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EFFECT OF THYMOL EXTRACT ON TRICHINELLOSIS COMPARED TO ALBENDAZOLE AMONG EXPERIMENTALLY INFECTED MICE

By
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Abstract

Trichinellosis is a zoonotic disease of public health concern since it caused human outbreaks in many countries. Traditional therapy has many adverse effects in addition to the developing resistance. So, this necessitates finding effective natural alternatives. The study assessed the effect of Thyme extract (active constituent of thyme) on *Trichinella spiralis* in experimentally infected mice compared with Albendazole®.

One hundred and twenty Albino mice were classified into four groups of 30 mice each. G1: non-infected control, G2: infected untreated control, G3: albendazole treated, and G4: thyme extract treated. Mice were sacrificed on the 7th day post infection for intestinal phase and on the 49th & 60th days post infection for muscular phase. Efficiency of treatment was assessed by parasitological and histopathological examinations.

The results showed significant decrease in intestinal worms in all treated mice relative to untreated control group. Reduction rates regarding the intestinal phase was 96.7% in albendazole treated mice, followed by 33.4% in Thyme extract treated ones. Histological showed a significant decrease in muscle larvae relative to positive control mice showed by reduction rates of 86%, & 45.1% for albendazole and thyme respectively on 49th dpi, and 99.9 %, & 68.6% respectively on 60th dpi. There was improvement in intestinal and muscular architecture in all treated mice compared to positive control one, which was markedly best in albendazole treated ones.

Keywords: Mice, Trichinellosis, Thyme extract, Albendazole, Parasitological evaluations.

Introduction

Trichinosis or trichinellosis is a nematode infection primarily transmitted via ingestion of improperly prepared pig meat and its by-products as the primary infection sources, which is potentially fatal, but commonly a self-limiting disease (Furhad and Bokhari, 2022). *Trichinella spiralis* was internationally categorized among the top of 10 food-borne parasites (El Temsahy *et al*, 2015). Other common hosts are synanthropic animals like cats, dogs, brown rats, walrus and armadillos (Zarlenga *et al,* 2016).

The Egyptian human trichinellosis was few reported in the fresh and processed pork (Siam *et al,* 1979). But, the trichinosis was reported in pigs (Azab *et al,* 1988), as well as in rodents around abattoirs in Suez Canal zone (Morsy *et al,* 1980) and in Alexandria (Loutfy *et al,* 1999). Light infections may be asymptomatic, but intestinal invasion may be accompanied by gastrointestinal symptoms (diarrhea, abdominal pain, vomiting).

Larval migration into muscle tissues (a week after infection) may cause periorbital and facial edema, conjunctivitis, fever, myalgias, splinter hemorrhages, rashes, and peripheral eosinophilia. Occasional life-threatening pictures include myocarditis, CNS involvement, and pneumonitis (CDC, 2017).

The anti-parasitic drugs (Albendazole® or Mebendazole®) may help in preventing progression of trichinellosis by killing the adults and stopped further release of larvae. Once larvae became established in skeletal muscle, usually by 3 to 4 weeks post infection, treatment may not completely eliminate the infection and associated symptoms. The treatment with either was recommended, if not initiated within the first several days of

infection, prolonged or repeated treatment courses must be necessary (CDC, 2020). Both were considered relatively safe, however, were associated with side effects including bone marrow suppression (García *et al*, 2014), and contra-indicated in pregnancy and children less than two years (Kocięcka, 2000). So, there was an increasing need for safe and effective drugs, especially those derived from the medicinal plants or herbs with neither toxicity nor no adverse effects (Basyoni and El-Sabaa, 2013).

