Research Journal of Parasitology

ISSN 1816-4943

Academic Journals Inc.

www.academicjournals.com

Research Journal of Parasitology 6 (5): 151-159, 2011
ISSN 1816-4943 / DOI: 10.3923/jp.2011.151.159

Efficacy of Garlic and Mirazid in Treatment of the Liver Granuloma in Mice Infected with *Schistosoma mansoni*

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ABSTRACT

This study has investigated the potency of traditional medicinal plants such as *Allium sativum* (garlic) and *Commiphora molmol* derivative (Mirazid) for treating schistosomiasis and the liver granuloma formation. The effects of garlic and mirazid on *Schistosoma mansoni* infected mice were examined by liver enzymes, counting worm burden, schistosome egg load, modulation of the granuloma and histopathology of the liver injury. The use of garlic or mirazid or combination of them led to marked reduction in worm load (43, 52 and 70%, respectively), ova deposition (43, 59 and 83%, respectively), size and number of granuloma (38, 46 and 67%, respectively) in the liver of mice infected with schistosoma. Also, these treatments ameliorated the histopathological injury changes of the liver and physiological assays of AST, ALT and total bilirubin. These findings indicate that garlic, mirazid and a combination of them have anti-schistosomal activity in mice. All of them significantly reduced the production of eggs output in *S. mansoni* infected mice. In addition, garlic and mirazid combination clearly reduced the liver granuloma size which was reflected by the degree of improvement in the status of the liver. To our knowledge, this study is the first in using the combination of garlic and mirazid in schistosomal treatment in mice.

Key words: *Schistosoma mansoni*, granuloma, garlic, mirazid, *Commiphora molmol*

INTRODUCTION

Schistosomiasis is a tropical disease caused by a parasite infection with worms from the Schistosomatidae family e.g., *Schistosoma mansoni*, *S. haematobium* and *S. japonicum*. It is estimated that the disease affects 200 million people worldwide, being endemic in 74 countries in tropical regions of Africa, Asia and South America (Chitsulo *et al*., 2000). The main species that causes infection in Egypt are *S. mansoni* and haematobium (Khaled *et al*., 2010). *Schistosoma* causes liver fibrosis and cirrhosis by deposition of eggs in the liver venules. Adult worms of *S. mansoni* and *S. intercalatum* reside in the inferior mesenteric veins (draining the colon) and release eggs into the portal blood flow. The eggs are trapped in submucosa of the intestine or carried to the liver by blood stream. The signs and symptoms of schistosomiasis are due to the host's immune response to schistosome eggs trapped in the tissues. The eggs secrete antigens that excite eosinophilic inflammatory and liver granuloma formation, which leads to stages of the disease are replaced by fibrosis (Gryseels *et al*., 2006). The natural progress of the disease leads to hepatic, splenic and intestinal injury, caused mainly by the immune response against *S. mansoni* eggs that

are deposited in these sites; so, hepatosplenomegaly, liver cirrhosis, portal hypertension and gastroesophageal varices are commonly associated with schistosomiasis (Manzella *et al*., 2008; Andrade, 2009).

When the schistosomal egg emerges from the female worm, it is still immature and causes no tissue reaction. It takes about 5-6 days for an embryo of the miracidium to differentiate and to start eliminating lytic and antigenic secretions through micro pores in the egg-shell. Moreover, the eggs that do not successfully pass through the intestinal mucosa towards the lumen are usually carried by the portal vein blood flow to the liver until they stop inside small pre-sinusoidal vessels. In a histological section, the granuloma appears to be located within the liver parenchyma. Around the ova, granuloma is formed in a allowing host when a mature egg is trapped within the alive tissue. It is an essentially inflammatory and fibrosis, that soon becomes encapsulated by the formation of concentric collagen rings at its periphery (Andrade, 2009).

