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RESEARCH ARTICLE

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EFFICACY OF MANDARIN (*CITRUS RETICULATA*) PEEL EXTRACT IN THE CONTROL OF *SCHISTOSOMA MANSONI* LARVAL STAGES AND THEIR INTERMEDIATE HOSTS

ABSTRACT:

Little information is known about using citrus plants in the eradication of snails and infective stages of *Schistosoma mansoni*. The present study was conducted to evaluate the effect of mandarin (*Citrus reticulata*) peel extract on *Biomphalaria alexandrina* snails and *S. mansoni* cercariae as well as the ability of mandarin peel extract-attenuated cercariae to infect mice with schistosomiasis. The molluscicidal effect of various concentrations of air-dried mandarin peel extract was increased by increasing exposure time, and the more effective concentration was 500 ppm. This concentration led to 35, 60, 95 and 95 % mortality after 24, 48, 72 and 96 hours, respectively. Exposure of *S. mansoni* cercariae to ascending concentrations of mandarin peel extract (1000-2500 ppm) for different time intervals (15-120 min) displayed a significant level of cercaricidal potential in a concentration-time relationship pattern. Attenuation of schistosomal cercariae using 1/10 LC_{50} of mandarin peel extract for different time periods (30-150 min) impaired the cercarial ability to infect mice, even for a short time. In addition, the relative spleen and liver weights of mice exposed to mandarin peel extract-attenuated *S. mansoni* cercariae were significantly reduced compared to those of the non-attenuated infected control mice. Taken all together, it can be concluded that air-dried mandarin peel extract may be considered as a promising candidate for the control of *S. mansoni* transmission as it has molluscicidal and cercaricidal activities. These effects may be due to active flavonoids and other constituents that cause death and attenuate the ability of cercariae to infect the final host.

KEY WORDS:

Biomphalaria alexandrina, cercariae, *Citrus reticulate*, flavonoids, *Schistosoma mansoni*.

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INTRODUCTION:

Schistosomiasis, a chronic and debilitating parasite disease, affects approximately 200 million people in the developing world causing tremendous loss of national economy and manpower, despite the continuous control efforts (Wang *et al.,* 2004). In addition to the treatment of the infected individuals with anti-schistosomal drugs such as praziquantel, snail and cercarial control using synthetic products has been one of the methods of choice to reduce the risk of schistosomiasis transmission (WHO, 1993). However, there is a renewed interest in finding natural products that could be cheaper and locally available alternative to synthetic products used in the control of schistosomiasis snails and cercariae.

Citrus plants (Family: Rutaceae) are well known for their pharmacological and economic importance. They are rich in flavonoids, and the pharmacological effects of flavonoids are mainly due to their antioxidant activity and their inhibition of certain enzymes (Amic *et al.,* 2007; Moein *et al.,* 2008). Recent studies point to a possible protective effect of citrus flavonoids against inflammation and cardiovascular diseases (Benavente-Garcia and Castillo, 2008) as well as leishmanial activity (Quintin *et al.,* 2009). Citrus peel contains a variety of essential oils that inhibit the growth of or kill pathogenic bacteria (Nannapaneni *et al.,* 2008). Effective anticancer and anti-corpulence compounds are also isolated from the peel of citrus plants (Hirata *et al.,* 2009; Purushotham *et al.,* 2009).

Regarding schistosomiasis, El-Lakkany *et al.* (2004) and Hamed and Hetta, (2005) studied the anti-schistosomal effects of grapefruit juice and mandarin root extracts in mice. They found that these extracts improved hepatic enzyme activities with notable reduction in ova count and worm burden, as well as enhanced degeneration of eggs and accelerated healing of the pathological granulomatous lesions. Other flavonoid-

containing plants were studied for their molluscicidal, cercaricidal, and schistosomicidal activities. Topical application of a chloroform extract of *Millettia thonningii* seeds to mouse skin appeared to be effective in preventing subsequent infection with *S. mansoni* cercariae (Perrett *et al.,* 1995). Robustic acid and alpinumisoflavone mixture from the dichloromethane extract of *M. thonningii* seeds also killed *S. mansoni* cercariae and adult schistosomes *in vitro* (Lyddiard *et al.,* 2002). Chloroform extract of *Iris germanica* rhizomes showed significant molluscicidal activity against *B. alexandrina* snail, and *S. mansoni* cercaricidal potential in a time- and concentration-relationship (Singab *et al.,* 2006).