Thymus vulgaris (thyme, German thyme or garden thyme), is a species of flowering plant in the mint family Lamiaceae very popular traditional medicinal plant widely used worldwide; especially the Middle East Countries (Kindersley, 2008). Thyme extract showed significant effect against myiasis producing flies (Morsy *et al,* 1988)*, Trichomonas gallinae* (Nasrabadi *et al*, 2012), echinococcosis scolices (Moazeni *et al*, 2012), and cysts (Pensel *et al*, 2014), chronic toxoplasmosis (Eraky *et al*, 2016) regulated renal redox, oxidative stress, anti-oxidant levels, and inflammation-associated genes at molecular, biochemical, and cellular immunohistochemical levels (soliman *et al,* 2022).

Besides, thyme treats gastro-intestinal disturbaances (Chander *et al*, 2010), has antispasmodic, expectorant, anti-jussive, anti-inflammatory, analgesic, anti-helminthic, carminative, diuretic, sedative, anti-oxidant activities (Orłowska *et al*, 2015), relief toothache (Matthew and Natalya, 2016), anti-tumor activities (NagoorMeeran *et al*, 2017), antimicrobial, preservative, and antiseptic (Hasan *et al*, 2019). Soliman *et al.* (2021) added that $NaNO_2$-induced hepatic injury significantly reduced by pretreatment with *T. vulgaris* extract protected against hepatic oxidative stress and its associated genes at biochemical, molecular, and cellular levels.

This work aimed to detect the effect of thyme extract of *Thymus vulgaris* (Thyme) on *Trichinella spiralis* in experimentally infected Albino mice as compared with the traditional Albendazole®.

Materials and Methods

This experimental study was carried out in Theodor Bilharz Research Institute, Biological Unit) from July to October 2021.

Animals: One hundred and twenty laboratories bred male Swiss Albino mice; 8-10 weeks old; ~20-25g in weight were used. They were maintained under suitable light, 24ºC temperature and provided with suitable diet and water. Mice were provided from European Country Farms in Egypt and were housed and maintained at the Schistosome Biology Supply Center (SBSC) Theodor Bilharz Research Institute, Giza, Egypt. Mice handling and treatment were done according to internationally valid ethical guidelines.

Parasite: *Trichinella spiralis* strain was isolated from infected pigs' diaphragm in El-Bassatine Governmental Abattoir, Cairo. The parasite was kept by regular repeated passages. Muscles of mice heavily infected with *T. spiralis* were cut and digested in a solution of 1% pepsin & 1% hydrochloric acid in warm water bath. After an overnight incubation at 37°C, larvae were extracted using sedimentation technique, washed several times in normal saline, and number of larvae/ml was counted by the hemocytometer. Each mouse was infected orally by 200 larvae by a blunt tuberculin syringe (Wassom *et al,* 1988).

Treatment: 1- The thyme extract (active one) was kindly supplied by Faculty of Pharmacy, 6 October University and was given orally as 2.5ml/kg/day. 2- Albendazole was purchased as 20mg/ml suspension from the Egyptian International Pharmaceutical Industries Company (EIPICO) and was given orally as 50mg/kg /day.

Experimental design: Mice were classified into four main groups: G1: Neither infected nor treated mice (negative control). G2: Infected non-treated mice (positive control), which was subdivided into four subgroups. Each one was sacrificed on the 7^{th}, 30^{th}, 49^{th}, and 60^{th} day post-infection (pi). G3: Albendazle treated group was subdivided into three subgroups*:* SGA: received 50mg/kg st-

arted from the 3rd day pi for three successsive days. They were sacrificed on the 7th day pi to evaluate drugs' efficacy on adult day started from the 31st day pi for seven successive days. They were sacrificed on the 49th day & 60th day pi respectively. SGB & SGC: received 50mg/kg albendazole. G4: Thyme extract treated mice was subdivided into 3 subgroups. SGA: received 2.5ml/kg thyme extract started from the third day pi for three successive days. They were sacrificed on the 7th day pi. SGB & SGC: received 2.5ml/kg thyme extract started from the 31st day pi for seven successive days. They were sacrificed on the 49th day & 60th day pi respectively.