Most of the control program strategy of schistosomiasis includes use of drugs for Mollusca to eliminate the intermediate host. Education of the population associated with sanitation improvement and chemotherapy. The development of a vaccine is an important alternative strategy for the control of schistosomiasis. So, no suitable drug has yet been developed that to consult more than not complete protection against infection (Frantz *et al*., 2011; Araujo *et al*., 1996). Because of the great need to develop new anti-schistosomal agents, the aim of this study was designed to test the potency of traditional medicinal plants such as *Allium sativum* and *Commiphora molmol* for treating schistosomiasis and the liver granuloma formation. Where, Ancient Egyptians accomplished the benefits of garlic as a remedy for a diversity of ailments. In recent times, garlic has been shown to have multiple beneficial effects such as antimicrobial, antithrombotic, hypolipidemic, hypoglycemic and antitumor activities (Thomson and Ali, 2003). While *Commiphora molmol* known in folklore medicine as "myrrh" is one of the most common herbs in the Saudi society, which *Commiphora molmol* has been used in the treatment of hypertension, hyperlipidemia, respiratory infections, ulcer and cancer. For many years, myrrh (molmol) has been used for its healing quality benefits during injuries. Clearly, the importance of using the oleo-gum resin, *C. molmol*, in the Middle East System of Medicine have been proven in scientific studies (Haffor, 2010). So, the present study aimed to investigate the potency of traditional medicinal plants such as garlic and *Commiphora molmol* derivative (Mirazid) for treating schistosomiasis and the liver granuloma formation.

MATERIALS AND METHODS

Infection of mice: *S. mansoni* cercariae and clean CD1 male albino mice were supplied from Theodor Bilharz Research Institute, Imbaba, Giza, Egypt. Male CD1 albino mice were infected with 80±10 *S. mansoni* cercariae per mouse by the tail immersion method, modified by Olivier and Stirewalt (1952). The ethical obligations to experimental animals were followed. The experiments were carried out at Zoology Department, Faculty of Science, Fayoum University, Fayoum, Egypt.

Experimental design: 40 mice were divided into four groups of 10 mice each. Group I was infected with *S. mansoni* cercariae and non-treated. Group II was infected with *S. mansoni* cercariae and treated with oral dose of garlic, 150 mg kg^{-1} (TOMEX, ATOS Pharma, Egypt); Group III was infected with *S. mansoni* cercariae and treated with oral dose of Mirazid (600 mg kg^{-1} b.wt., the oleoresin extract from Myrrh of the *C. molmol* tree, family: Burseraceae) is a product of Pharco Pharmaceutical Company, Egypt. Mirazid was given for three consecutive days on the empty

stomach, at least 1 h before a meal regarding to the concentration of Mirazid illustrated in the brochure of the drug (Haridy *et al*., 2003) and group IV was infected with *S. mansoni* cercariae and treated with garlic and mirazid together. All mice were sacrificed at the end of the 8th week of the experiment (starting from the day of infection).

Parasitological parameters

Worm burden: Perfusion of adult worms from the liver and portomesenteric system was performed 8 weeks after infection according to Duvall and DeWitt (1967) and Smithers and Terry (1965). The percentage reduction in worm numbers after treatment was calculated by the method of Tendler *et al*. (1986) as follows:

$$P = \frac{C-V}{C} \times 100$$

where, P = % of protection, C = Mean number of parasites recovered from infected animals and V = Mean number of parasites recovered from treated animals.

Tissue egg load: The number of eggs per gram tissue (liver) was studied according to the procedure by Cheever (1968). Worm counts were made at 10-weeks post-infection after perfusing the hepatic portomesenteric vessels of the animal (Duvall and DeWitt, 1967) according to the procedure outlined by Smithers and Terry (1965). The whole livers of both the treated and infected control mice were weighed and 0.5 g was removed, blotted between two filter papers and placed in a test tube containing 5 mL of 5% KOH solution at 37°C for 24 h until completely digested (Cheever, 1968).

Biochemical measurement: Blood was collected and serum was separated by centrifugation at 3000 rpm (10 min) for estimation of total bilirubin concentrations by colorimetrically measured (Shimadzu-CL 770 spectrophotometer), whereas aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations were measured using the enzyme-kinetic method.