Although the molluscicidal and cercaricidal potencies of many plants were extensively studied allover the world (de S'Luna *et al.,* 2005; Goel *et al.,* 2007), a little considerable attention was given to citrus plants. Therefore, the present study was conducted to evaluate the effect of mandarin (*Citrus reticulata*) peel extract on *Biomphalaria alexandrina* snails and *S. mansoni* cercariae, as well as the ability of mandarin peel extract-attenuated cercariae to infect mice with schistosomiasis.

MATERIAL AND METHODS:

Mandarin peel extraction:

Mandarin (*Citrus reticulata)* fruits were purchased from a local market of Tanta city, Egypt. Fresh, oven-dried and air-dried peels were carefully blended, and standard stock extracts were freshly prepared by soaking the mandarin peels in dechlorinated tap water (1g/1l, 1000 ppm) for 24 hours at room temperature. The peel extracts were filtered and water was added to the filtrate to adjust the final volume. Stock extracts were kept in refrigerator and renewed weekly to avoid deterioration. Experimental concentrations were prepared from the stock solution by further dilutions.

Experimental animals:

Laboratory bred *S. mansoni*-infected *B. alexandrina* snails; cercariae of *S. mansoni* and adult male Swiss albino mice (CD1 strain) were purchased from the Schistosome Biological Supply Program, Theodore Bilharz Research Institute, Cairo, Egypt. Snails were acclimatized to the laboratory conditions for at least one month before experimentation. They were maintained in 30 liter-plastic aquaria under constant and continuous aeration at the room temperature. A diet of fresh lettuce leaves was supplied *ad libitum.* The cercariae were collected from *S. mansoni* infected snails according the method described by Christensen *et al*. (1984). A concentrated suspension of freshly shed cercariae was prepared and adjusted with dechlorinated tap water to about 200 cercariae/ml. Mice (weighing 20 g in average at the beginning of the experiments) were kept under normal laboratory conditions and provided with free access of water and standard pellet diet for two weeks before infection with *S. mansoni*.

Determination of molluscicidal activity of mandarin peel extracts:

B. alexandrina snails were maintained in 2 liter-plastic containers by a capacity of 20 snails/box. Snails were exposed to graded concentrations of mandarin peel extract in dechlorinated tap water under pH 7.2 and room temperature of 23 ± 2°C. The number of dead snails was recorded at 12 hours intervals over a period ranged from 24-96 hours for 3 times. The values of the lethal concentrations (LC_{10}, LC_{50}, and LC_{90}) for each extract were calculated from the linear equations produced from the mortality percentage against tested concentrations after a particular time period, or from the statistical Probit analysis according to the method of Finney (1971).

Determination of cercaricidal activity of mandarin peel extracts:

To determine the cercaricidal activity of mandarin peel extracts, about 20-30 freshly shed cercariae of *S. mansoni* were exposed to graded concentrations of mandarin peel extracts in small Petri dishes for 2 hours. At 15 minutes intervals, the number of dead or immobilized cercariae was counted using a dark field-dissecting microscope. In each case, the percentage of the mortality rates of cercariae was determined. The values of the lethal concentrations (LC_{10}, LC_{50}, and LC_{90}) were calculated using the linear equations.