Parasitological examination for intestinal phase for isolation and counting of adults: Adults were collected from the small intestine counted, and worm load was expressed as the total number of intestinal worms per mouse. Muscle larvae were recovered from infected mice's bodies after scarification by artificial digestion according to the accepted standard procedures. Muscle larval load was determined by counting all larvae in a carcass digest aliquot, and expressed as a total number of encysted larvae per mouse

Histopathological examination of small intestine samples from mice sacrificed on the 7th dpi (Nasseff *et al,* 2018). The muscular specimens were taken from sacrificed mice on 49th, and 60th dpi. Sections from intestine, diaphragm and thigh muscles were fixed in 10% buffered formalin solution, dehydrated, cleared, and embedded in paraffin blocks. Paraffin sections 5µm-thickness were mounted on clean glass slides, and stained with H & E for histopathological examination to detect pathological changes caused by adults in intestine and larvae in muscles (Drury and Wallington, 1980).

Ethical considerations: The protocol of was approved by Research Ethics Committee, Faculty of Medicine, Benha University, Egypt. Mice were handled according to National Institutes of Health (NIH) guidelines for animal experimentation. All experiments were carried out according to the Clinical and Laboratory Standards Institute (CLSI) guidelines and were approved by an institution responsible for animal ethics concerning care for animals and safe disposal of their wastes at Theodor Bilharz Research Institute. These all went with the guidelines of Helsinki (2000)

Statistical analysis: Data were coded then entered and analyzed using SPSS version 26 (Statistical Package for the Social Science; IBM Corp, Armonk, NY, USA) for Microsoft Windows 10. Analysis was conducted by one-way ANOVA Test followed by Duncan post hoc adjustment. Reduction percenttage of (efficacy) was calculated between treated groups and control ones in the same column by: Reduction rate (%) = $\frac{B-A}{A} x100$.

Results

Parasitological results: A significant decrease in intestinal worms was found in all treated groups compared to infected controls. Worms' number recovered from albendazole treated mice showed least mean count value (3.5±1.6) as compared with infected control (104.60±7.3) with P <0.001, as compared with thyme treated ones (P <0.001). Worms' number recovered from thyme treated mice (69.7±5.4) showed significant decrease in intestinal number as compared with positive control ones (P <0.001). Reduction % in worms' number was higher in albendazole treated mice followed by thyme treated ones (96.7% & 33.4% respectively) with a significant difference (p<0.001).

Muscular phase in all treated mice showed a significant decrease in muscle larvae relative to infected untreated control on the 49th dpi, Larvae number recovered from albendazole treated mice showed least mean count value (1074.20 ±362.19) as compared with infected control mice (8077.50±301.88, P <0.001); and as compared with thyme treated mice (P <0.001). Larvae recovered from thyme treated mice showed significant decrease in number (4438.30±1576.81) as compared with infected control (P <0.001). On 49th day pi, reduction in larval count was significantly high in albendazole treated mice

(86.7%) with significant difference as compared to thyme treated ones (45.1%), with P<0.001. At 60th day pi larval number recovered from albendazole treated mice showed the least mean count when compared to positive controls and thyme treated mice (P<0.001). Worms recovered from thyme treated mice showed significant decrease in intestinal number as compared with positive control (P<0.001). But, on 60th day pi reduction in larval count was significantly higher in albendazole treated mice (99.9%) with significant difference as compared to thyme treated ones (68.6%) with P<0.001.

Histological analysis of small intestine of control positive mice on 7th day pi showed distorted villous pattern (short broad villi) with scattered many adults in mucosa with moderate inflammation, inflammatory infiltrate composed mainly of lymphocytes and plasma cells. Intestinal sections of mice treated with albendazole showed mild inflammation in core of villi and mildly distorted villous pattern without worms, while thyme extract treated mice showed distorted villous pattern with scattered adults in mucosa, inflammatory infiltrate composed mainly of lymphocytes and plasma cells in both.

