, ALT, urea, creatinine and CPK levels but total proteins increased in all treated mice compared to corresponding infected non-treated ones. Ivermectin gave the best results and continued for treating *T. spiralis.*

Key words: Trichinellosis, Experimental mice, Treatment

Introduction

Trichinellosis is one of the wordwide zoonotic disease (Kaewpitoon *et al*, 2008). It is due to ingestion of the first-stage larvae of the nematode of the genus *Trichinella* in raw or undercooked meat of pigs and/or bear (CDC, 2015).

*Trichinella spirali*s is the first discovered species (pozio, 2007). It is the most virulent and pathogenic species in human. Although, 8 other species were reported all over the world in other mammals (Hosking *et al*, 1996), its higher pathogenicity compared with other species is due to large number of larvae produced by a gravid female (Pozio *et al*, 1992) and the intense immune reaction occurred in human relative to the other genotypes (Bruschi *et al*, 1999; Gomez Morales *et al*, 2002).

Trichinella infection in human host consists of two phases, an intestinal phase and a muscular phase. Following ingestion of infected under cooked meat, larvae are released upon gastric digestion in the new host, and the first-stage larval parasite reaches the intestine and develops into adult worm. After mating adult female sheds larvae that migrate to other organs through lymphovascular system. Developing larvae invade skeletal muscles forming nurse cell complex (Gottestein *et al*, 2009).

The pathology is characterized by inflammatory reaction in the tissues and other organs which manifested by high fever, diarrhea, myalgia, periorbital edema and serious complications as myocarditis. Death was due to inflammatory reaction in heart, lung and CNS (Capo and Despommier, 1996).

Albendazole and mebendazole are the main antihelminthic drugs used for treatment of trichinellosis (Gottstein *et al*, 2009). They inhibited the microtubule polymerization through selective binding to beta-tubulin monomer of the parasite, with little effect on

binding of host tubulin (Aguayo-Ortiz *et al*, 2013). Albendazole has an advantage over the mebendazole as its recommended plasma levels are achieved in most patients so don't need monitoring ,while that of mebendazole vary among patients ,so need monitoring and dosing(Gottstein *et al*, 2009). They have limited bioavailability, a high degree of resistance and weak action against encysted larvae (Caner *et al*, 2008). An alternative effective and safe drugs was claimed (Yadav and Temjenmongla, 2012).

Ivermectin is the most essential anti helminthic drug available nowadays (McCavera *et al*, 2007). Ivermectin is a derivative that shows great potency and low toxicity (Fisher and Mrozik, 1992). The anti-*Trichinella* effects were by interfering with nervous system and muscle function, mainly by improving the inhibitory neurotransmission (Yates and Wolstenholme, 2004). Ivermectin in treatment of trichinellosis shows an effective action against the intestinal adult worms (De Muth, 2009), and larvicidal effect on migrating and muscular larvae (Arena *et al*, 1992).

Medicinal plant, *Nigella sativa* showed wide range of effects, as an anxiolytic, anti-inflammatory, immunomodulatory, hypoglycemic, hypolipdemic, wound healing and diabetic embryopathy protective effects (Ahmed *et al*, 2017).

The present study assessed the effectiveness of *Nigella sativa* (black seeds), Ivermectin® and Albendazole® against different stages of *Trichinella spiralis* in mice.

Materials and Methods

Experimental study: One hundred and twenty eight Swiss albino mice (20-25gm) were obtained from Schistosomal Biological Unit, Theodor Bilharz Research Institute (TBRI). The mice were kept on a standard commercial pelleted diet with free accessible water all over the time of study. *Trichinella spiralis* (*T. spiralis*) was obtained from laboratory bred infected albino mice in Parasitology Department Faculty of Medicine, Tanta University.

