



**IN VIVO EVALUATION OF ORANGE PEEL OIL AND ITS MAJOR COMPONENT
HESPERIDIN AGAINST ENTERAL PHASE OF *TRICHINELLA SPIRALIS***

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ABSTRACT

Background: Trichinellosis is widespread zoonosis for which no effective drug treatment is available at this time; anthelmintics such as albendazole are commonly used to treat human trichinellosis, although it is not fully effective. This study was carried out to assess, for the first time, the effect of orange secondary metabolites for treatment of this disease. **Objective:** In this study the effect of different orange peel components against enteral phase of *Trichinella spiralis* in mice was evaluated for the first time. This includes: orange oil prepared either by solvent extraction (OIL I), its non-volatile solid residue (OIL IR), or the oil prepared by steam distillation (OIL II) and hesperidin (HSP) **Materials and Methods:** For each fraction two doses 300 mg/kg and 600 mg/kg were investigated in mice. The effectiveness is compared to mice treated with the common commercial drug albendazole and infected non-treated mice. **Results:** The least mean adult count was found in group B treated with albendazole (18.0 ± 4.6) with efficacy of 84.6%. All treated groups with 600 mg/kg showed significant increase in the reduction of adult *T. spiralis* more than the treated groups by 300 mg/kg. The decrease in the mean number of the adult worms was highly significant in all treated groups (*p* value < 0.001) except group E (I) treated with OIL II at a dose of 300 mg/kg was only significant (*p* value < 0.05). **Conclusion:** Orange peel has a significant anti-*Trichinella* activity. This activity is attributed mainly to its content of hesperidin, OIL I and OIL IR.

KEYWORDS: Enteral phase, Hesperidin, Orange oil, Orange peel, *Trichinella spiralis*.

INTRODUCTION

Trichinella spiralis is an important cause of human disease. Its high infectivity for laboratory animals provides valuable *in vivo* models for basic biological, pathological and immunological studies.^[1] Rapid treatment may kill adult worms and thereby stop further worsening of symptoms. Infection is typically treated with albendazole or mebendazole but both medications have been associated with many side effects.^[2-4] In recent years, there has been a growing interest in developing newer anthelmintics from medicinal plants, particularly the ones used in traditional medicine throughout the world.^[5]

Citrus peel is one of the main by-products in fruit industry. However, they are rich source for diversity of active metabolites including essential oils, flavanone glycosides, coumarins, furanocoumarins and polymethoxyflavones.^[6,7] Orange peel mainly contains orange essential oil,^[8,9] hesperidin (HSP) as its main flavanone glycoside,^[10] and polymethoxylated flavonoids.^[6,7] These metabolites are of great importance for their industrial and pharmacological applications.^[10,11]

In traditional medicine orange peel is usually employed for treatment of intestinal parasites. In other parts of the world especially in Central America orange peel decoction is a remedy for treatment of dysentery and other intestinal worms.^[12]

Recently, several experimental studies reported the efficacy of orange peel in the treatment and control of various parasitic diseases. For example, HSP displays anti-parasitic activity against adult worms of the trematode *schistosomiasis mansoni*.^[13-15] Additionally, orange oil emulsion was potentially effective against the ruminant gastrointestinal nematode *Haemonchus controtus*^[16] and has lethal effect on *Trypanosoma evansi* and *Trypanosoma brucei brucei*.^[11,17]

There are many studies which have investigated the effect of different plant extracts against the intestinal phase of *T. spiralis* in experimental animals.^[2,4,18-21] To our knowledge, no data dealing with the action of orange peel extracts investigates its anti-*Trichinella* activity. This initiates our interest to study the effect of different components of orange peel including: orange essential oil prepared by solvent extraction (OIL I), non-volatile solid residue of OIL I (OIL IR), orange oil prepared by

steam distillation (OIL II), orange peel methanolic extract (Met. ext.) and purified HSP against adult worm of *T. spiralis* (enteral phase) in infected mice.