Stained skeletal muscles of control positive mice at 49th dpi showed many cysts with intact capsule and contents infiltrating skeletal muscle bundles. Albendazole treated mice showed few cysts with mostly degenerated capsule and contents infiltrating skeletal muscle bundles. However, thyme treated mice showed many cysts with partially intact capsule and contents infiltrating skeletal muscle bundles with patchy inflammation.

Muscles of control positive mice at 60th dpi showed few cysts with degenerated capsule and contents infiltrating skeletal muscle bundles. Albendazole treated mice showed few remnants of cysts with mostly degenerated capsule and contents infiltrated skeletal muscle bundles. Occasionally, thyme treated mice showed cysts with degenerated capsule and contents infiltrating the skeletal muscle bundles.

Details were given in tables (1, 2, 3 & 4) and figures (1, 2, 3, 4, 5, 6, 7, 8, 9, 10 & 11).

Table 1: *Trichinella spiralis* adults' number recovered from intestine among groups on 7th dpi (n=10).

Variants	Infected controls	Albendazole treated	Thyme treated	p-value
Mean ±SD	104.60±7.3[a]	3.5±1.6[c]	69.7±5.4[b]	<0.01*
Minimum	95.00	1.00	60.00	
Maximum	117.00	6.00	78.00	

[abc] superscript different letters means significant different values, *p-value ≤0.01 = significant

Table 2: *Trichinella spiralis* larval count among groups on 49th dpi (n=10)

Variants	Infected controls	Albendazole treated	Thyme treated	p-value
Mean ±SD	8077.50 ±301.88[a]	1074.20±362.19[c]	4438.30±157.81[b]	<0.01*
Minimum	7645.00	686.00	2375.00	
Maximum	8509.00	1625.00	6500.00	

[abc] superscript different letters means significant different values, *p-value ≤0.05 = significant

Table 3: *Trichinella spiralis* larval count among the examined groups on 60th dpi (n=10/group)

Variants	Infected controls	Albendazole treated	Thyme treated	p-value
Mean ±SD	5921.20 ±467.88[a]	2.90 ±1.79[c]	1857.20 ±156.47[b]	<0.01*
Minimum	3571.00	0.00	1665.00	
Maximum	7600.00	6.00	2125.00	

[abc] superscript different letters means significant different values,*p-value ≤0.01=significant

Table 4: Mean values of *Trichinella spiralis* adult worms' and larval counts among groups (n=10/group)

Variants	Adults on 7th dpi	R%	Muscle larval on 49th dpi	R%	Muscle larval 60th dpi	R%
Infected controls	104.60 ±7.3[a]	-	8077.50 ±301.88[a]	-	5921.20 ±1467.88[a]	-
Albendazole treated	3.5 ±1.6[c]	96.7	1074.20 ±362.19[c]	86.7	2.90 ±1.79[c]	99.9
Thyme treated	69.7 ±5.4[b]	33.4	4438.30 ±1576.81[b]	45.1	1857.20 ±156.47[b]	68.6
p-value	<0.001*		<0.001*		<0.001*	

[abc] superscript different letters means significant different values, R%: Reduction, *p-value ≤0.05 = significant

Discussion

Trichinellosis is a severe meat-borne zoonosis worldwide spread with marked significance in several developing countries (Bai *et al,* 2017), but sylvatic trichinellosis was prevalent in the Mediterranean and African

regions (Youssef and Uga, 2014). Besides, zoonotic trichinellosis outbreaks were described many times in Lebanon from to small series in the 20th century (Khalil *et al,* 2022). Also, outbreaks were reported in other Mediterranean Countries, such as Greece, Italy, and Spain, during the few last years (Turiac *et al,* 2017). Trichinellosis belongs to encapsulated parasites and causes most human infections and deaths (Farid *et al,* 2019).

Nowadays, the primary drug treatment for trichinosis is antihelminthics such as albendazole & mebendazole. Albendazole reaches adequate plasma levels and without monitoring whereas mebendazole plasma levels varied from patient to patient requiring individual monitoring and dosing (Yadav and Temjenmongla, 2012) But, it gave diminished effectiveness against *T. spiralis* encysted larvae (Pozio *et al,* 2001).