Histopathological study: Liver tissue specimens were fixed in 10% buffered formalin and then embedded with paraffin. Sections (4 μm thick) were stained with hematoxylin and eosin (H and E) to measure hepatic granulomas and to examine the associated histopathological changes. The size of *Schistosoma* granulomas at ×10 was measured per section using an ocular micrometer. Only lobular granulomas containing an egg in the center and confluent were measured. The diameters of the 10 largest granulomas in each section were measured and mean granuloma diameter (±SD) calculated for each group of mice according to Lichtenberg (1962). Only granulomas appearing as circular in section were measured. Granulomas adjacent to areas of hepatocyte necrosis were excluded from diameter measurement.

Statistical analysis: Comparison was performed between the treated groups and untreated control using SPSS11 windows program (ANOVA test). The data were considered significant if p-values were less than 0.05.

RESULTS

Results in Table 1 showed the significant reduction (43, 51.5 and 70%) in the mean number of *S. mansoni* adult worms in the groups of infected mice treated with garlic, mirazid and mixture of

Table 1: Effects of mirazid (*C. molmol* derivative) and garlic on worm load in mice infected with *Schistosoma mansoni*

Animal groups	Worm load	Worms reduction (%)
Untreated control (I)	25.60±0.55	---
Garlic (II)	14.39±0.43	43*
Mirazid (III)	12.40±0.23	52*
Garlic+mirazid (IV)	8.18±0.09	70*

The data presented as Mean±SD, *p<0.05 vs. untreated control

Table 2: Effects of myrrh derivative (mirazid) and garlic on egg load in mice infected with *Schistosoma mansoni*

Animal groups	Hepatic ova	Ova reduction (%)
Untreated control (I)	6856±52.4	---
Garlic (II)	3920±18.4	43*
Mirazid (III)	2801±10.3	59*
Garlic+mirazid (IV)	1200±8.2	83*

The data presented as Mean±SD, *p<0.05 versus untreated control

Table 3: Effects of myrrh derivative (mirazid) and garlic on liver functions in mice infected with *Schistosoma mansoni*

	Liver functions		
Animal groups	AST (IU)	ALT (IU)	Total bilirubin
Untreated control (I)	109.15±19.20	53±10.02	1.5±0.07
Garlic (II)	60.12±8.05	45±5.12	1.3±0.03
Mirazid (III)	55.10±10.3*	40±6.01*	1.3±0.05*
Garlic+mirazid (IV)	45.03±3.2*	35±3.02*	1.08±0.01*

The data presented as Mean±SD, *p<0.05 versus untreated control, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase

garlic and mirazid, respectively when compared to the infected controls (p<0.01, 0.009, 0.005, respectively) where, the number of worm loaded in infected mice with schistosoma was 25.6±0.55 which reduced into 14.39±0.43 in garlic treatment, 12.4±0.23 in mirazid treatment and 8.18±0.09 in garlic and mirazid treatment groups.

Moreover, in the Table 2, significant reduction in the mean number of ova/gram tissue of liver was detected in the groups treated with garlic (3920 ova), mirazid (2801 ova) and mixture of garlic and mirazid (1200 ova) compared to infected group (6856), (p<0.01, 0.007 and 0.005, respectively).

The Activities of Serum AST, ALT and total bilirubin of normal controls and treated groups are represented in Table 3. Serum ALT and AST activities in infected mice (group I) were significantly increased compared to group II (infected group received garlic) during the experiment. On the other hand, serum ALT and AST activities in the infected mice receiving mirazid (group III) were decreased and significantly decreased in the group IV (infected group received garlic and mirazid).

After 8 weeks post *S. mansoni* infection, typical hepatic bilharzial lesion is observed in sections of infected mouse liver. Schistosome eggs are surrounded by inflammatory cells and schistosome fibrotic changes are evident in this area. Histopathological examination of the liver of infected non-treated mice revealed chronic granulomatous inflammation characterized by inflammatory infiltrate and numerous granulomas. Granulomas were marked by concentric fibrosis with many fibroblasts encircled the trapped eggs. They were surrounded by a cuff of aggregated lymphocytes, epithelioid

