Original Article

Tegumental changes induced in adult *S. mansoni* by limonin: animal experimental and electron microscopic studies

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ABSTRACT

Background: Praziquantel (PZQ) is the drug of choice recommended by the World Health Organization for the treatment and control of human schistosomiasis. The fact that schistosomes are developing resistance to PZQ indicates the urgent need for new effective treatment compounds. Herbal medicinal products, either as pure compounds or as standardized plant extracts, have been employed successfully for the development of new drugs. Limonin which is a citrus fruit compound has been reported to possess a wide range of biological activities.

Aim of the work: To investigate *in vitro* the therapeutic efficacy of limonin as an alternative anti-schistosomal compound.

Material and Methods: The *in vitro* assay was carried out on adult *Schistosoma mansoni* worms (Egyptian strain) using four concentrations of limonin in RPMI-1640 culture medium (15.6, 31.2, 62.5, 125.0 µg/mL). Schistosomes recovered from all groups were processed for ultrastructural investigation by scanning electron microscopy.

Results: Limonin at a concentration of 125.0 µg/mL reduced the worm motor activity and caused death of all male schistosomes, which were more susceptible. The product had a concentration-dependent response effect within a smaller period of exposure. Female worms showed less prominent effects (death) at higher tested concentrations associated with reduction in the motor activity. This effect decreased with reduction of concentration . On the ultrastructural level, limonin at higher concentrations induced massive tegument destruction exposing the sub-tegument tissues.

Conclusion: Obtained data confirm that limonin is effective against adult *S. mansoni in vitro* and presents a potential for the development of a candidate anti-parasitic agent.

Key Words: *in vitro*, limonin, orange dried seeds, scanning electron microscopy, *S. mansoni*.

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INTRODUCTION

Schistosomiasis is listed as one of the neglected tropical diseases. It affects over 250 million people worldwide and presents a major public health problem in 78 tropical and subtropical countries. The majority (up to 90 %) of the cases are located mainly in sub-Saharan Africa[1,2]. Praziquantel is the major chemotherapeutic agent for treatment of schistosomiasis as well as other helminthesiasis[3-5]. In view of the growing resistance to this therapy, attempts are made to explore other new anti-schistosomal options[6]. There is now a promising tendency for the use of natural compounds derived from plant extracts as drugs against *Schistosoma* spp., being safe and with less medical side effects[7-9]. A review article was published in 2013 and in its first part, the reviewer focused on all drugs recommended from WHO for treatment of schistosomiasis during the last 6 decades, as well as all herbal medicines tested in several studies in comparison with PZQ. It also dealt with PZQ resistance in several African countries and its mechanism(s), mentioning methods for measurement of susceptibility or resistance of *Schistosoma* spp. to PZQ. The second part of the review discussed informatics and new technology in *Schistosoma* spp. genomics and proteomics that allowed scientists to develop new anti-schistosomal drugs. Finally, several studies that utilized the fast progress in molecular technology conducted during the period from 2000-2012 were summarized[10].

Citrus fruits are regarded for their content of certain compounds (limonoids) with extremely bitter taste mainly accumulated in seeds. The term limonoids was derived from limonin, the first tetranortriterpenoid obtained from citrus bitter principles[11]. Limonin is a highly oxygenated triterpenoid dilactone, which is particularly abundant in the seeds of citrus fruits as the Rutaceae and Meliaceae families[12]. Limonoids have recently been found to possess multiple *in vitro* and *in vivo* biological functions[13]. Other biological activities include insecticidal, insect anti-feeding and growth regulating activities in insects, as well as being antiviral, anticancer, cholesterol-lowering and antioxidant[14-19]. Limonoids as limonin and nomilin have been found to induce increased activity of the detoxifying enzyme glutathione-S-transferase. The increased enzyme activity was found to correlate with the ability of these compounds to inhibit chemically induced carcinogenesis

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in laboratory animals[20,21]. Toxicological studies reported possibility of hepatotoxicity due to the use of herbal medicine. Another recent study in 2014 on rats showed that limonin proved to have hepatoprotective effects against liver toxicity. This occurs either directly through antioxidant effects, or indirectly through inhibition of TNF-α production and subsequent suppression of eosinophils infiltration in D-galactosamine-induced liver injury[22].

Several researchers studied the alterations in the surface topography of schistosomes by scanning electron microscopy (SEM). This was employed for the evaluation of several drugs/compounds, since the tegument of schistosomes is an important target for such drugs[23-25].