In the present study, a significant decrease in the number of intestinal adult worms was found in all treated mice compared to the positive control ones on the 7th day pi. The number of worms recovered from albendazole treated mice was few as compared with both control positive, and thyme extract treated one. But, intestinal adults recovered from thyme extract treated ones were significant less than in control positive ones. Also, reduction % in adults was significantly higher among albendazole-treated mice followed by the thyme treated ones (96.7% & 33.4% respectively). This agreed with Attia *et al.* (2015) in Egypt who reported that the effect of myrrh (*Commiphora molmol*) and thyme (*Thymus vulgaris*) against intestinal phases of *Trichinella spiralis* in mice compared with albendazole were more or less significantly decrease in the mean adult number with all drugs. However, the least count was found in albendazole treated mice with efficacy of 94.2% followed by myrrh treated ones with efficacy of 90.3% and least reduction was in thyme-treated mice with efficacy of 79.4%.

Siriyasatien *et al.* (2003) in Thailand reported that 20 mg/kg albendazole for 15 days against the early stage of *T. spiralis* infection (7 days pi) resulted in 100% efficiency. The high efficacies of albendazole, mebendazole, and benzimidazole derivative were reported in Korea (Chung *et al*, 2001), in India (Yadav and Temjenmongla, 2006), and in Egypt (Shalaby *et al*, 2010).

Moreover, Nassef *et al.* (2018) in Egypt evaluated the chitosan nanoparticles singly or combined with albendazole to treat trichinellosis experimental infected mice reported that the lowest mean of adult count was in those received chitosan nanoparticles loaded with albendazole (1.8±1.03) with best effectiveness (99.1%) of *T. spiralis* worms.

In the current study, larval count of *T. spiralis* larvae in the skeletal muscles among mice groups on the 49th day and the 60th day pi showed a significant decrease in muscle larvae relative to positive control ones. The larval number recovered from albendazole treated mice showed the least mean number as compared with either positive control or thyme treated ones, but with more significant decrease than in positive control ones. The reduction in larval count on the 49th day & the 60th day pi was significantly higher in albendazole treated mice (86.7% & 99.9% respectively) followed by thyme treated ones (45.1% & 68.6% respectively). This agreed with Attia *et al.* (2015) who reported a significant decrease in the mean larvae count in all treated groups, with the best reduction of larvae in albendazole treated mice 90.9%, followed by myrrh treated ones 79.6%, and the least in thyme treated mice with efficacy of 71.3%. However, lower efficacies of albendazole against encysted larval stages were reported in Egypt by Shoheib *et al*. (2006) and Shalaby *et al*. (2010) as well as in Thailand by Siriyasatien *et al.* (2003). The last authors explained the differences in albendazole efficacies against muscular stages depended on dose, time and duration treatment.

Aguayo-Ortiz *et al*. (2013) in Mexico reported that the albendazole inhibited microtubule polymerization by selective binding to parasite's beta-tubulin monomer with little

effect on host tubulin binding.

In the present study, histopathology of the intestine on the 7th day pi, of positive control and thyme treated mice showed distorted villous pattern with scattered adults within the mucosa. Albendazole treated mice showed mildly distorted villous pattern with occasional adults in the intestinal mucosa. Attia et *al.* (2015) reported that intestinal sections of positive controls on the 7th day pi showed adults in the intestinal mucosa with chronic inflammatory cells infiltrating the mucosa and submucosa. They added that intestinal sections of treated mice, compared with A showed a marked decrease in inflammatory infiltrate in group B, but mild to moderate cellular infiltration was in groups C & D. Also, Nada *et al.* (2018) in Egypt reported that in 7th day pi small intestine section of positive control showed epithelial hyperplesia, inflammatory reaction in lamina propria and edema. They added that small intestine positive control showed necrosis and villi atrophy. But, in small intestine of albenazole treated mice showed mild inflammation with eosinophils infiltrate.