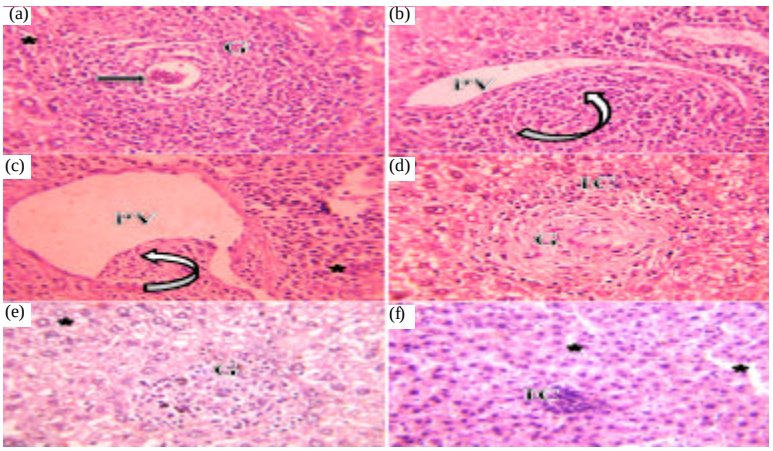

Fig. 1(a-f): Hematoxylin and Eosin stain Photomicrographs of a liver section of *S. mansoni*-infected, (a, b and c): Non-treated mice showing formation of granuloma (G) with trapped central eggs (arrow) surrounded by concentric rings of epithelioid cells and lymphocytes, The disorganized hepatic strands, dilated sinusoids (*), some necrotic hepatocytes and mass of lymphocytes and fibrotic projection into the lumen of the portal vein (curved arrows). (d and e): Photomicrographs of a liver section of *S. mansoni*-infected and treated with Mirazid and garlic mice, respectively showing a reduced granuloma size, accumulation of inflammatory cells surrounding the granuloma (IC), vacuolated hepatocytes and some hypereosinophilic hepatocytes. (f): Photomicrograph of a liver of *S. mansoni*-infected, garlic and mirazid mixture treated mouse showing a remarkably reduced granuloma and ameliorated hepatocytes. However, there are some inflammatory cells (IC) and some dilated sinusoids (*)

Table 4: Granuloma diameters (μm) in the liver of the treated and non-treated animal groups

Animal groups	Granuloma diameter (μm)	Reduction of diameter (%)
Untreated control (I)	800±50	---
Garlic (II)	500±55	38*
Mirazid (III)	438±40	46*
Garlic+Mirazid (IV)	275±45	67*

The data presented as Mean±SD, *p<0.05 versus untreated control

cells, eosinophils and collagenous fibers. Perigranulomatous hepatocytes revealed hypereosinophilia, vacuolar degeneration and necrotic foci characterized by nuclear pleomorphism. Portal veins were dilated and infiltrated with mass of inflammatory cells.

Examination of the liver of garlic, mirazid and a combination of garlic and mirazid treated mice showed a significant reduction in the number and size of granulomatous inflammation compared with infected non-treated ones. The hepatic lobular architecture restored its normal feature and most hepatocytes showed normal appearance. However, some sinusoids were still dilated and infiltrated with lymphocytes (Fig. 1). The mean diameter of the hepatic granuloma was significantly smaller in infected and treated mice than in infected and non-treated mice ($p<0.05$, Table 4).

DISCUSSION

During the past decades, the intensive use of chemical anti-helminthic (AHs) has unfortunately led to the widespread development of resistant in worm populations. Considerable attention has been given recently to bioactive plants which can be used as non-conventional AHs.

The search for bioactive plants which can be used as non-conventional antihelmintics (AHs) has received considerable attention in recent times because of the increasing, worldwide development of resistance to chemical anti-helminthic in worm populations. However, scientific evidence to validate the use of plants remains limited (Hoste *et al*., 2008). Thus, this study was oriented to evaluate the protective and curative capacity of garlic against *S. mansoni* through different means. The assessment of the influence of garlic administration on infected mice has been attained by comparing the results of treated infected mice to that of the corresponding Mirazid (Myrrh) treated-infected mice (control group).