Swiss Albino mice were divided into six groups. GA: 8 normal healthy non infected mice, GB: 24 infected controls, GC: 24 treated with *N. sativa* before infection (prophylactic), GD: 24 infected and treated with *N. sativa*, GE: 24 infected and treated with ivermectin and GF: 24 infected and treated with albendazole. Gs: B to F were subdivided into three equal subgroups: SGI were sacrificed on 7 dpi to see effects on intestinal phase, SGII were sacrificed on 35 dpi to detect effects on muscular phase, SGIII were kept for 30 dpi blood samples to explore trichinosis effect and treatment on serum enzymatic activities.

Ethical aspects: Mice were maintained under convenient conditions following the recommendations of the National Institutes of Health Guidelines for Animal Experimentation. The study was approved by the ethical committee of Faculty of Medicine, Zagazig University.

Larval extraction and preparation of inoculums (Dunn and Wright, 1985): Digestion of muscles of infected mice (Five weeks post infection) by immersion in artificial gastric juice formed by adding 1% pepsin and 1% concentrated HCL in warm tap water. Incubation of the mixture at $37C^{o}$ for 2 hours during with continuous agitation by electric stirrer was done. The resulting filtrated fluid was by sieve ($50mesh/cm^2$), then by sieve ($200mesh/cm^2$). The collected larvae were washed two to three times with tap water and suspended in a conical flask for half an hour to help sedimentation. Supernatant fluid was discarded and sediment larvae were microscopically counted using hemocytometer. Concentration of counted larvae in fluid was adjusted to each 0.25 ml. containing 200 living larvae, the recommended dose for infection of every mouse. Before infection, mice were starved for 12 hours, then oral infected by 0.25ml. *T. spiralis* larvae using a tuberculin syringe fitted with 18 gauge blunt needle to introduce infective larvae into mice stomach (Wassom *et al*, 1988).

Drugs: *Nigella sativa* extract was available as Baraka capsule (Pharco Co., Alex.) disso-

lved in 250ml ethanol 3% to give 5ml/ kg/ bodywt/day orally for 4 weeks before and post infection from 2nd infection (Abu El Ezz, 2005).

Ivermectin as Ivomec 1% injection (Dawaya, Egypt) given as a single subcutaneous dose 200µg/kg/bodywt at 1st dpi.

Albendazole (Alzental) suspension (EIPICO) as 20mg/ml was given in dose 50mg/kg orally for 3 successive days starting from the 3rd dpi (Attia *et al*, 2015).

Isolation and counting of adults in intestine of mice (Wakelin and Lioyed, 1976): In brief, intestine of mice in all subgroups I was opened longitudinally along its entire length and cut into two centimeters pieces and placed in normal saline at 37C^{o} for three to four hours. Intestine was shaken well in saline, rinsed in saline, removed and discarded. Adult sedimentation was done by standby the container for half an hour. Discard the supernatant fluid, adults in least amount of fluid was poured into a petri-dish and counted under a dissecting microscope.

Counting number of muscle larvae (Dunn and Wright, 1985): For all the subgroups Π, process was the same as that for preparing muscle larvae for infection, but here both dead larvae and living larvae were counted.

Histopathological examination (Drury and Wallington, 1980): One cm from mid intestinal region was taken at 7 dpi and a piece of diaphragm of infected mice was taken at 35 dpi, fixed in 10% formol-saline, dehydrated in ascending grades of ethanol, and cleared in xylol. Impregnation was done in pure soft paraffin for 2 hours at 55^{o}C then hard paraffin sections of 5µ thickness were cut by microtome. Sections were stain in Hematoxylin and Eosin stain.

Biochemical assessment: Blood samples were withdrawn at 30 dpi from retro-orbital vein using disposable capillary tube from mice in GA & all subgroups III. Sera were separated and used to determine therapeutic effect of *N. sativa*, ivermectin and albendazole on serum biochemical parameters as renal parameters (urea, creatinine), liver parameters (total protein, albumin, globulin, AST & ALT) and muscle enzyme (CPK).