In spite of this wide range of therapeutic activities, little information is available about the anti-helminthic effects of limonoids. Hamed and Hetta reported that treatment with *C. reticulata* (Mirazid) improved succinate dehydrogenase (SDH), lactate dehydrogenase (LDH) and its isoenzymes, glucose-6-phosphatase (G-6-Pase), acid phosphatase (AP), 5'- nucleotidase, and liver function enzymes activities in *S. mansoni* infected mice with a noticeable reduction in ova count and worm burden[26]. Co-administration of grapefruit juice with artemether achieved complete protection of the host from damage induced by schistosomal infection[27]. Therefore, the present study investigated, whether limonin, which is the most abundant limonoid in citrus fruit, has an anti-schistosomal activity against mature *S. mansoni in vitro*.

MATERIAL AND METHODS

The present experimental descriptive study was carried out at Schistosome Biological Supply Center (SBSC), Theodor Bilharz Research Institute (TBRI), Giza, Egypt during the period from December 2015 to March 2016.

Plant material and extraction procedure: Bitter sweet orange (*Citrus aurantium var. bigaradia Hook f.*, *Citrus bigaradia Risso* and *Poit, C. bigaradia Loisel*; *Rutaceae*) was used in this study. The selection of this plant was made on the basis of information gathered about its use in traditional medicine. Dried seeds were obtained from the Research Center of El-Qanater El-Khireya, Qalubyia, Egypt. The seeds were collected from ripe fruits, cleaned, dried in the shade and ground into powdered form.

Preparation of the plant extract: Limonin with molecular formula: C26H30O8 (Figure 1) was prepared in Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University according to the method described by Mahmoud *et al*.[22]. A voucher specimen (P-80, 81) was deposited in the herbarium of the Faculty of Pharmacy, Zagazig University. Briefly, the dried powder was defatted with light petroleum and extracted with methanol. This concentrated methanolic extract was then suspended in water and extracted with CH2Cl2. The solvent was evaporated under vacuum to give a brownish yellow oily residue.

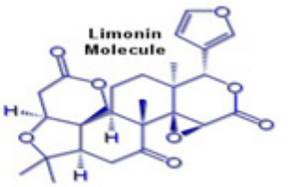

Fig. 1: Chemical structure of limonin (7,16-Dioxo-7,16-dideoxylimondiol)

Isolation of the compounds: The dry mixed initial zone of the dichloromethane fraction was chromatographed over a silica gel column, eluted using cyclohexane then the polarity was increased gradually using ethyl acetate followed by methanol. Fractions 250 mL each were collected, concentrated under vacuum and monitored by thin layer chromatography (TLC) using solvent systems[28].

Spectral analysis using liquid chromatography electrospray mass spectrometry (LC-ESI/MS) for limonoids: Chromatographic separation was carried out using RP C-18 LiChro CART (Merck- Darmstadt) column to identify the minor limonoids in the dichloromethane fraction. The purity of limonin used in the present study and tested using high performance liquid chromatography (HPLC) was > 97 %. Spectral data for limonin was as described by Hamdan *et al*.[28] while, the procedure used for LC-ESI/MS of limonoid was as illustrated by Mahmoud *et al*.[22].

As a benchmark, PZQ was purchased from Sigma Aldrich. Both limonin and PZQ were dissolved in 0.5% dimethyl sulfoxide (DMSO, Sigma-Aldrich) immediately before use.

Animals and parasites: Five adult male Syrian golden hamsters (*Mesocricetus auratus*), 100-120 g each, were purchased from SBSC, TBRI, Giza, Egypt. They were maintained under environmentally-controlled conditions (temperature 25°C; humidity 70%; 12 h light and 12 h dark cycle) and acclimatized for one week before infection. Three hundred *S. mansoni* cercariae (Egyptian strain) were used to infect each hamster by abdominal skin exposure according to the infection protocol of SBSC, TBRI.

