

ANTIPARASITIC ACTIVITY OF MYRRH CRUDE EXTRACT AND MYRRH VOLATILE OIL COMPARED TO ALBENDAZOLE AGAINST TRICHINELLA SPIRALIS MUSCULAR LARVAE IN VITRO

By

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Abstract

Trichinellosis is a foodborne zoonotic disease caused by *T. spiralis* affecting human and animals. Treatment with commercially available drugs has not been satisfactory due to rapid development of drug-resistant particularly against encapsulated larvae. There is an increasing need to discover alternative anthelmintics agents from medicinal plants. The present study evaluated the in vitro antiparasitic activities of myrrh crude extract and myrrh volatile oil on *T. spiralis* larvae compared to albendazole to estimate their sublethal concentrations on the infectivity of *T. spiralis* larvae in mice. The *in vitro* effect of these agents was evaluated by assessing mortality rate and by a scanning electron microscopic analysis of ultrastructural changes in the cuticle of the larvae. The surface changes induced by crude myrrh extract and albendazole were more severe than those observed after exposure to myrrh volatile oil. All responses to the substances were time dose-dependent and highly significantly different from the control group ($p < 0.001$). 100% mortality rate of larvae occurred on the 4th day at 3, 5, & 7mg/ml of myrrh crude extract, on the 7th day with volatile oil while total larval death occurred on the 1st day of exposure in to 5, 10, 15 & 20µg/ml of albendazole. In vitro exposure to sublethal dose of albendazole, crude myrrh extract and volatile oil extract resulted in infectivity reduction by 100%, 98% & 88% respectively in intestinal phase and 100%, 98% & 59% respectively in muscular phase.

Keywords: In vitro, *Trichinella spiralis*, Albendazole, Myrrh crude, Myrrh volatile oil

Introduction

Trichinellosis is a foodborne zoonotic disease caused by *Trichinella spiralis* and affecting many mammals, including humans (Despommier *et al*, 1974). About 10,000 people were infected per year with a 0.2% mortality rate (Ashour and Elbakary, 2011; García *et al*, 2014). Millions of people were chronically infected with muscle larvae suffered from the muscular pain (Dupouy-Camet, 2000). Consequently, trichinellosis represents a public health threat not only affecting human health but also lead to enormous economic losses in porcine animal industry and food safety (Gottstein *et al*, 2009). *Trichinella spiralis* was internationally categorized among the top 10 foodborne parasites (Temsahy *et al*, 2015). Infection of humans occurs with the consumption of encysted *Trichinella* larvae in the under cooked pork meat (Pozio, 2007). Trichinellosis was divided into two phases: an intestinal (or enteral) ph-

ase and a muscular (or parenteral or systemic) phase. Light infection is usually asymptomatic, while heavy infection manifested by gastroenteritis with diarrhea and abdominal pain and represents the acute intestinal phase. Later, the migratory larvae and their metabolites initiate an inflammatory and allergic reaction. Pyrexia, myalgia, eyelid and facial edema, and eosinophilia are the commonest manifestations. Sometimes, case might be complicated by thromboembolic disease, myocarditis and encephalitis. Chronic trichinellosis represented by muscle weakness, numbness and conjunctivitis (Gottstein *et al*, 2009). Hence, the administration of efficient anthelmintic drugs at the early stage of intestinal phase is essential for effective therapy (Yu and Qi, 2015). At the present time there is no adequate treatment to properly control this parasitic disease because the intestinal phase cannot always be diagnosed.

Anthelmintic drugs, Albendazole[®], Mebendazole[®] and Thiabendazole[®] are the principal drugs used to treat trichinosis (Sampbell and Cuckler, 1965; Chug *et al*, 2001). Because of emerging of high resistance degree and/or reduced anti-parasitic activity of these drugs particularly encapsulated larvae (Caner *et al*, 2008), there was a need to search for alternative anthelmintics agents from the traditional medicinal plants (Abu El Ezz, 2005).

Myrrh is a natural treatment obtained from the aloe-gum resin of *Commiphora molmol* (Atta and Alkofahi, 1998). It contains 57%-61% gum, 25%-40% resin, 7%-17% volatile oil and 3%-4% impurities and (Massoud *et al*, 2001). The gum consists of polysaccharides and proteins, while the volatile oil retains sterols, steroids and terpenes. Furanos-quiterpene component is responsible for the characteristic odor of myrrh (Hanus *et al*, 2005).