Testing the ability of mandarin peel extract-attenuated cercariae to infect mice:

Cercariae were attenuated with 1/10 cercarial LC_{50} of mandarin peel extract for 30, 60, 90, 120, and 180 min before infection. Five groups of mice (n=10) were individually exposed to the attenuated cercariae (200 cercariae/mouse) for two hours using tail immersion method as previously described (Greene *et al*., 1983). Mice of the control group were individually exposed to 200 cercariae in dechlorinated tap water. All mice were housed in the laboratory for consecutive 8 weeks to attain a complete infection. At the end of experiments, mice were individually weighed, sacrificed and dissected. For the worm count, the portal blood vessels of the dissected animals were perfused with buffered saline solution as described by Christensen *et al*. (1984), and the number of dead and live worms recovered from each mouse was calculated. For the egg count, all viable eggs in the liver tissue were counted and classified according to the criteria previously described by Pellegrino *et al*. (1962). Haemobiotic

organs including spleen and liver were excised, removed out and weighed. The relative weight of each organ was determined according to the following formula: organ's relative weight = organ's weight (g)/ body weight (g) X 100

RESULTS:

Molluscicidal activity of mandarin peel extracts:

Figure 1 shows the effect of fresh, oven-dried and air-dried mandarin peel extracts (1000 ppm) on the mortality percentage of *B. alexandrina* snails at different time intervals. Air-dried mandarin peel extract showed a maximum mortality after 48 hours. The time period required for 50 % mortality was 35 hours of snails exposed to such peel extract.

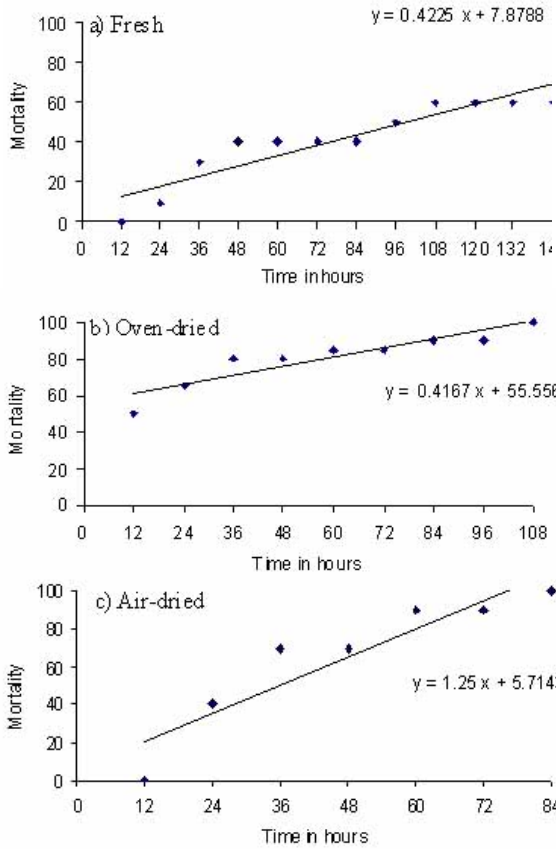


Fig. 1. Correlation between exposure time and mortality percentage of a) fresh, b) oven-dried and c) air-dried mandarin peel extract (1000 ppm) against *B. alexandrina* snails.

Figure 2 shows the effect of various concentrations of air-dried mandarin peel extract on the mortality percentage of *B. alexandrina* snails at different time intervals. After 24 hours, it gave 35 % mortality of *B. alexandrina* by applying 500 ppm of air-dried mandarin water peel extract (Fig. 2a). After 48 hours, it gave 60 % mortality with the same concentration, 500 ppm (Fig. 2b); the LC_{50} was calculated as 727.78 ppm after the same period of exposure. After 72 hours, the mortality pecentage was increased with all concentrations used of air-dried mandarin peel extract (Fig. 2c). The maximum mortality percentage was 95 % with 500 ppm, the LC_{50} value was calculated as 163.79 ppm. The same effects were resulted after 96 hours (Fig. 2d), where 95 % mortality was observed with 500 ppm, the value of LC_{50} was calculated as 107.54 ppm.