Statistical analysis: Data were tabulated and analyzed using the SPSS program version 18.0. A probability of less than 0.05 was considered significant (Finney 1971).

Results

Adults count in small intestine at 7 dpi in mice with treated different drugs showed highest percentage reduction in mean adult count with ivermectin (97.4%) followed by albendazole (83.6%), *N. sativa* prophylactic (45.9%), and least with *N. sativa* (26.5%)

Table 1: Adult worm count (Mean ± SD) in small intestine at 7dpi.

Group	Adult	R %	F. test	P. value
GB (n=8)	38.63 ± 4.69 [a]		155.79	<0.001**
GC (n=8)	20.88 ± 4.97 [b]	45.9%		
GD (n=8)	28.38 ± 3.25 [c]	26.5%		
GE (n=8)	1.0 ± 0.3 [d]	97.4%		
GF (n=8)	6.3± 2.1[e]	83.6%		

**Highly significant from infected control group at P <0.001

The larval count in skeletal muscle of all groups at 35 dpi: Treated mice showed high reduction in mean larval count of *T. spiralis* was with the ivermectin (99.4%) followed by albendazole (80%), *N. sativa* (65.3%), and lastly *N. sativa* prophylactic (43.7%).

Table 2: Mean larval count of *T. spiralis* in both control and treated groups at 35dpi.

Group	larvae: Mean ± SD	R %	F. test	P. value
GB (n=8)	205830±8999 [a]		185.69	<0.001**
GC (n=8)	115880 ± 4433 [b]	43.7%		
GD (n=8)	89795±6068 [c]	65.3%		
GE (n=8)	1191.2±74.28[d]	99.4%		
GF(n=8)	41166±2783[e]	80%		

Biochemical study at 30dpi (infected control) showed a significant increase in serum activates of AST, ALT, urea, creatinine, globulin & CPK (P<0.001) as compared with healthy control. There was a significant decrease in serum levels of total protein and albumin (P<0.001) as compared with the healthy control. All treated groups showed significant decrease in serum activates of AST, ALT, urea, creatinine, globulin and CPK (P<0.001) when compared with their corresponding infected control group. A significant serum levels elevation of the total protein and albumin (P<0.001) was noticed in all treated groups as compared with corresponding infected control.

Table 3: Serum parameters (AST, ALT, globulin, total proteins, albumin, urea, creatinine and CPK in groups at 30 dpi:

Group	GA (n=8)	GB (n=8)	GC (n=8)	GD (n=8)	GE (n=8)	GF (n=8)	F. test	P. value
AST: (U/ML)	123.2±4.2 [a]	178.2±4.3[b]	158.2 ± 4.3 [c]	161.2 ± 4.2 [c]	130.2 ± 3.4 [a]	130.3 ± 3.5 [a]	242.6	<0.001**
ALT: (U/ML)	30.8±1 [a]	58.6±2.2 [b]	49.0 ± 2.2 [c]	42.0 ± 2.2 [c]	38.0 ± 4.1 [a]	40.0 ± 4.2 a	88.98	<0.001**
Urea:(mg/dl)	49.2±2.1 [a]	67.3±2.7 [b]	57.2 ± 2.7 [c]	58.2 ± 2.7 [c]	51.4 ± 2.3 [a]	51.5 ± 3.1 [a]	51.61	<0.001**
Creat: (mg/dl)	1.6±0.1 [a]	2.6±0.2 [b]	2.2 ± 0.1 [c]	2.3 ± 0.1 [c]	2 ± 0.1[c]	2.1 ± 0.1 [c]	59.02	<0.001**
Protein: (g%)	5.0±0.1[a]	3.3±0.3 [b]	4.5± 0.2 [c]	4.3±0.2 [c]	4.9±0.2 [a]	4.8±0.2 [a]	119.5	<0.001**
Albumin: (g%)	3.5±0.1[a]	1.1±0.1 [b]	3.3±0.1 [a]	3.4±0.1 [a]	3.2±0.2 [a]	3.1±0.2 [a]	33.67	<0.001**
Globulin: (g%)	3.0±0.1 [a]	3.6±0.1 [b]	3.2±0.1 [a]	3.15±0.1 [a]	3.04±0.1 [a]	3.09±0.1 [a]	36.14	<0.001**
CPK: (IU/L)	53±4.6 [a]	457±30.4 [b]	252±23 [c]	242±21 [c]	120±13 [d]	122±11 [d]	86.7	<0.001**