MATERIALS AND METHODS

Experimental animals and parasite

Eighty male parasitic free BALB/c mice (25-30 g) aged six-eight weeks were used. They were obtained from the animal house, Faculty of Medicine, Assiut University, Assiut, Egypt, and maintained under standard environmental conditions and rodent diet. Seventy of them were infected orally with about 300 larvae per mouse while the remaining ten mice were kept as the non-infected non-treated control group. The strain of *T. spiralis* used was originally isolated from the diaphragms of infected pigs obtained from El Bassatine Abattoir, Cairo. It had been routinely maintained in the laboratory of the Faculty of Medicine, Assiut University, by consecutive passage through BALB/c mice. Briefly, the heavily infected diaphragms of the pigs were minced and digested in 1% pepsin-hydrochloride. After overnight incubation at 37 °C, larvae were collected using the sedimentation technique, washed in physiological saline (0.85%) several times, and the number of larvae per ml was counted.^[22,23]

Reference drug

Albendazole was supplied as suspension, 20 mg/ml, from the Egyptian International Pharmaceutical Industries Co.

Plant materials and preparation of different orange peel fractions

Orange fruits (*Citrus sinensis*) were purchased from local market and peeled using knife. The obtained orange peel was air dried and grinded well. 100 g of the dried powder were extracted by dichloromethane using Soxhlet apparatus to get oil, fats and non-polar compounds. The Soxhlet was allowed to return its cycle for three times each one took five hours. The resulting extract was concentrated under vacuum to yield oil prepared by solvent extraction (OIL I, 2 g.). Oil I was obtained as reddish brown liquid (9 ml) with a strong orange odor. To study the non-volatile solid residue of OIL I, the oil was totally evaporated and the remaining non-volatile solid residue was labeled as (OIL IR, 200 mg). The defatted powder was further extracted by methanol and filtrated. The filtrate was collected and concentrated under vacuum to dryness to yield methanolic extract (met. ext., 110 g) (Figure 1).

Precipitation and Crystallization of crude Hesperidin

HSP was isolated and purified as previously reported.^[24] Crude HSP (5.5 g) was precipitated from met. ext. using 6% aqueous acetic acid. Crystallization using a mixture of DMSO and distilled water at (60-80 oC) yielded HSP as white crystals (HSP, 2.9 g). The product was analyzed and characterized by spectroscopic methods (1H NMR and 13C NMR). 1H NMR examination of isolated compound showed (DMSO-*d*₆, 400 MHz) δ : 12.01 (1H, *br s*, 5-OH), 9.09 (1H, *br s*, 3' OH), 6.94 (1H, *br. s*, H-

2'), 6.93 (1H, *d*, *J* = 8.0 Hz, H-5'), 6.9 (1H, *d*, *J* = 8.0 Hz, H-6'), 6.15 (1H, *d*, *J* = 2.0 Hz, H-6), 6.13 (1H, *d*, *J* = 2.0 Hz, H-8), 5.53-5.49 (1H, *dd*, *J* = 11.0, 3.0 Hz, H-2), 4.98 (1H, *d*, *J* = 7.2 Hz, H-1'''), 4.52 (1H, *s*, H-1''''), 3.78 (3H, *s*, 4'-OCH₃), 3.10–3.55 (10 H, *m*, H-2'' to H-6''), 3.20–3.60 (3H, *m*, H-2''' to H-5'''), 3.11 (1H, *dd*, *J* = 17.0, 11.0 Hz, H-3a), 2.80-2.77 (1H, *dd*, *J* = 17.0, 3.0 Hz, H-3b), 1.09 (3H, *d*, *J* = 6.0 Hz, H-6'''). 13C NMR (DMSO-*d*₆, 100 MHz) δ : 197.46 (C-4), 165.56 (C-7), 163.47 (C-5), 162.92 (C-9), 148.42 (C-4'), 146.86 (C-3'), 131.29 (C-1'), 118.46 (C-6'), 114.55 (C-2'), 112.45 (C-5'), 103.77 (C-10), 101.00 (C-1''), 99.89 (C-1'''), 96.84 (C-6), 96.02 (C-8), 78.87 (C-2), 76.67 (C-5''), 75.95 (C-3''), 73.41 (C-2''), 72.52 (C-4''), 72.13 (C-4'''), 70.71 (C-3'''), 70.05 (C-2'''), 68.76 (C-5'''), 66.44 (C-6''), 56.12 (4'-OCH₃), 42.45 (C-3), 18.24 (C-6''') (Figure 2).