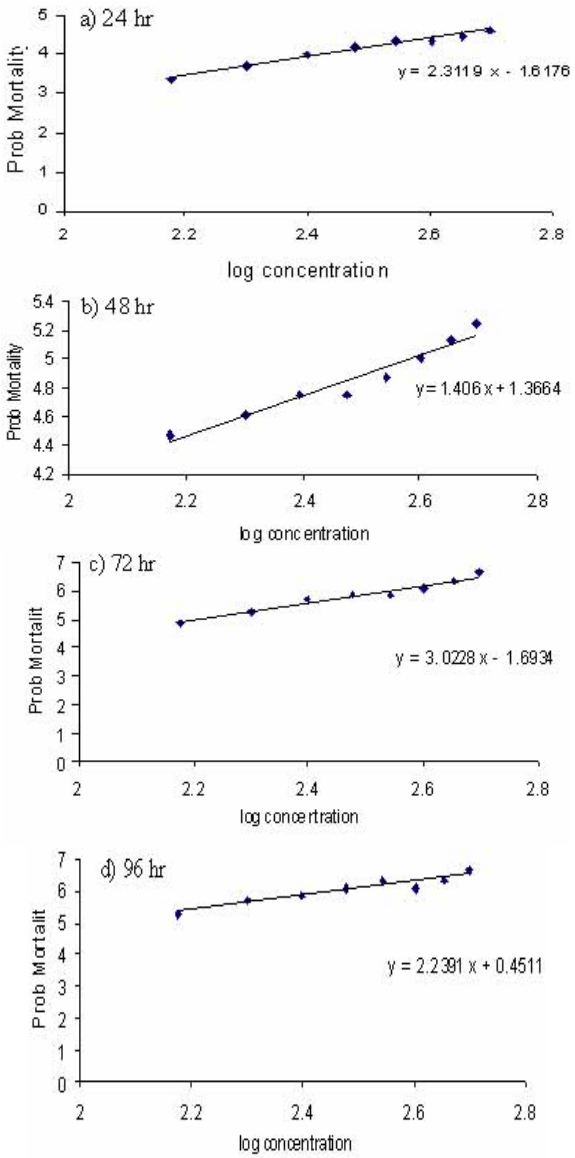
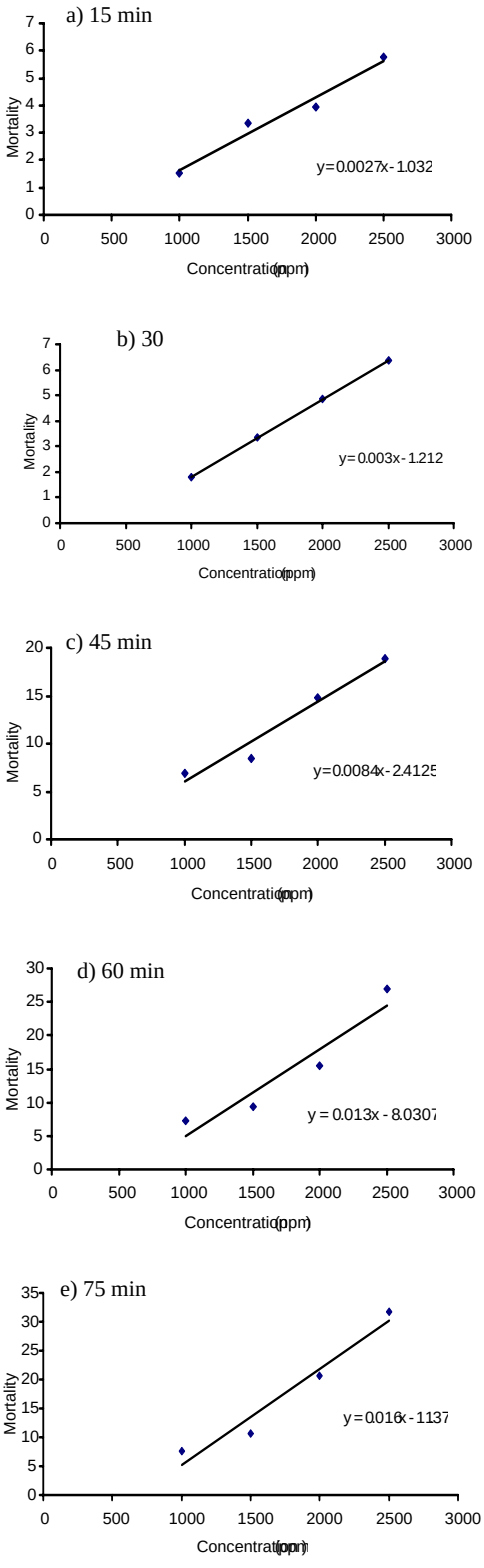


Fig. 2. Correlation between different concentrations of air-dried mandarin peel extracts and the mortality percentage of *B. alexandrina* snails after (a) 24 hr, (b) 48 hr, (c) 72 hr, and (d) 96 hr of exposure.