Discussion

Generally speaking, during the initial infection of *T. spiralis*, invasion of the intestines could result in diarrhea, abdominal pain, and vomiting (Gari-Toussaint *et al*, 2005). Migration of larvae to muscle, which occurred about a week after being infected, could cause swelling of the face, inflammation of the whites of the eyes, fever, muscle pains, and a rash. Minor infection might be without symptoms (Bein *et al*, 2012) The complications might include inflammation of heart muscle, central nervous system involvement, and inflammation of the lungs (Bruschi and Dupouy-Camet, 2014).

The present study showed that using *N. sativa* for 4 weeks as prophylactic resulted in significant reduction in the mean adult worm count (45.9%) at 7 dpi., and significant reduction in mean larval count at35 dpi (43.7%). Using *N. sativa* for 4 weeks post infection showed mild reduction in mean adult count of *T. spiralis* (26.5%) at 7 dpi with significant reduction in mean larval count (65.3%) at35 dpi. Thus, using *N. sativa* as prophylactic gave better results than as therapeutic in lowering the mean adult count, but as therapeutic gave better results in lowering the mean larval count than prophylactic. The anti-*Trichinella* effect of *N. sativa* when used for 4 weeks before infection caused significant reduction in mean adult and larval count by the stimulatory the immune system resulted in abortion of larval stages with stoppage of adult development. This result agreed with Mohamed *et al.* (2005) used *N. sativa* as treatment and found significant reduction in adult count (30%), and reduction of larval count (74.7%) when used *N. sativa* during intestinal phase but, mild reduction (29.5%) during migratory phase. Abu El-Ezz (2005) found a significant reduction in adult (45.3%) and larval count (43.3%) as prophylactic more than treatment 40 dpi., and as treatment gave (21.7%) reduction in adult and (56.9%) in larval count. The mechanism was due to spasmolytic action through saponin-like effect that enhanced adult expulsion by intestinal movement (Boskabady *et al*, 2004). The immune mediated action involved hypersensitivity including secretion of IgE or protective IgA on intestinal mucosa surface (El Shazly *et al*, 2002). *N. sativa* stimulates immune cells and bone marrow by rising T4:T8 ratio and natural killer cells activity that led to increase in interferon production & antibodies producing B cells inducing humoral and cellular immune responses (Al-Ghamdi, 2001).

The present results showed that ivermectin in a single dose 200ug/kg S.C. at 1st day of infection gave significant reduction in mean adult (97.4%) and larval (99.4%). This

agreed with El-Azzouni (1997) and Basyoni and El-Sabah (2013) they found reduction in adult count (98.5%) and encysted larvae (76.5%). But, Song-Mingxin *et al*. (2002) used ivermectin in dose 0.3ml/kg found less effective on total adult count with reduction between (47.5% & 58.1%), and ivermectin more effective against encysted larvae (at 40dpi) with reduction in larval count ranging between (72.0% & 82.8%). Ivermectin interferred with nervous system and muscle function by improving inhibitory neurotransmission and binding to glutamate-gated chloride channels in membranes of nerves and muscle cells led to increase permeability to chloride ions causing cellular hyperpolarization, followed by paralysis and death (Yates and Wolstenholm, 2004). El-Azzouni (1997) found that ivermectin effected adults by topographic destruction and degeneration with subsequent reduction in larval count. In the present study, albendazole in dose 50mg/kg for 3 successive days from the 3rd day post infection caused significant reduction in mean adult (83.6%) and larval count (80%) compared to infected control. This agreed with Attia *et al*. (2015) who used albendazole in the same dose and found significant reduction in both adult (94.2%) and larval (90.9%). However, lower efficacies of albendazole against the encysted larval stages were reported (Shoheib *et al*, 2006; Shalaby *et al*, 2010). The differences in albendazole efficacies against both intestinal and muscular stages depend on dose, time, duration of treatment (Siriyasatien *et al*, 2003). Its mode of action is the inhibition of microtubule polymerization through selective binding to beta-tubulin monomer of the parasite, with little effect on binding of the host tubulin (Aguayo-Ortiz *et al*, 2013).