In the current study, histopathology of muscular stage, in positive controls at the 49th day pi showed many *Trichinella* cysts with intact capsule with infiltrating the skeletal muscle bundles and moderate inflammation with infiltration by mononuclear inflammatory cells. In albendazole treated ones in muscles at 49th day pi showed few *Trichinella* cysts with mostly degenerated capsule with infiltrating the skeletal muscle bundles. But, in thyme treated mice muscles at 49th day pi showed many *Trichinella* cysts with partially intact capsule and infiltrating the skeletal muscle bundles with patchy inflammation. Attia *et al.* (2015) reported that skeletal muscle sections of *T. spiralis* infected mice on the 21st, 30th, 49th, & 60th day pi of positive control showed a massive number of *T. spiralis* encysted larvae diffused in the muscles sarcoplasm and a massive number of chronic inflammatory cells in form of lymphocytes, plasma cells, eosinophils, and histiocytes infiltrating muscle bundles surrounding the encysted larvae. But, thyme treated mice at 49th day showed encysted larvae surrounded by thick intact capsule and intense inflammatory cellular infiltrate. The albendazole treated mice at the same day showed homogenized larvae, vacuolation and splitting of capsule into thin layers with diffuse inflammatory cellular infiltration surrounding and invading capsule. For myrrh treated mice at the same day showed homogenised larvae with broken down incomplete capsule that completely invaded and surrounded by inflammatory cells. Nada *et al.* (2018) reported that at 35th day pi, sections in diaphragm muscle of positive control showed multiple larval depositions with marked muscle inflammation. However, Albendazole treated mice showed single larval deposition surrounded by muscle inflammation.

In the present study, on the 60th day pi muscles sections of positive control showed few *T. spiralis* cysts with degenerated capsule and with infiltrating the skeletal muscle bundles. Albendazole treated mice showed few remnants of cysts with mostly degenerated capsule and with infiltrating the skeletal muscle bundles whereas thyme treated mice showed occasional *T. spiralis* cysts with degenerated capsule and with infiltrating the skeletal muscle bundles. This agreed with Attia *et al.* (2015) who found that albendazole treated mice on day 60th day pi the muscular sections showed much fewer numbers of encysted larvae with degenerative changes, areas of thinning and splitting of capsule into thin layers, areas of breakdown, vacuolization and invasion by inflammatory cellular infiltrate. In thyme treated mice muscles section showed a fewer number of encysted larvae than in positive control with heavier inflammatory cellular infiltration surrounding them, and capsule in majority of larvae were thick and complete.

Conclusion

This study concluded that thyme extract was effective against the intestinal and muscular stages of *T. spiralis* in experimentally

infected mice but it was less effective than albendazole. The histopathology was a good tool for assessment of the drug efficacy in trichinosis treatment.