Experimental design: The *in vitro* schistosomicidal assay was conducted according to the method described by Yousef *et al*.[29] and the modification carried out by El Beshbishi *et al*.[30]. Briefly, *S. mansoni*, mature parasites were recovered by perfusion from portal and mesenteric

veins of 8 w infected hamsters. Freshly recovered viable adult schistosomes were washed three times in PBS then washed again in RPMI 1640 culture medium then placed in fresh medium and stored in an incubator chamber at 37°C. Adult worms were incubated in a 24-well culture plate (TPP, St. Louis, MO, USA), placing 3-6 pair of worms in each well, containing the RPMI 1640 medium at 37°C in a 5% CO2 atmosphere. Limonin was dissolved in DMSO 0.5% and tested at concentrations of 15.6 to 125.0 µg/ml (15.6, 31.2, 62.5, 125.0 µg/ml) in culture plates with a final volume of 2 ml. The control worms were assayed in RPMI 1640 medium alone, and in RPMI 1640 medium containing 0.5% DMSO without drugs as negative controls groups and 10.0 µg/ml PZQ (reference drug) as positive control group. Each test was performed in duplicate and repeated at least two times. The parasites were kept for 96 h and monitored using an inverted microscope every 24 h. The effect of the drug was assessed with emphasis on changes in worm viability, worm motor activity and alteration in the tegument as previously described[31]. Parasites showing no body contractions for at least 1 to 2 min of observation were considered dead (i.e. no worm movement after 1-2 min without motor activity)[32-33]. In addition, worms were also examined using scanning electron microscopy.

SEM study: To observe the morphological changes in the tegument of the adult parasites, schistosomes were monitored using SEM following standard procedure as described by Hassan *et al*.[34]. Briefly, samples of *S. mansoni* adult worms understudy were, washed twice in PBS for 10 min then fixed in 3% glutaraldehyde buffer solution over night at 4ºC. Worms were then washed of any trace of fixative by keeping them over night at 4ºC in PBS, then post-fixed in 1% Osmium tetroxide (OsO4) for one hour. Samples were then washed and dehydrated in ascending grades of ethyl alcohol (30%, 40%, 50%) for 15 min each. Worms were then kept in 70% ethyl alcohol until examination. Before examination, they were washed twice for 30 min in 80% and 90% ethyl alcohol respectively. The last wash was for one hour in 95% ethyl alcohol, after which worms were mounted on stainless steel holders and put in a drier for about 30 min and then subjected to sputter coat of gold. Different parts of worms were examined using Joel JEM-1200 scanning electron microscope, fitted with a camera. Areas in the worms that showed specific changes were examined and photographed mainly suckers and tegumental tubercles[35].

Statistical analysis: Data were analyzed using statistical program SPSS version 20 (USA). Quantitative data was represented by using mean and standard deviation, and analyzed using correlation coefficient (r) and ANOVA test (F value). The first was used to measure the closeness of the association between two continuous variables. Linear regression: is the standard method for determining whether 2 variables are correlated; i.e. X=Y, 2X=2Y, so X and Y are correlated. Regression coefficient = coefficient of correlation = r which indicates the degree of correlation between X and Y, while value of 1 indicates perfect correlation and value of 0 indicates no correlation at all. ANOVA test was used to compare mean of more than two groups of quantitative data with post hoc multiple comparison (LSD). Significance level was considered if $P < 0.05$.

Ethical considerations: The present study was approved by the Ethical Committee of Faculty of Medicine, Benha University, Egypt. The experimental animal studies were conducted in accordance with the ethical guidelines approved by the Ethical Committee of the Federal Legislation and National Institutes of Health Guidelines in the USA and were approved by the Medical Ethical Committee of TBRI. All the animals were handled in strict accordance with good animal practice as defined by the "Animals Use Ethics Committee" of Faculty of Medicine, Benha University, Egypt. The maintenance and care during experimentation of animals was compliant with international guidelines for the human use of laboratory animals.

RESULTS

The results of the current *in vitro* study with adult *S. mansoni* worms exposed to limonin at concentrations of 15.6, 31.2, 62.5 and 125.0 µg/ml and control groups are summarized in tables (1,2), and figure (2). At 125.0 µg/ml concentration, limonin was lethal to 100% of male adult parasites after 72 h of *in vitro* exposure. With the same concentration and within the same time period, little mortality was observed in female worms, accompanied with significant reduction of motor activity. At concentrations less than 62.5 µg/ml, reduction in the schistosome motility was the only observed finding. In the negative control groups (RPMI 1640 medium only and RPMI 1640 medium containing 0.5% DMSO without limonin), no impact was observed on worm motor activity or survival. The schistosomes showed normal motor activity and had no observed mortality. In the 10.0 µg/ml PZQ treated control group, there was complete loss of motor activity associated with death of all parasites within the first 24 h post incubation. The present study also showed significant correlation between increasing limonin concentration (from 15.6 µg/ml up to 125 µg/ml) and both mortality of male worms and reduction in worms motor activity (Table 2).