The present results showed significantly increased in levels of AST, ALT, urea, creatinine, CPK and globulin with significantly decrease in levels of total proteins and albumin in infected control group as compared with non-infected healthy mice. This change might be due to hepatic and renal damage caused by the migrating larvae (Gamble *et al*, 1997). This agreed with Mikhail (1979). While the decrease in total proteins, albumin levels may be due to damage of liver parenchyma by migrating larvae or hepatic affection by the parasite metabolic products (Mikhail *et al*, 1978). Globulin elevation may be due to the compensatory reaction to restore osmotic pressure in serum reduced due to low albumin level or to increased formation of antibodies against the parasite or its metabolic products (Reinhold, 1955). The results agreed with Soliman *et al*. (2011) and Basyoni and El-Sabah (2013).

In the present study, marked biochemical parameters improvement to normal values occurred in all mice treated with *N. sativa*, Ivemectin and Albendazole. This agreed with Soliman *et al.* (2011) they reported highly significant reduction in AST & ALT, decrease in urea and creatinine levels and increase in total proteins and albumin after the early treatment with ivermectin as a single dose of 0.2mg/kg S.C. injection at 4 dpi which assessed the biochemical parameters at 30 dpi. They attributed this due to drugs larvicidal activity as animals were experimentally infected with *T. spiralis* and free from other parasites. The results also agreed with Basyoni and El-Sabah (2013).

In the present study, all treated mice showed mild increase in inflammatory cellular reaction in mid-intestinal region. Diaphragmatic muscle showed decrease in larval deposition with mild inflammatory reaction around. In infected control, there was necrosis and atrophy of intestinal villi with marked inflammatory reaction mainly eosinophils in lamina propria. The diaphragmatic muscle showed multiple larval deposition surrounded by marked inflammation. This agreed with Abu El Ezz (2005) and Soliman *et al.* (2011) they showed a high number of migrating larvae to diaphragm of untreated rats. Ivermectin as a single subcutaneous dose of 0.2mg/kg/bodywt at 10th day showed a reduction in number of larvae encysted in diaphragms of infected rats. Aver-

mectin for field use gave significant larvicidal activity (Arena *et al,* 1992).

Conclusion

The outcome data showed that ivermectin gave the best results at dosage level and formulations. This recommended the effectiveness of *N. sativa* extract as trichinellosis effective treatment in experimental mice.

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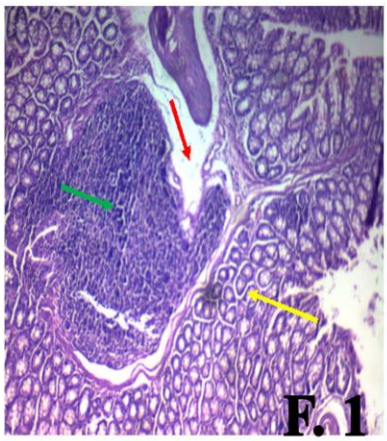
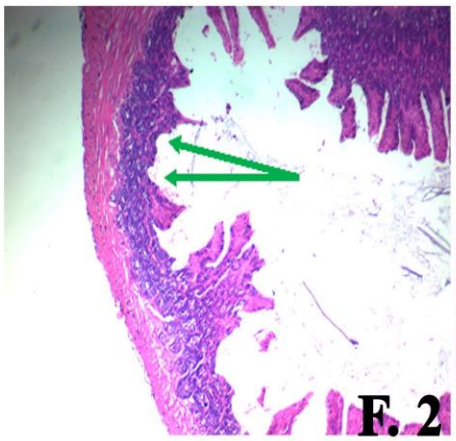
Explanation of Figures