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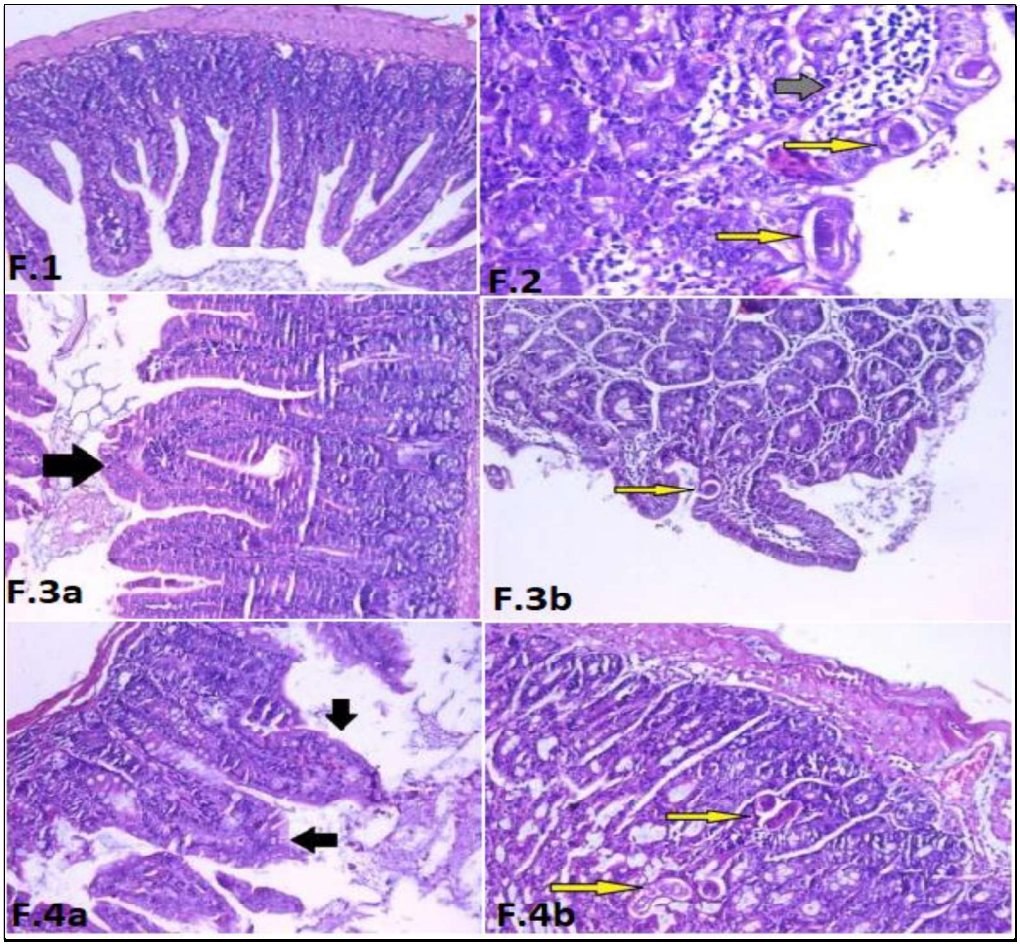
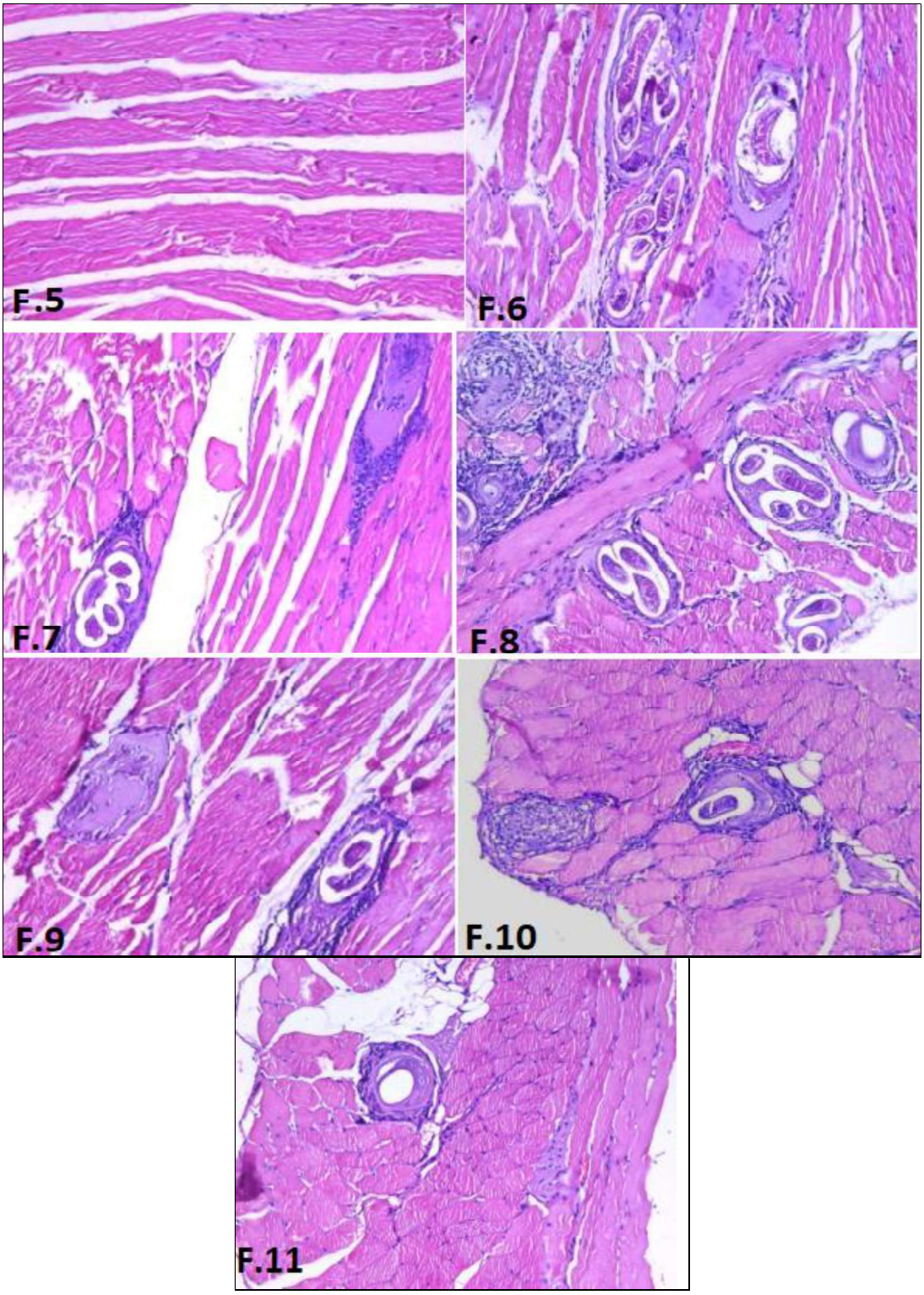
Explanation of figures