Myrrh is widely used in traditional treatment of some infectious diseases. Extracts were used topically for management of ulcers, abscess and wound (Samuelsson *et al*, 1992; Lans *et al*, 2006), relieved headaches and backaches (Bagatti, 1946). There were many studies on myrrh oleoresin as antimicrobial agent (Romero *et al*, 2005; Rahman and Gibbons, 2007; Alzahran *et al*, 2011; Kuete *et al*, 2012), anti-parasitosis as anti-fascioliasis (Soliman *et al*, 2004; Abo-Madyan *et al*, 2004), anti-monieziasis *expansa* (Haridy *et al*, 2004), anti-heterophyiasis (Massoud *et al*, 2007), and anti-cryptosporidiosis (Abou-el-Nour *et al*, 2016). Besides, Attia *et al*. (2015) reported strong efficacy of myrrh extract against both phases of *T. spiralis* when used in experimentally infected mice.

This study aimed to evaluate the in vitro lethal effect of crude myrrh extract and myrrh volatile oil on *T. spiralis* larvae compared to the larvicidal effect of albendazole as a reference drug. Also, the effect of the sub-lethal concentrations on the infectivity of *T. spiralis* larvae in mice was assessed. From

the available literature, myrrh and essential oil of myrrh have been in vitro tested for the first time against *T. spiralis* larvae.

Material and Methods

Parasites material: *Trichinella spiralis* was originally collected from a naturally infected pig slaughtered in El-Bassatine Abattoir, Cairo and was maintained by consecutive infection of BALB/c mice at the animal house of Assiut University. Every mouse was orally infected by 300 muscle larvae considered as the specific pathogen-free conditions. Larval muscle were recovered from the carcasses of infected mice after 30 days post infection by incubating minced skinned mouse with artificial digestive fluid in conical flask at 37°C overnight. Larvae were filtered using thieve to remove bones and hair and then washed in PBS. Larvae collected from the conical flask bottom were washed several times with BPS, and their number/ ml was counted under a light microscope x40 (Gamble, 2016).

Plant extracts preparation: 1- Myrrh methanol extract: It was prepared (Evans, 2002). Briefly, 250g of myrrh was pulverized to fine powder and soaked in 1L of methanol for 24hr. The mixture was sonicated (Crest Ultrasonics powersonic TM, USA) for 1hr to speed up the extraction process and enhance the yield. Later, the produced extract was filtered by using Whatman filter paper No. 1. All methanolic extracts were collected and evaporated by a rotary evaporator (Heidolph VV2000, Schwabach, Germany) under reduced pressure at 40°C to obtain a dried residue representing the alcohol-soluble portion of the used exudate (namely, essential oil and alcohol-soluble resins). All solvents and reagents used were of analytical grade and received with no further purification. 2- Volatile oil preparation: Myrrh extract of essential oil was achieved via the hydro-distillation process (Hanus *et al*, 2005). In brief, 250 g of fine powdered myrrh was placed in a 2L glass flask containing 1L of distilled water and then connected to the Clevenger apparatus. A heating mantle was used as a source of heat. The resultant water

vapor, charged with the desired essential oil, passes to the condenser of the apparatus, where it was condensed led to the target oil recovery from water.

Reference drug: Albendazole[®] was supplied as suspension (Alzentale) from the Egyptian International Pharmaceutical Industries. The concentrations used were prepared by dissolving albendazole tablet in PBS or saline supplemented with 1% dimethylsulfoxide (DMSO) and different concentrations (0.5, 1, 2, 5, 10, 15 & 20µg/mL) were prepared (Tomar and Preet, 2017).

Study design: Muscular larvae obtained from experimentally infected mice were added to Rapid Prototyping and Manufacturing Institute (RPMI)-1640 medium contained 10% fetal calf serum and antibiotics (200U/ml penicillin & 200µg/ml streptomycin). Then about 50µ from larval suspension contained about 100 larvae (counted by light microscope) was added to 1ml from myrrh different concentration of crude (1, 3, 5, & 7 mg/ml), myrrh volatile oils (1, 3, 5, & 7 mg/ml) and albendazole (0.5, 1, 2, 5, 10, 15 & 20µg/ml). Control group consisted of larvae incubated with the equivalent volume of PBS. All experiments were achieved using sterile 96-well microtitre plates. Plates were then sealed and incubated at 37°C in an atmosphere of 5% CO₂ for 1, 4, 24, 48, 72 & 96hr. Tests were performed in 3 replicates. Samples of larvae were taken after 24h of incubation with 7mg/ml of total myrrh extract, 7mg/ml myrrh volatile oil and 20µg/ml of albendazole and processed for electron microscope examination.