Preparation of orange oil by steam distillation (OIL II)

4 kg of orange fruits were peeled off carefully with the help of knife. The white flesh under the rind was removed. The rind (500 g fresh sample) was cut into smaller pieces and rinsed with distilled water. The oil was extracted using hydro-distillation method for 8 hours using Clevenger-type apparatus. The resulting oil was isolated as a colorless liquid (17.5 ml oil), dried over anhydrous Na₂SO₄, and reserved at 4 oC until analysis.

GC/MS analysis of orange oil

The analysis of the oil samples was performed using GC/MS by injection into a DB-5 column (Agilent; 30 m length, 0.25 mm internal diameter, 0.25 μ m film thickness) on a 7890N gas chromatograph coupled to a 5975B mass spectrometer using conditions previously reported.^[25]

LC-ESI-MS analysis of non-volatile solid residue of oil prepared by solvent extraction (OIL IR)

The HPLC-ESI-MS analysis of OIL IR was performed with a Waters Corporation, Milford, MA01757 U.S.A., mass spectrometer. ESI-MS positive ion acquisition mode was carried out on a XEVO TQD triple quadrupole instrument. Sample was dissolved in acetonitrile and analyzed using: ACQUITY UPLC-BEH reversed phase C18 column (1.7 μ m particle size, 2.1 mm \times 50 mm i.d.). The mobile phase consists of linear gradient of eluent A (H₂O acidified with 0.1% formic acid) and eluent B (MeOH acidified with 0.1% formic acid). The gradient was applied starting from (90:10, v/v) to (10:90, v/v) in 25.0 min. and from (10:90, v/v) to (90:10 v/v) in 5 min. at flow rate of 0.2 ml/min.

Study design

Groups of animals

To evaluate the anthelmintic activity of the tested compounds, infected mice were classified into seven main groups; A, B, C, D, E, F and G of ten animals each. Group A was infected untreated as a control group. Group B received albendazole (as a reference drug) while groups C, D, E, F and G received oral doses of

OIL IR, OIL I, OIL II, met. ext. and HSP respectively. The efficacy of the tested components against the adult worms was assessed by direct count of surviving worms. Taking the worm burden of infected control animals as the reference, the percentage worm reduction was determined to compare the plant extract efficacy.^[4,26]

Dose schedule

Each main group of the treated animals C, D, E, F and G was subdivided into two subgroups (I and II), each subgroup having 5 mice. Animals of subgroup I received 300 mg/kg of OIL IR, OIL I, OIL II, met. ext. and HSP respectively, while animals of subgroup II received a dose of 600 mg/kg for the same extracts starting from the third day post-infection (dpi) for three successive days.^[27] While animals of group B received 50 mg/kg albendazole. To assess the efficacy of the extracts on adult worms (intestinal phase), all animals were sacrificed on the seventh dpi. The number of adult worms in the gut were isolated and counted following the method previously described.^[4,28] Briefly, the intestine was opened, washed and then incubated in 10 ml saline at 37°C for 2 hr to allow worms to leave the intestine to the container. Washing was done several times till the fluid become clear. Then, the fluid was collected and centrifuged at 1,500 rpm for 5 min. The supernatant was decanted and the sediment was reconstituted in a few drops of saline to be examined drop by drop under the dissecting microscope for counting the adults.

Statistical analysis

The collected data were analyzed by Statistical Package for Social Sciences v.20 for Windows. All values were expressed as mean \pm standard deviation. The significance of differences between the groups was calculated using the ANOVA test. The percentage of reduction was calculated between the treated groups and the control groups. The significance of the differences between experimental and control groups were calculated using Student's *t* test. A *p* value of < 0.05 vs control was considered statistically significant (*). A *p* value of < 0.001 vs control was considered statistically highly significant (**). The significance of using two different concentrations was calculated using Student's *t* test.