Fig. 1: Section in intestine of control negative mice showed regular villous pattern (H & E, X200)
Fig. 2: Section in intestine of control positive (infected) group of mice in 7th dpi showed distorted villous pattern (black arrow) with scattered adult worm sections within the mucosa (yellow arrows) (H & E, X200)
Fig. 3: Section in intestine of albendazole 7th dpi mice showed mildly distorted villous pattern (black arrow) occasional adults in mucosa [yellow arrow (b)] (H & E, X200)
Fig. 4: Section in intestine of thyme extract 7th dpi mice showed distorted villous pattern (black arrow, a) with scattered adult sections in mucosa (yellow arrows, b) (H & E, X200)
Fig. 5: Section in muscles of control negative group of mice showed normal pattern and arrangement of skeletal muscle bundles (H & E,, X200)
Fig. 6: Section in muscles of Control +ve in 49th dpi mice showed many *Trichinella* cysts with intact capsule and infiltrated skeletal muscle bundles (H & E, X200)
Fig. 7: Section in muscles of albendazole 49th dpi mice showed few *Trichinella* cysts with mostly degenerated capsule and infiltrated skeletal muscle bundles (Hematoxylin and Eosin stain, X200)
Fig. 8: Section in muscles of (thyme extract 49th dpi) group of mice showed many *Trichinella* cysts with partially intact capsule and infiltrated skeletal muscle bundles with patchy inflammation (H & E, X200)
Fig. 9: Section in muscles of control +ve 60th dpi mice showed few *Trichinella* cysts with degenerated capsule and infiltrated skeletal muscle bundles (H & E, X200)
Fig. 10: Section in muscles of albendazole 60th dpi mice showed few remnants of *Trichinella* cysts with mostly degenerated capsule and infiltrated skeletal muscle bundles (H & E, X200)
Fig. 11: Section in muscles of thyme extract 60th dpi mice showed occasional *Trichinella* cysts with degenerated capsule and infiltratedskeletal muscle bundles (H & E, X200)

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