EMS: Larvae were washed several time in PBS and then placed in freshly prepared fixative solution of 2.5% glutaraldehyde. Larvae were washed again in PBS for 5 min, post-fixed for 1 hour in a 2% osmium tetroxide in sodium cacodylate buffer. Post fixed larvae were then dehydrated using graded ethanol series (30%, 50%, 70%, 90% & 100%), air dried, mounted, coated with gold and then examined under a Jeol-Jsm-5400 lv SE microscope (Bughdadi, 2013).

In vitro assay of the antiparasitic activity of myrrh crude extract, myrrh volatile oils and albendazole: At the end of each incubation period, larvae (both dead and living) in the wells were collected and counted with a stereo-microscope. Parasite's viability rate was calculated as follows: Viability percent = No of viable parasite/total parasite 100%.

Assessment effects of myrrh crude extracts and myrrh volatile oils compared to albendazole on the infectivity of larvae in experimental animals: *T. spiralis* larvae were treated by sub-lethal dose of myrrh crude and volatile oils of myrrh (dose of 3 mg/ml) to detect their effect on infectivity of muscular larvae in relation to 0.5µ/ml albendazole (Bolás-Fernandez, 2002).

Controls included larvae incubated with the appropriate PBS volume. Larval suspensions were incubated at 37°C and 5% CO₂ for 24hr. Viability of the larvae and its appearance was assessed using a light dissecting microscope. The larvae were collected for oral infecting 6 Swiss female mice, 8 wks old for each treatment and corresponding control group. Five days p.i. 3 infected mice of each group were sacrificed and small intestines removed to recover and count adult worms. After 30 days p.i. the remained 3 mice in each group were sacrificed to reveal muscular larvae and counting them.

Statistical analysis: Data were analyzed by Statistical Package for Social Sciences v.20 for Windows. Chi-square for trend analysis was used to compare the proportion of viable larvae in relation to control. P value of < 0.001 was considered significant.

Result

SEM of *T. spiralis* larvae of control untreated group manifested the characteristic pattern of cuticle with longitudinal ridges and transverse striations (Fig A1). While the cuticle of treated larvae revealed multiple degenerative changes. The cuticle of myrrh crude extracts and albendazole treated larvae showed considerable damage in form of haziness with loss of striation, occurrence of blebs, multiple vesicles and focal sloughing

(Fig 2:B1 & 2 & Fig 3:C1 & 2 respectively). Larvae treated with myrrh essential oil were less affected, only cuticle normal pattern loss in some areas was blunt (Fig 4:D1 & 2).

In the current study, the effect of myrrh crude extract and myrrh volatile oils on the viability of *T. spiralis* muscular larvae in relation to albendazole was evaluated. Crude myrrh extract reduced the viability of *T. spiralis* larvae in time and dose dependent manner, 100% efficacy against *T. spiralis* larvae achieved on the 4th day at concentrations of 3, 5, & 7mg/ml, while at conc. of 1mg/ml total death occurred on the 5th day. There was significant difference ($P < 0.001$) in larvicidal effect in different conc. of crude myrrh extracts treated larvae with reference to untreated control one (Tab. 1). But, essential oil extract of myrrh had relatively lower larvicidal effect compared to crude myrrh extracts effect; larvicidal effect of myrrh essential oil extracts was demonstrated from the 3rd day ongoing even with the highest used conc (7mg/ml). The 100% mortality rate was on the 7th day of different concentrations exposure. So, antiparasitic activity was directly proportional with concentrations and exposure time to the volatile oil extract. Differences between all volatile oil conc., and control group started on 3rd day were highly significant ($P < 0.001$) (Tab. 2).