Ethical consideration

The experimental animal studies were conducted in accordance with the international valid guidelines approved by the Institutional Animal Care and Use Committee and were maintained under convenient conditions at the Animal House, Faculty of Medicine, Assiut University.

RESULTS

Preparation of hesperidin (HSP) and orange oils (OIL I and OIL II)

Orange peel is known for its diversity of active constituents. The epicarp of the fruits that represents the most outer layer of the rind is characterized by the presence of oil glands. These glands are responsible for

the production of orange essential oil. On the other hand the internal spongy white layer (mesocarp) is characterized by the presence of pectin, flavanone glycosides and polymethoxyflavones where HSP is the major flavanone glycoside. To study the effect of these different metabolites on trichinellosis of infected mice, various fractions were prepared. At first, orange oil was prepared by two different ways either by solvent extraction using dichloromethane as a solvent giving OIL I or by steam distillation giving OIL II as described in the experimental part and illustrated in Figure 1. OIL I was obtained as a reddish brown oily liquid with strong orange odor. Upon complete evaporation of the oil, non-volatile solid residue was precipitated. This residue was labeled as non-volatile solid residue of OIL I (OIL IR). However, OIL II was obtained as a colorless liquid with faint orange odor. The composition of OIL I and OIL II was established by GC/MS analysis of both samples while the composition of OIL IR was studied by LC-ESI-MS analysis as described in the following sections. Additionally, the met. ext. was prepared. Met ext. contains the more polar metabolites mainly HSP that was isolated and purified as prescribed in experimental part. HSP was isolated and purified and its structure was identified by spectroscopic analysis (¹H NMR and ¹³C NMR).

Analysis of orange oil by GC/MS

Both OIL I and OIL II was subjected to GC/MS analysis to analyze their major volatile constituents. The major five identified components of OIL I and OIL II, their retention time (tR) and their relative mass fractions are presented in table 1. Limonene represents the major component in both OIL I and OIL II. However, its percentage in OIL II is about 35% which is almost twofold its amount in OIL I that was found to be 65%. Other components identified in case of OIL I are: β -myrcene (2.5%), linalool (2%), valencene (2%) and citronellol (0.7%). For OIL II the other main components are β -myrcene (10%), linalool (4%), α -pinene (3%) and sabinene (1.5%) (Table 1).

LC/MS analysis of non-volatile residue of OIL I (OIL IR)

OIL I is expected to contain other metabolites beside the detected volatile constituents. Consequently, OIL IR that represents the non-volatile solid residue of OIL I was prepared and subjected to HPLC-ESI-MS analysis as illustrated in the experimental part. Five major peaks were detected in the total ion current (TIC) chromatogram of the tested sample. The tR, the detected molecular ion peak [M+H]⁺, as well as the expected structures based on the comparison with literatures^[6,29-31] are shown in table 2. Based on comparing the resulting molecular ion peak for each of the five detected peaks with literature they were identified as polymethoxyflavonoids including: two isomers of pentamethoxyflavones with molecular ion peak at [M+H]⁺ 373 with retention time tR at 15.5 min, 17.2 min. respectively; two isomers of heptamethoxyflavones

with molecular ion peak at $[M+H]^+$ 434 showing tR at 16.4 min, 16.6 min. respectively. In addition to hexamethoxyflavone (tR 15.8 min) with molecular ion peak at $[M+H]^+$ 403.

Effect of orange oils and hesperidin on enteral phase of *Trichnella spiralis*

All treated groups with 600 mg/kg showed significant increase in the reduction of adult *T. spiralis* more than the treated groups by 300 mg/kg. The decrease in the mean number of the adult worms was highly significant in all treated groups (p value < 0.001) except group E (I) treated with orange oil II at a dose of 300 mg/kg was only significant (p value < 0.05).