Cercaricidal activity of mandarin peel extracts:

To study the cercaricidal activity of air-dried mandarin peel extract, healthy *S. mansoni* cercariae were exposed to ascending concentrations of mandarin peel extract (1000-2500 ppm) for 15, 30, 45, 60, 75, 90, 105, and 120 minutes. Accordingly, linear relationships were existed between the cercarial mortality and the ascending concentrations of mandarin peel extract after exposure time intervals (15-120 minutes). No cercaricidal effect was observed

before applying 1000 ppm of the peel extract at all exposure time periods. The mortality percentage of cercariae was increased at each time interval by increasing the concentration of mandarin peel extract. At each time period, the mortality percentage of the exposed cercariae was concentration-dependent. (Fig. 3a-h).



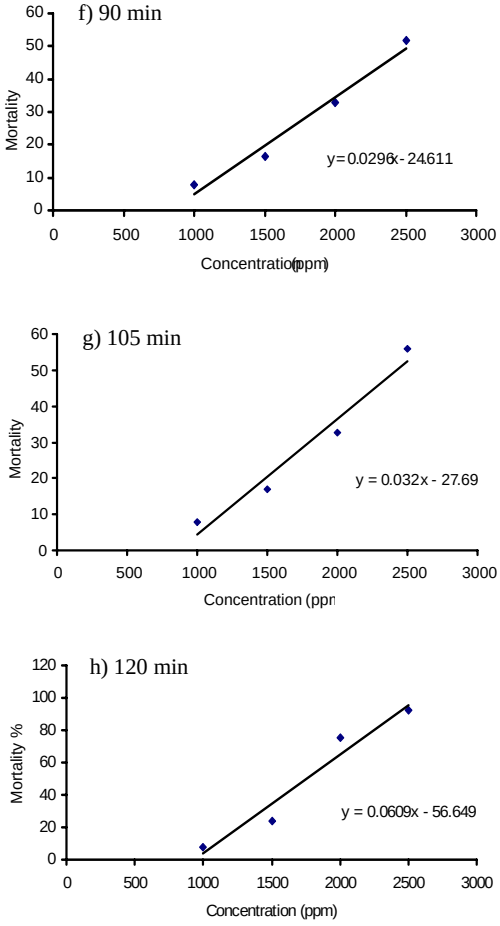


Fig. 3. Correlation between mortality percentage of *S. mansoni* cercariae and different concentrations of air-dried mandarin peel extract after (a) 15 min, (b) 30 min, (c) 45 min, (d) 60 min, (e) 75 min, (f) 90 min, (g) 105 min and (h) 120 min of exposure.

The values of lethal concentrations (LC_{10}, LC_{50}, and LC_{90}) of air-dried mandarin peel extract were calculated from the produced linear equation (Table 1). By increasing the exposure time from 15 to 120 minutes, the value of LC_{10} was reduced from 4085 to 1094 ppm, the value of LC_{50} was reduced from 18900 to 1751 ppm, and the value of LC_{90} was greatly reduced from 33714 to 2408 ppm.

Table 1. The effect of lethal and sublethal concentrations of air-dried mandarin peel extract on *S. mansoni* cercariae at different time intervals

Exposure time	Lethal concentration		
	LC_{10}	LC_{50}	LC_{90}
15 min	4085	18900	33714
30 min	3737	17070	30404
45 min	1477	6240	11001
60 min	1386	4464	7540
75 min	1285	3695	6104
90 min	1163	2519	3874
105 min	1177	2428	3677
120 min	1094	1751	2408

Ability of attenuated cercariae with mandarin peel extract to infect mice with *S. mansoni*:

To test the potency of mandarin peel extract on the ability of *S. mansoni* cercariae to infect mice, 1/10 LC_{50} was used to attenuate cercariae for 30, 60, 90, 120, and 150 minutes, respectively. As shown in table 2, the mean number of worms of control mice exposed to cercarial suspension (200 cercariae) reached 47.50 ± 6.74, and the mean egg count in the liver was 78.1, of which 30.35 eggs (38.9 %) were found alive and 47.75 eggs (61.1%) were dead. Attenuation of cercariae with 1/10 LC_{50} of mandarin peel extract for 30, 60, 90, 120, and 150 minutes led to the complete weakness of these cercariae to infect mice with *S. mansoni*. In this case, both worm and egg counts recorded zero.