As to albendazole, larvicidal 100% mortality occurred on the 1st day at conc. of (5, 10, 15 & 20 μ g/ml.), on the 2nd day at 1 μ g/ml and total death of larvae were recorded on

the 3rd day at the lowest used of albendazole (0.5 μ g/ml). Significant ($P < 0.001$) time-dose dependent reduction in larvae viability was observed with albendazole (Tab.3).

In the present study, sublethal dose of myrrh crude extract, essential oil extract and albendazole were used to identify their effects on the muscular larvae infectivity. After larval incubation with definite concentration of each agent for one day, the treated larvae were used to infect mice. The highest rate of inhibition of intestinal phase occurred in 0.5 μ g/ml albendazole that caused 100% inhibition of adult stage followed by 3mg/ml myrrh crude extract producing 98.5% reduction and the lowest inhibition perceived in 3mg/ml myrrh volatile oil (88.47% inhibition rate). There were significant difference between them and control one ($P < 0.001$), also a significant difference between albendazole, myrrh crude extract and myrrh volatile oils ($P < 0.001$).

Concerning inhibitory effect on muscular phase, the 0.5 μ g/ml albendazole caused 100% larval reduction. While 3mg/ml myrrh crude extract caused 98.02% reduction of larval stage. Finally, 3mg/ml myrrh volatile oil produced 59.41% reduction. There were significant differences between all conc. and control group (P value < 0.001), also there were significant difference between albendazole, myrrh crude extract and myrrh volatile oil (P value < 0.001).

The details were given in tables (1, 2 & 3) and figures (1, 2, 3 & 4).

Table 1: Effects of Myrrh Crude Extract on viability of Muscular Larvae of *Trichinella Spiralis*

Dose	1 st hr	4 th hr	1 st day	2 nd day	3 rd day	4 th day	5 th day	6 th day	7 th day	P value
1mg/ml	95	95	40	20.5	15.3	7.5	0	0	0	< 0.001
3mg/ml	95	95	35	17.89	13.68	0	0	0	0	< 0.001
5mg/ml	95	95	29.5	15.5	11.57	0	0	0	0	< 0.001
7mg/ml	95	95	25.6	14.7	11.5	0	0	0	0	< 0.001
Control	95	95	90	85	85	70	50	35.29	20	< 0.001
P value	=1.000	=1.000	=0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	

Table 2: Effects of Myrrh Volatile Oil on viability of Muscular Larvae of *Trichinella Spiralis*

Dose	1 st hr	4 th hr	1 st day	2 nd day	3 rd day	4 th day	5 th day	6 th day	7 th day	P value
1mg/ml	95	95	89.47	76.31	70.52	64.6	50.6	25.5	0	< 0.001
3mg/ml	95	95	88.07	67.3	67.3	60.4	40.7	20.3	0	< 0.001
5mg/ml	95	95	86	65	45.5	33.67	25.8	10.66	0	< 0.001
7mg/ml	95	95	85	65	40.3	29.5	15	7.5	0	< 0.001
Control	95	95	90	85	85	70	50	35.29	20	< 0.001
P value	=1.000	=1.000	=0.648	=0.018	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	

Table 3: Effects of Albendazole on viability of Muscular Larvae of *Trichinella Spiralis*

Dose	1 st hr	4 th hr	1 st day	2 nd day	3 rd day	4 th day	5 th day	6 th day	7 th day	P value
0.5µg/ml	95	85	50	10	0	0	0	0	0	< 0.001
1µg/ml	95	80	30	0	0	0	0	0	0	< 0.001
5µg/ml	95	78.5	0	0	0	0	0	0	0	< 0.001
10µg/ml	95	66.6	0	0	0	0	0	0	0	< 0.001
15µg/ml	95	50	0	0	0	0	0	0	0	< 0.001
20µg/ml	95	45	0	0	0	0	0	0	0	< 0.001
Control	95	95	90	85	85	70	50	35.29	20	< 0.001
P value	=1.000	=0.021	= 0.005	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	