The least mean adult count was found in group B which was treated with albendazole (18.0 ± 4.6) with efficacy

of 84.6% followed by group C (II) treated with OIL IR at a dose of 600 mg/kg (18.5 ± 1.7) with efficacy with 84.2% followed by group G(II) treated with HSP at a dose of 600 mg/kg (19.3 ± 3.0) with efficacy of 83.6% then group D (II) treated with OIL I at a dose of 600 mg/kg (22.5 ± 2.9) with efficacy of 80.8%. The least reduction in the number of adult worms was observed in group E (I) treated with OIL II at a dose of 300 mg/kg with efficacy of 46.8% (Table 3).

A p value of < 0.05 for 600 mg vs 300 mg for the same fraction was considered significant only for OIL IR, orange OIL I and HSP. However, using higher concentrations for orange OIL II and met. ext. was not significantly different.

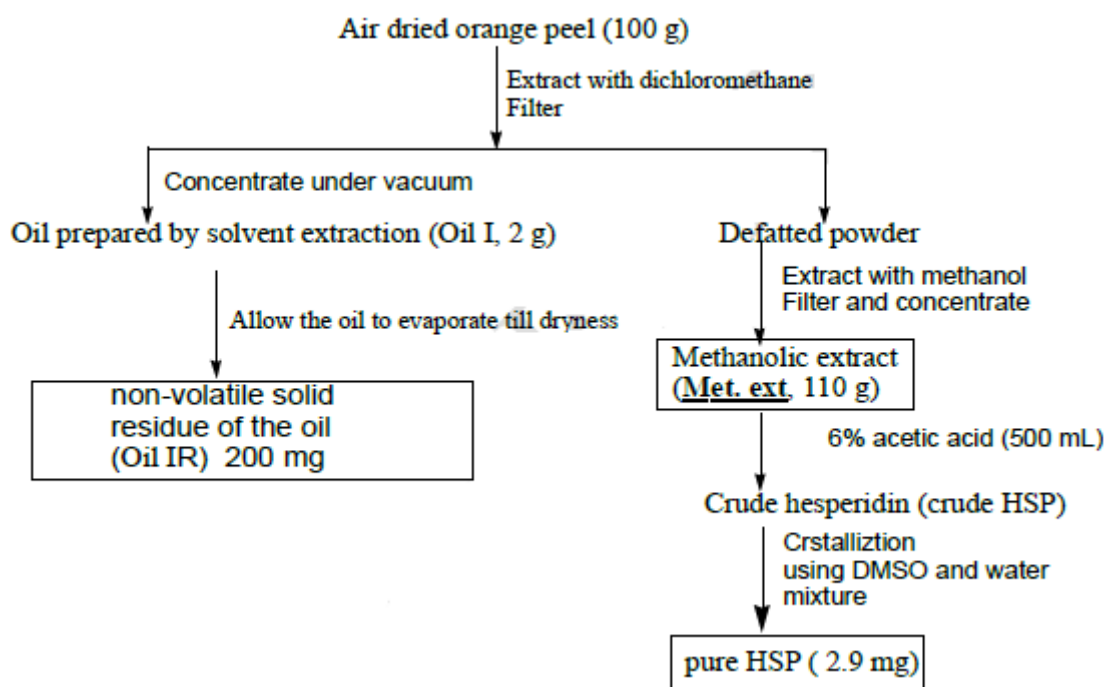


Figure 1: Flow chart for extraction and preparation of different fractions of orange peel: oil prepared by solvent extraction (OIL I), non-volatile solid residue of OIL I (OIL IR), methanolic extract (Met. ext.) and pure hesperidin (HSP).

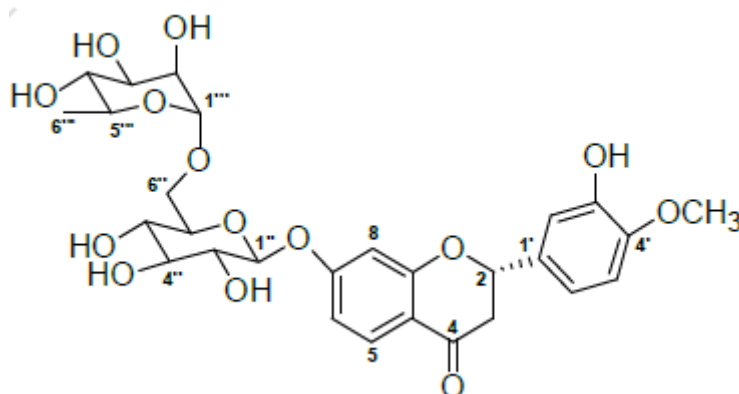


Figure 2: Chemical structure of hesperidin (HSP).