Table 2. Change percentage of worm burden and egg load (total, viable and dead) in mice challenged with 1/10 LC_{50} of air-dried mandarin peel extract-attenuated *S. mansoni* cercariae for different exposure time periods (30-150 min).

Mice groups	Worm burden		Egg load					
			Total		Viable		Dead	
	Mean ± SE	%	Mean ± SE	%	Mean ± SE	%	Mean ±SE	%
	47.5 ± 6.7		78.1 ± 11.5		30.4 ± 5.2		47.7 ± 7.2	
Group 1 (30 min)	0	0	0	0	0	0	0	0
Group 2 (60 min)	0	0	0	0	0	0	0	0
Group 3 (90 min)	0	0	0	0	0	0	0	0
Group 4 (120 min)	0	0	0	0	0	0	0	0
Group 5 (150 min)	0	0	0	0	0	0	0	0

Data are expressed as Mean ± Standard error.

Changes in the relative weights of spleen and liver of mice infected with 1/10 LC_{50} mandarin peel extract-attenuated *S. mansoni* cercariae for 30, 60, 90, 120, and 150 minutes were recorded in table 3. There was significant decreases in the relative spleen and liver weights of mice exposed to 1/10 LC_{50} mandarin peel extract-attenuated *S. mansoni* cercariae for different time intervals compared to those of the non-attenuated infected control mice. The percentage of decrease in the relative spleen weight ranged from -7.7 to -47.3 % while the percentage of decrease in the relative liver weight ranged from -8.9 to -34.1.

Table 3. Changes in the relative weights of spleen and liver in mice challenged with 1/10 LC_{50} of air-dried mandarin peel extract-attenuated *S. mansoni* cercariae for different exposure time periods (30-150 min)

Mice groups	Relative spleen weight (Mean ± SE)	Change		Relative liver weight (Mean ± SE)	Change	
		%	P value		%	P value
Control (0 min)	0.91 ± 0.05			7.85 ± 0.59		
Group 1 (30 min)	0.57 ± 0.08*	-37.4	0.03	5.76 ± 0.56*	-26.6	0.02
Group 2 (60 min)	0.64 ± 0.05*	-29.7	0.01	6.16 ± 0.45*	-21.5	0.06
Group 3 (90 min)	0.48 ± 0.03*	-47.3	0.01	5.17 ± 0.38*	-34.1	0.01
Group 4 (120 min)	0.84 ± 0.11	-7.7	0.88	5.74 ± 0.36*	-26.9	0.03
Group 5 (150 min)	0.84 ± 0.06	-7.7	0.39	7.15 ± 0.56	-8.9	0.06

Data are expressed as Mean ± Standard error.
*: Significantly different in comparison with the control group at $P < 0.05$

DISCUSSION:

Flavonoids are natural plant compounds increasingly used in therapeutic applications. Some of which, e.g., hesperidin, naringin, and polymethoxylated flavones, are characteristic of citrus fruits and others such as rutin and quercetin are common in plant kingdom (Nogata *et al.,* 2006). Their large spectrum of activities depends on their structures and cellular targets. Flavonoids have long been recognized to possess anti-inflammatory, hepatoprotective, antioxidant, and anticarcinogenic activities (Theoharides *et al.,* 2001; Pradeep *et al.,* 2008; Renugadevi and Prabu, 2009; Xiao *et al.,* 2009). Most recent research showed that they are promising drugs for controlling human and animal parasitic diseases (Kerboeuf *et al.,* 2008). Other compounds, chalcones, have been isolated from leaves of mandarin collected from Egypt (Abdel-Alim *et al*., 1998). Several potential properties of chalcones were exhibited as anti-HIV, anti-tuberculosis, cytotoxic and antimalarial activities (Cheenprach *et al.,* 2006; Sivakumar *et al.,* 2007; Fang *et al.,* 2008; Dominguez *et al.,* 2009).