Discussion

The present study showed that crude extracts and volatile oil of myrrh have good anti-parasitic activity against *T. spiralis* larvae in vitro with albendazole as a reference drug. There were no in vitro studies on the effect of myrrh crude extract or its volatile oils on *T. spiralis* muscular larvae. In vitro studies preferred the in vivo ones due to low cost, simplicity and rapid turnover (Zenebe *et al.*, 2017). The current SEM study showed that *T. spiralis* muscular larvae treated with crude myrrh extract caused more extensive cuticle damage than that obtained by its essential oil. The degenerative changes were blebbing, vesicle formation and focal sloughing. The results agreed with Massoud (1999) who showed minute tegumental changes in *S. mansoni* males recovered from experimentally infected mice treated with myrrh volatile oil. Also, Hassan *et al.* (2003) reported that in vitro exposure of *S. mansoni* adults to Mirazid[®] (*Commiphora molmol*) caused tegumental disruption in the form of edematous with severe distraction of the inter-papillary areas and sensory bulbs with loss of spines covering tubercles. Likewise El-Sayed *et al.* (2017) who found marked tegumental damages and loss of *S. mansoni* male spines recovered after in vivo Mirazid[®] treatment of experimentally infected murine model. But, these present results disagreed with Massoud *et al.* (2012) who reported more extensive degeneration of *Fasciola gigantica* adults' cuticle treated with crude myrrh oil than that produced by Mirazid[®] itself. Also, Abdelaal *et al.* (2017) reported that the *in-vitro* *Fasciola hepatica* treated with myrrh, egg production became abnormal with distinct changes in the ovary and Mehlis' gland. These differences may be attributed to difference in my-

rrh dosage, extract preparation methods and/or to *Fasciola* different species.

In the current study, there was significant association between graded concentrations of extracts, exposure time interval and larval stage of *Trichinella* mortality rate. Crude myrrh extract was more effective as larvicidal agent than the volatile oil extracts. This agreed the *in-vitro* studies of Hassan *et al.* (2003), and Bakr *et al.* (2009) who reported a strong antiparasitic activity of Mirazid on *S. mansoni* adults resulted after 24hr exposure. Also, Karamustafa *et al.* (2011) reported lethal effects of Mirazid on *S. mansoni* adults and cercariae. The present result agreed with the analogues study conducted by Ozkoc *et al.* (2009) on the *in-vitro* effect of resveratrol (natural phytoalexin form mainly in grapes) against larvae and adults of *T. spiralis*, and found that resveratrol has antiparasitic activity on larval and adult stages of *T. spiralis* in a dose-time dependent manner.

In the present study, *T. spiralis* larva infectivity was suppressed after exposure to sublethal doses of crude myrrh extracts, myrrh volatile oil and albendazole. The highest inhibition rate of both intestinal and muscular phase was gained with 0.5µg/ml albendazole followed by 3mg/ml of myrrh crude extract and its volatile oil.

Bolás-Fernandez (2002) studied the ability of *Trichinella spiralis* (L₁) muscle larvae survival in different cell culture media and found that infectivity was intensely reduction of larvae incubated in media under 5% CO₂ and microaerobic conditions. Infectivity was completely reserved when larvae were cultured under anaerobic media conditions. But, García-Rodríguez *et al.* (2015) found a strong *in-vitro* activity of *Artemisia absinthium* essential oil against *T. spiralis* larvae

with reduced infectivity between 72 & 100% at doses of 0.5 to 1mg/ml. compared with other parasitic infectivity. Abd El-Ghaffar *et al.* (2018) showed that the *S. mansoni* cercariae infectivity was inhibited after exposure to sublethal concentrations (LC₅₀) of methanol extracts of *Solanum nigrum* and *Callistemon citrinus* leaves and marked reduction in the worm burden in mice as compared with the control.

Conclusion

The outcome data showed that myrrh either as crude extracts or volatile oil has a promising *in-vitro* larvicidal activities against *T. spiralis* larvae. But, albendazole may be used as it's a well-known drug with broad anti-parasitic activity. Myrrh crude extracts fractionations to clarify its active anti-*T. spiralis* efficacy and to explore biological pathways are ongoing and will be published later.

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Explanation of figures

Fig A1: SCM showed normal cuticle of control *T. spiralis* ML. Short arrow (transverse creases) and long arrow (longitudinal ridge)

Fig 2: (B1-B2): SCM of *T. spiralis* larvae in vitro treated with myrrh crude extract showed opacity and degenerative changes of cuticle with loss of striation.

Fig 3: (C1-C2): SCM of *T. spiralis* larvae in vitro treated with albendazole showed destruction and deformity of cuticle.

Fig 4 (D1-D2): SCM of *T. spiralis* larvae in vitro treated with myrrh volatile oils showed mild opacity with loss of striation.