Table 1: The five major components of orange volatile oils prepared either by solvent extraction (OIL I) or steam distillation (OIL II). Each component is expressed as percentage of its mass fraction and its retention time (t_R).

Major components for Oil I	tR (min.)	Percentage (%)	Major components for Oil II	tR (min.)	Percentage (%)
Limonene	13.6	35	Limonene	13.6	65
β -Myrcene	12.7	2.5	β -Myrcene	12.7	10
Linalool	14.6	2	linalool	14.6	4
Valencene	21.5	2	α -pinene	11.8	3
Citronellol	16.6	0.7	sabinene	12.5	1.5

Table 2: Major common peaks detected by HPLC-ESI-MS analysis in non-volatile solid residue of oil prepared by solvent extraction (OIL IR).

Compound no.	tR (min.)	Detected molecular weight in positive mode [M+H] ⁺	Expected compound
1	15.5	373	Pentamethoxyflavone
2	15.8	403	Hexamethoxyflavone isomer
3	16.4	434	Heptamethoxyflavone isomer
4	16.6	434	Heptamethoxyflavone isomer
5	17.2	373	Pentamethoxyflavone isomer

Table 3: Efficacy (as percentage of reduction) of the studied orange peel fractions and albendazole against adult worms of *Trichinella spiralis*.

Animal group	Subgroup I (300 mg/Kg) for the studied fractions		Subgroup II (600 mg/Kg) for the studied fractions	
	No. of worms/mouse (Mean \pm S.D.)	Efficacy (% of reduction)	No. of worms/mouse (Mean \pm S.D.)	Efficacy (% of reduction)
Infected non-treated (A)	117.5 \pm 17.1		0	
Albendazole in dose 50 mg/kg (B)	18.0 \pm 4.6**		84.6	
OIL IR (C)	32.5 \pm 6.0**	72.3	18.5 \pm 1.7**	84.2
OIL I (D)	40.0 \pm 5.3**	65.9	22.5 \pm 2.9**	80.8
OIL II (E)	62.5 \pm 14.4*	46.8	55.5 \pm 5.8**	52.7
Methanolic extract (F)	26.0 \pm 3.4**	77.8	28.3 \pm 8.5**	75.9
Hesperidine(G)	37.8 \pm 11.6**	67.8	19.3 \pm 3.0**	83.5

* $P < 0.001$ versus control considered significant, ** $P < 0.001$ versus control considered highly significant.

DISCUSSION

Recently plant-based traditional medicine has received considerable attention for the discovery and development of finding some alternative drugs for an effective management of trichinellosis.^[5,21] Traditional treatment of the parasitic infestations has their drawbacks. Many drugs are ineffective at the recommended dose, beside the incomplete elimination of the parasites and the development of resistant parasite strains against drugs. Many studies reported that anti-*Trichinella* drugs are insufficient.^[32]

There are many studies that investigated the effect of different plant extracts against *T. spiralis* in experimental animals.^[2,18,34] In related studies, as the study done by Shalaby et al. 2010 had shown that methanolic extract of *Balanites aegyptiaca* fruits has high efficacy against parental stages of *T. spiralis*.^[21] The study by Basyoni and El-Sabaa, 2013 revealed that myrrh was effective and could be a promising drug against the Egyptian strains of *T. spiralis* with results nearly comparable to ivermectin,^[35] also the study done by Attia et al., 2015

assessed the effectiveness of myrrh and thyme extracts against different stages of *T. spiralis* in mice compared with the common commercial drug albendazole.^[20]