Regarding the molluscicidal potential of mandarin peel extract on *B. alexandrina* snails, the present study showed that the more effective concentration of mandarin peel extract was 500 ppm, where it gave 35 % mortality after 24 hours. The mortality of snails increased in a time-concentration relationship pattern to reach 95 % after 72 hours. Similar molluscicidal effects were observed in other flavonoid-containing plants. Water extract of *M. thonningii* seeds showed molluscicidal activity against *Bulinus trunctatus* snails (Evans *et al.,* 1986). Chloroform extract of *M. thonningii* seeds also showed molluscicidal activity against *B. glabrata* snails. The molluscicidal compounds in *M. thonningii* seeds have been identified as isoflavones (Perrett, 1994; Perrett and Whitfield, 1996). Leaves and wood bark extracts of two flavonoid-containing medicinal plants were active against *B. glabrata* snails (de S'Luna *et al.,* 2005). Singab *et al.* (2006) isolated two new flavonoids from rhizome extract of *Iris germanica* and found that these flavonoids showed significant molluscicidal activities against *B. alexandrina* snails. Ethanolic extract of *Dalbergia sissoo* fruits and roots exhibited promising molluscicidal activities against *B. pferfferi* snails (Adenusi and Odaibo, 2008). The active ingredients in this plant are isoflavonoid glycosides (Farag *et al.,* 2001) and flavonoids (Shrestha *et al.,* 2008). Barsoum *et al.* (2006) screened the molluscicidal activity of chalconoids against *B. alexandrina* snails. Most of the prepared chalcone as well as pyrazoline derivatives showed moderate molluscicidal properties. The bispyrazolines 4 exhibited better

molluscicidal properties than those of their starting bispropenones 3. Moreover, the substituents attached to the phenyl group have been found to be of great importance, enhancing the observed biological activity.

With respect to cercaricidal effect of mandarin peel extract, the present study showed that exposure of *S. mansoni* cercariae to ascending concentrations of mandarin peel extract (1000-2500 ppm) for different time intervals (15-120 min) displayed a significant level of cercaricidal potential in a time-concentration relationship pattern. Similar cercaricidal effects were also observed in other flavonoid-containing plants. *M. thonningii* seed extract displayed cercaricidal activity against *S. mansoni* cercariae (Squire and Whitfield, 1989). Alpinumisoflavone and 4-methylalpinumisoflavone may be responsible for this cercaricidal effect (Lyddiard *et al.,* 2002). Fraction B prepared from the chloroform extract of *Iris germanica* rhizomes also displayed a significant level of *S. mansoni* cercaricidal potential in a time-concentration relationship pattern (Singab *et al.,* 2006).

Concerning cercarial infectivity, the present study showed that attenuation of schistosomal cercariae using 1/10 LC_{50} of mandarin peel extract for different time periods (30-150 min) impaired the cercarial motility to infect mice, even for a short time. These results are consistent with the results of Perrett *et al.* (1994) who reported that exposure of *S. mansoni* cercariae to different concentrations of *M. thonningii* seed extract was associated with a concentration-dependent decline in worm establishment at 55 days post-infection. The present results also confirm the results of Perrett *et al.* (1995) who reported that topical application of chloroform extract of *M. thonningii* seeds to mouse skin 2 and 24 hours prior to *S. mansoni* cercarial exposure appeared to be effective in preventing subsequent establishment of infection. The compound responsible for the activity is thought to be the isoflavonoid alpinumisoflavone.