Fig. 1: Section in small intestine of infected control showed epithelial hyperplesia (yellow arrow), inflammatory reaction in lamina propria (green arrow) and edema (red arrow) (X100, H&E).
Fig. 2: Section in small intestine of infected control showed necrosis and atrophy of villi (green arrow) (X100,H&E).
Fig. 3: Section in small intestine (lamina propria) of *Ni.sativa* prophylactic showed mild increase in inflammatory cellular reaction (red arrow) (X100, H&E).
Fig. 4: Section in small intestine of Ivermectin treated showed mild inflammation with eosinophils infiltrate (red arrow) (X100, H&E).
Fig. 5: Section in small intestine of Albenazole treated showed mild inflammation with eosinophils infiltrate (red arrow) (X100, H&E).
Fig. 6: Section in muscle of diaphragm of infected control showed multiple larval deposition (red arrow) (X100, H&E).
Fig. 7: Section in muscle of diaphragm of infected control showed multiple larval deposition (red arrow) and marked muscle inflammation (black arrow). (X100, H&E).
Fig. 8: Section in muscle of diaphragm of *N. sativa* treated showed single larval deposition (black arrow) surrounded by mild muscle inflammation (green arrow), (X400, H&E).
Fig. 9: Section in muscle of diaphragm of Ivermectin treated showed single larval deposition (red arrow) surrounded by mild muscle inflammation (black arrow), (X400, H&E).
Fig. 10: Section in muscle of diaphragm of Albenazole treated showed single larval deposition (red arrow) surrounded by muscle inflammation (black arrow), (X400, H&E).