Table 1: Effect of different limonin concentrations on mature *S. mansoni*

Group	Incubation Period (h)	% of dead worms		% of motor activity reduction	
		Male	Female	Male	Female
Control (-ve) RPMI 1640 medium	24 h	0%	0%	0%	0%
	48 h	0%	0%	0%	0%
	72 h	0%	0%	0%	0%
	96 h	0%	0%	0%	0%
RPMI 1640 medium containing 0.5% DMSO	24 h	0%	0%	0%	0%
	48 h	0%	0%	0%	0%
	72 h	0%	0%	0%	0%
	96 h	0%	0%	0%	0%
Control (+ve) (PZQ 10.0 µg/ ml)	24 h	100%	100%	100%	100%
	48 h	100%	100%	100%	100%
	72 h	100%	100%	100%	100%
	96 h	100%	100%	100%	100%
Limonin 125.0 µg/ ml	24 h	10%	0%	100%	30%
	48 h	60%	30%	100%	60%
	72 h	100%	50%	100%	100%
	96 h	100%	70%	100%	100%
Limonin 62.5 µg/ ml	24 h	0%	0%	60%	20%
	48 h	33.3%	10%	100%	50%
	72 h	50%	20%	100%	100%
	96 h	70%	40%	100%	100%
Limonin 31.2 µg/ ml	24 h	0%	0%	0%	0%
	48 h	0%	0%	0%	0%
	72 h	20%	10%	40%	20%
	96 h	40%	20%	100%	50%
Limonin 15.6 µg/ ml	24 h	0%	0%	0%	0%
	48 h	0%	0%	0%	0%
	72 h	0%	0%	0%	0%
	96 h	0%	0%	0%	0%

Table 2: Correlation between increase of limonin concentration from 15.6 µg/ml - 125 µg/ml and its effect on worms.

	Limonin	
	Correlation coefficient	*P* value
Dead worm (male)	0.608	0.036*
Motor activity reduction (male)	0.626	0.029*
Motor activity reduction (female)	0.624	0.03*

*=significant ($P<0.05$)

By SEM, prominent changes in adult *S. mansoni* male tegument were observed (Figure 2). The severity and pattern of these morphological alterations depended on the concentration of limonin. This effect progressively intensified with the increase of the limonin concentration and incubation period. Both adult males and females were affected even at lower concentrations where mild to moderate tegumental alterations were observed. Severely damaged worms were observed at higher concentrations as there was massive tegumental destruction exposing the sub-tegumental tissues. Abnormal body attitude, flattened spines, shrinking, corrugations, unfolding and widening of the gynecophoral canal, and sloughing and disintegration of the tegument were observed on the tegument of examined worms.