Orange peel is a good source of various secondary metabolites that have several pharmacological actions such as antibacterial, antifungal, antiparasitic and insecticidal activities. It has been used traditionally to treat many medicinal conditions.^[11,36] However, to our knowledge, no data dealing with the action of orange extracts investigates its anti-*Trichinella* activity. Therefore, the current research was carried out to assess, for the first time, the effect of orange oil prepared by solvent extraction (OIL I) and its non-volatile solid residue (OIL IR), orange oil prepared by steam distillation (OIL II), met. ext. and HSP, the main flavanone glycosides of orange peel against adult stage of *T. spiralis* in mice. Their effectiveness is compared to mice treated with the common commercial drug albendazole and infected non-treated mice. For each fraction two doses 300 mg/kg and 600 mg/kg were investigated.

As it is known, following infection, the encysted larvae of *T. spiralis* become adults in the gut of the host within 28-36 h.^[37] Therefore, the experimental animals were treated on the third day of infection and for three successive days post-infection to assess the efficacy of the extracts against the adult stage of the parasite. Once the *Trichinella* larvae have become encysted in muscle tissues, the therapeutic intervention is generally less feasible.^[38] Consequently, the elimination of the intestinal form is significant for early and successful therapy which is the main target of anthelmintics applied during the first three days after infection.^[33]

In the present study, albendazole-treated mice were given 50 mg/kg for three successive days starting from the third day of infection, which revealed a significant reduction (84.6%) in the number of adult worms. Similar efficacies of albendazole were detected by previous studies.^[19,20,39,40] In other studies, albendazole treatment resulted in complete eradication of *T. spiralis* worms in albino rats.^[21,41] The differences in the efficacy of albendazole against the intestinal stage depend on the time, dose, and duration of treatment.^[40] However, albendazole had low water solubility limiting its oral absorption and bioavailability. Albendazole treatment schemes are suitable for adults and children; however, they are contraindicated during pregnancy and are not recommended in children less than two years old.^[42,43]

The present results showed highly significant decrease in the mean number of the adult worms in all treated groups except group E (I) treated with OIL II at a dose of 300 mg/kg that was only significant. Interestingly, the effectiveness of some orange peel components: OIL IR, HSP and orange OIL I in doses of 600 mg/kg against enteral stage of *T. spiralis* was nearly similar to that of albendazole.

Mice groups treated either by OIL I or OIL II produced a significant decrease in the mean number of adult worms. However, Group treated with OIL I at 600 mg/kg showed efficacy of 80% while group treated with OIL II at the same concentration showed efficacy of 50%. This suggests that the antiparastic activity of orange oil is not directly related to their content of limonene and it might be related to other components. As shown from GC/MS analysis limonene is the major component of both oils. However, the percentage of limonene in OIL II is about 65% which is approximately double fold its percentage in OIL I that is found to be 35% (Table 1). These results indicate that the activity of OIL I might be attributed to the presence of other non-volatile constituents that is dissolved in the oil prepared by this way. For more understanding the nature of these compounds, the non-volatile solid residue of OIL I was prepared (OIL IR) and analyzed by LC-ESI-MS. The analysis showed the presence of five major compounds. Based on their molecular ion peaks and comparing with literatures,^[6,29,30,31] these compounds were identified as polymethoxylated flavonoids: pentamethoxyflavone (2

isomers), heptamethoxyflavone (2 isomers) and hexamethoxyflavone (Table 2). Further work to isolate and study the anti-*Trichinella* activity of these compounds is going on.

CONCLUSION

Our study established for the first time that orange peel could be promising natural source against the enteral phase of *T. spiralis*. This activity is attributed mainly to its major flavanone glycoside HSP, orange oil prepared by solvent extraction (OIL I) and its non-volatile solid residue (OIL IR). The effectiveness of orange oil might be attributed to their content of polymethoxylated flavonoids as established from LC-ESI-MS analysis. Our result provides a base for further exploration of this fruit to achieve an alternative, safe and effective natural compound to treat trichinellosis in man. Therefore, further work to isolate and test the effect of the pure compounds from orange peel against different stages of *T. spiralis* in experimental models is going on.

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