The multiple effects of flavonoids make it difficult to understand their modes of action; however, the bioactivity of flavonoids against *B. alexandrina* snails and *S. mansoni* cercariae seems to be related to their inhibition of mitochondrial electron transport. Lyddiard *et al.* (2002) suggested that robustic acid and at least 1 alpinumisoflavone compound from the dichloromethane extract of the seeds of *M. thonniogii* are responsible for some of the observed bioactivity of this extract against schistosomes. Fry and Jenkins (1984) studied the effects of mitochondrial inhibitors on the *in vitro* development of *Nippostrongylus brasiliensis* in free-living life-cycle stages. All mitochondrial inhibitors tested were highly effective in killing or retarding development of free-living stages of *N. brasiliensis*. Concentrations of inhibitors effective against free-living stages were consistent with their level of inhibition against isolated mitochondria from embryonated eggs and 3rd-stage of infective larvae. On balance, the flavonoids appear to be remarkably safe nutrients with a wide range of biochemical and pharmacological activities that strongly suggest their possible role as health-promoting and disease-preventing dietary supplements (Middleton *et al.,* 2000).

Taken altogether, it can be concluded that air-dried mandarin peel extract may be considered as a promising candidate for the control of *S. mansoni* transmission because of its molluscicidal and cercaricidal activities, as it has the capacity to prevent cercarial infectivity. These effects may be due to the presence of flavonoids and other constituents that cause death and attenuate the ability of cercariae to infect the final host. Accordingly, results of this study suggest that air-dried peel of mandarin fruits may be of economic and medical importance in schistosomiasis control.

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دراسة كفاءة مستخلص قشر ثمرة اليوسفى فى مقاومة الأطوار اليرقية لطفيلى البلهارسيا والقواقع الناقلة لها

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لا تتوافر معلومات كافية عن استخدام نباتات العائلة الليّمونية فى إبادة القواقع والأطوار اليرقية لطفيلى البلهارسيا. وقد أُجريت هذه الدراسة لتقدير فعالية مستخلص قشر ثمرة اليوسفي في مقاومة قواقع البيومفلاريا الكسندرينا (العائل الوسيط) والسركاريا (الطور المُعدى) للبلهارسيا المعوية، بالإضافة إلى دراسة درجة إصابة العائل الأساسي، ممثلاً في الفئران البيضاء، بالسركاريا المُضّعفة نتيجة تعرُضها ل (LC_{50} 1/10) من مستخلص قشر ثمرة اليوسفي لفترات زمنية متصاعدة. وقد أوضحت النتائج أن التأثير المُميت للقواقع لتركيزات مختلفة من مستخلص قشر ثمرة اليوسفي المُجفّف هوائياً قد زاد بزيادة زمن التعرض. وقد كان التركيز الأكثر فعالية هو 500 جزء فى المليون، حيث أدى إلى نسبة وفيات 35% و60% و95% و95% بعد 24 و48 و72 و96 ساعة، على التوالى. وقد أظهر تعرُض السركاريا لتركيزات مختلفة من مستخلص قشر ثمرة اليوسفي (1000-2500 جزء فى المليون) لفترات زمنية متصاعدة (15-120 دقيقة) تأثيرات مميتة تتناسب طردياً مع زمن التعرض ودرجة التركيز المستخدم. وقد تبين أيضاً أن الفئران التي تعرضت للسركاريا المُضّعفة بمستخلص قشر ثمرة اليوسفي لأي من الفترات الزمنية (30-150 دقيقة) لم تتم إصابتها بالمرض، وعلى العكس من ذلك، فقد أُصيبت جميع الفئران المُعرضّة لسركاريا سليمة غير مُضّعفة. وبالإضافة إلى ذلك، فقد اختُزل الوزن النسبى لكل من الطحال والكبد للفئران التي تعرضت للسركاريا المُضّعفة بمستخلص قشر ثمرة اليوسفي مُقارنة بالفئران المُعرضّة لسركاريا سليمة غير مُضّعفة. وخلصت هذه الدراسة إلى كفاءة مستخلص قشر ثمرة اليوسفي في إبادة القواقع وإضعاف السركاريا ووقف إصابة الفئران بمرض البلهارسيا المعوية، وذلك بتعريض السركاريا لتركيزات منخفضة من مستخلص قشر ثمرة اليوسفي. ويمكن إعزاء هذه النتيجة إلى وجود مركبات الفلافونات ذات الفوائد الطبية المتعددة.