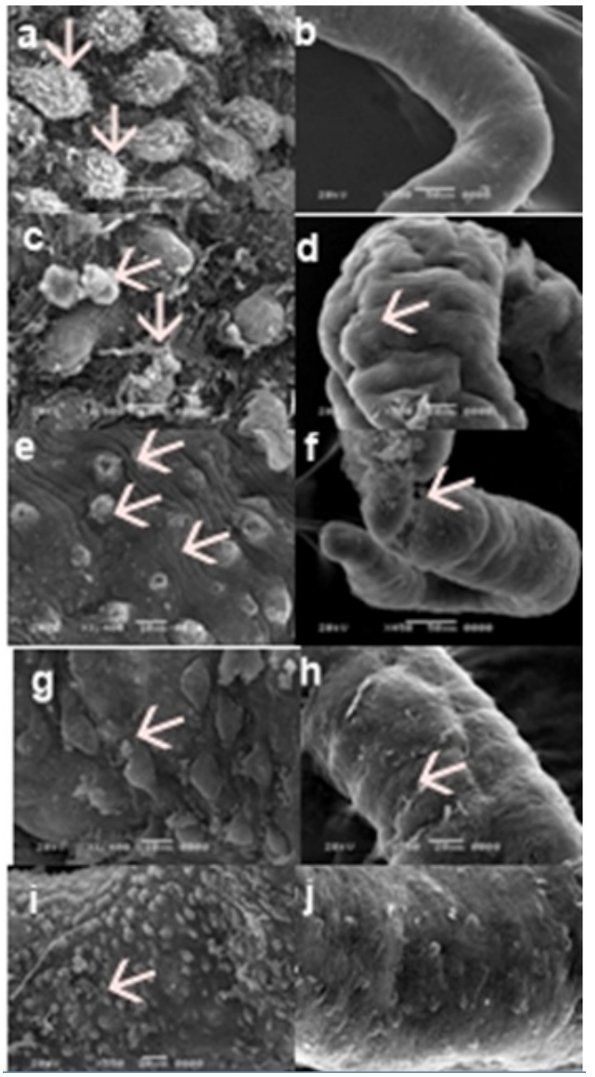

Fig. 2: Scanning electron microscopy of adult *S. mansoni* showing negative control worms with (a) normal tegument for adult male with normal tubercles covered by pointed spines with regular inter-tubercular spaces, (b) normal smooth intact tegument of female worm.
PZQ treated worms showing (c) severely damaged tegument of adult male following exposure to 1.0 µg/ml, (d) marked corrugation of female tegument. Exposure to 125.0 µg/ml limonin *in vitro* showing (e) destruction of tegument, rupture of tubercles with loss of spines and irregular inter-tubercular spaces in male worm, (f) distortion and destruction of female tegument.
Exposure to 62.5 µg/ml concentration showing (g) male worm reduction in number of tubercles and widening of inter-tubercular spaces, (h) moderate destruction of female tegument with multiple erosions and holes.
Exposure to 31.2 µg/ ml limonin (i & j) showing mild effects on the tegument.

DISCUSSION

Traditional chemical drugs are not fully effective against schistosomiasis due to the evolving drug resistant worm strains, so exploring new remedies derived from natural products is promising in combating schistosomiasis[36]. Limonin was reported to possess a wide range of biological activities that include antibacterial, anti-malarial, antiviral activities, in addition to its hepatoprotective effects[22] and anticancer efficacy[37]. In 2012, the larvicidal activity of citrus limonoids (limonin and nomilin) against *Aedes albopictus* larvae was reported to be due to anti-feeding and larvicidal activity[38]. Oral administration of limonin to *S. mansoni* experimentally infected mice resulted in worm burden reduction and significant reductions in both hepatic and intestinal tissue egg loads with elevated dead egg levels. Limonin also produced reduction in dimensions and number of hepatic granulomas[39]. In the present study, we investigated the *in vitro* schistosomicidal activity of pure limonin compound against adult *S. mansoni* worms. The observations in the negative and positive control groups are similar to previously described studies.

Investigations of the effect of PZQ and other natural compounds such as ginger extract (*Zinger officinale*)[43], and other natural products such as volatile organic components of *Ageratum conyzoides*[44] on *S. mansoni*, reported the greater susceptibility of adult male worms than female worms[43,45]. In contrast, artenusate showed higher survival rates of adult male than female worms[46]. Tegumental damage may not always result in death of worms[47], but the morphological alterations observed in our study are a result of the mechanism by which limonin kills the parasites. The resulting damage would expose hidden parasite antigens and consequently allow parasite attack by the host immune system. These tegumental alteration are similar to those induced by other drugs as PZQ[48] and other plant derived compounds[49]. No tegumental alterations were observed in the negative controls (RPMI and RPMI with 0.5% DMSO) until the end of the experiment (96 h), while the majority of schistosomes exposed to the reference drug (PZQ) showed pronounced tegumental changes similar to reports by previous studies[40-42].

As previously observed, finding a new compound that can kill parasites is not difficult, but the difficulty is to find a substance that can effectively kill the parasite without affecting the host[33]. Limonin is not only tolerable and safe but also exerts protective effects on liver toxicity associated with inflammation and tissue injury via attenuation of inflammation and reduction of oxidative stress[22]. Thus limonin may be a promising anti-schistosomal replacement therapy.

The exact mechanism by which the pure compound limonin exerts its effect on *S. mansoni* adult worm is still unclear. However, all the dead schistosomes showed massive tegumental affection. In addition, those still alive with affected motility also showed a variable degree of tegumental damage. Thus a relationship between the severity of tegumental damage and worm death was observed in the currently performed *in vitro* assay using different limonin concentrations.

In conclusion, we have reported here the *in vitro* anti-schistosomicial properties of limonin against adult *S. mansoni*. This study has highlighted *S. mansoni* as a possible new target for limonin therapy. However, further studies are required to confirm its action. Experiments to evaluate limonin *in vivo* with mice infected by *S. mansoni* are in progress.

Competing interest: The authors have declared that no competing interests exist.

Authors` contributions: GA Rashed, AA Elkholy conceived, designed, performed the experiments, analyzed the data and revised the manuscript. DI Hemdan contributed reagents/materials (purification of the plant materials). All authors revised and approved the final manuscript.

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