F. 1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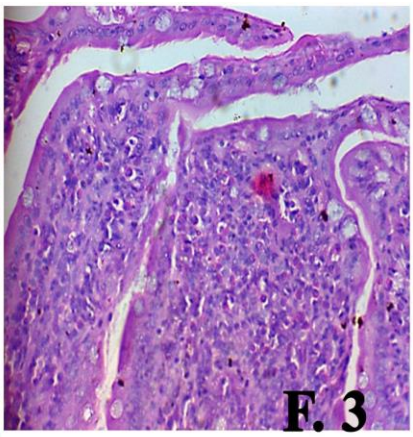
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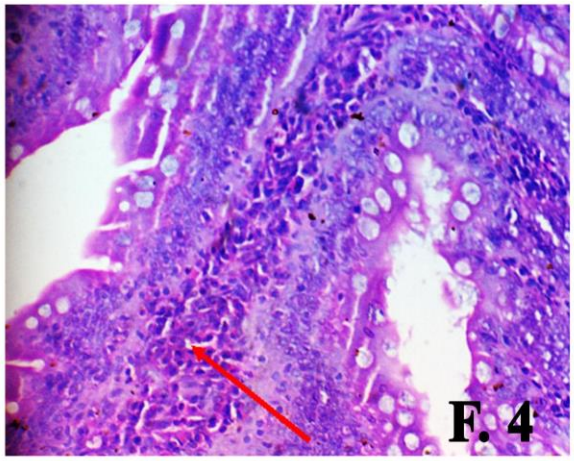
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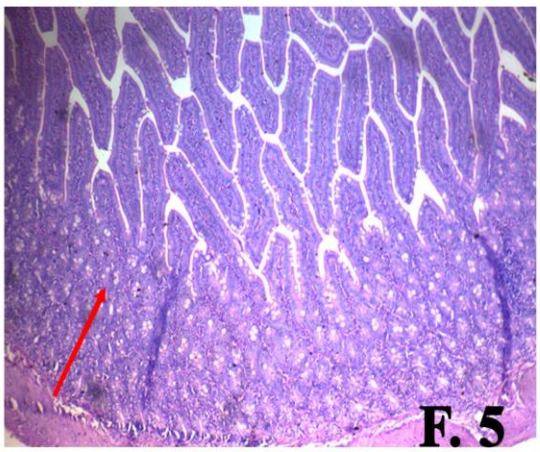
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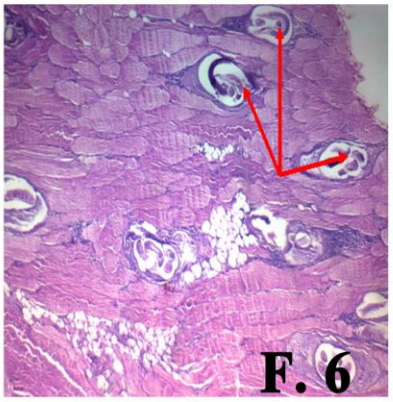
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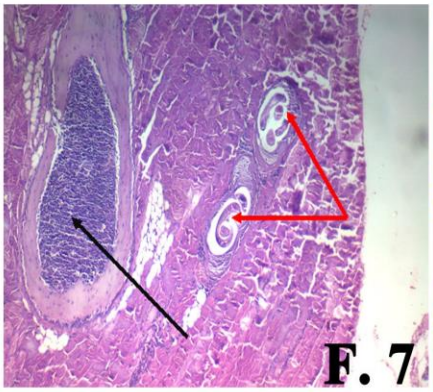
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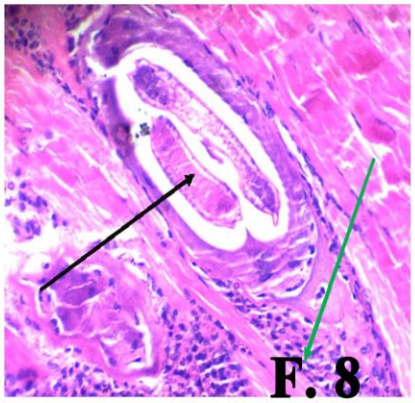
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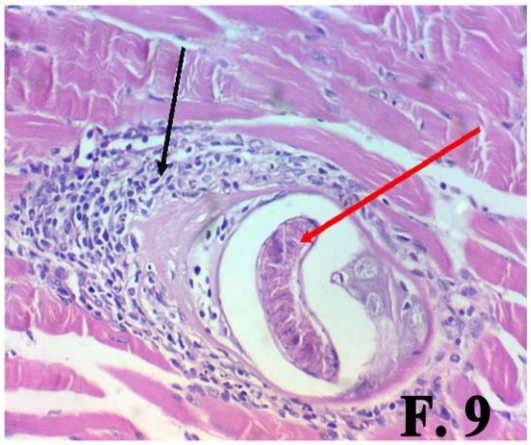
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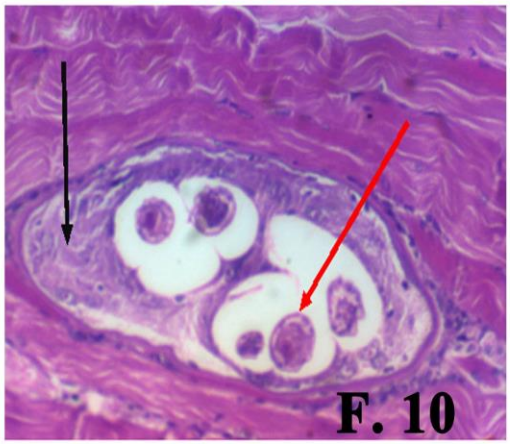
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