The anti-schistosomal drug Mirazid has been available in the local Egyptian market since 2001. Studies on its anti-schistosomal efficacy, whether in experimental animals or in humans, has been reported by the discoverers of these properties and no independent confirmation has appeared so far of these anti-schistosomal effects (El-Baz *et al*., 2003). Furthermore, a multicenter investigation of the potential anti-schistosomal activity of different derivatives of the resin, including the commercial preparation Mirazid was tested in experimental animals. The drug was found toxic for mice at high doses and produced modest or no worm reduction at lower doses (Botros *et al*., 2004). In the current study, the efficacy of myrrh in treating *S. mansoni* infections was tested and was compared with garlic treatment.

However, the results obtained showed that garlic or mirazid administration caused a highly significant reduction on egg loads in liver tissue.

The effect of garlic and mirazid may not be on ovum itself, since no effective anti-schistosomal drug acts on the eggs themselves. Eggs continue their development in the tissue up to maturation for a period of 12 days and elimination in the stools usually display no effect on the ova themselves. And ova depositions by worms continue their development in the tissue up to maturation. The mature ova remain alive in the tissues for a period of 12 days until their death and elimination in the stools (Standen, 1953). Accordingly, the significant reduction of ova might be due to the effect of garlic and Mirazid administration on worm fecundity, where some drugs seem to act initially on the reproductive organs of the worms. The reduction of ova loaded was occurred.

Riad *et al*. (2007) reported a significant reduction in the egg load after treating infected mice with aqueous garlic extract in acute and early chronic stages. El Shenawy *et al*. (2008) stated that aqueous garlic extract impaired the development and maturity of *Schistosoma* eggs, as the treatment resulted in the appearance of high numbers of dead eggs in the oogram assessment. This is possibly due to a positive linear relationship between the egg output and the worm burden, where the reduction of the number of worms is correlated with the reduction in the ova count. This is also suggested by Gryseels and Polderman (1991). However, several other factors may explain such reduction in schistosomal egg count. Probably, these factors diminished the fecundity of the worm pairs and increased the rate of egg excretion due to the egg death. Also, the present results recorded improvement levels of all enzymes under investigation after treatment of *S. mansoni* infected mice with mirazid and garlic. This amelioration was confirmed by a significant reduction of worm burden and ova count after treatment. Present data is confirmed by the previous reports by Hamed and Hetta (2005). They concluded that mirazid is an effective drug for treatment

of *Fasciola hepatica*, *S. mansoni* and *S. haematobium* through worm disruption, collapse of tubercles and reduction of worm burden as well as the reduction in ova count in urine and stool (Hamed and Hetta, 2005). Toxic substances and free radicals elaborated by worms consume antioxidants and may affect the capacity of the liver to detoxify or naturalize the effect of the toxic endogenous and exogenous compounds (Sheweita *et al*., 1998). This gives an additional support that the improvement pattern in liver enzyme after treatment with Mirazid resulted from its antioxidant capacity.

The liver plays an important role in the vital activities of the body where its hepatocytes show differences in the localization and concentration of some enzyme systems. Many of these enzymes served as marker enzymes for different cell organelles and any defect of them will be reflected to the enzyme activity itself (Hamed and Hetta, 2005). Hence, studying changes in these enzymatic activities could be helpful in evaluating the possible side effects of different treatments on different cell organelles after *S. mansoni* infection and the improvement occurring in such enzymes after treatment.

Serum AST activity is more useful in assessing the histological severity of liver disease probably because this cytosolic and mainly mitochondrial enzyme is present in larger quantities in the liver compared to the cytosol and thus more is released when tissue damage is more severe (Aranda-Michel and Sherman, 1998). These enzymes are commonly employed as biological markers for hepatic cell damage and impaired cell membrane permeability or due to heavy *Schistosoma* egg deposition. There was significant difference ($p<0.05$) between the effect of Aged Garlic Extract (AGE) on AST, ALT and ALP (El Shenawy *et al*., 2008). So, In the present study, serum total bilirubin, ALT and AST activities increased progressively in *S. mansoni* infected mice in group I, this attributed to greater destructive changes in liver compared with the group II, III and IV.

Results showed that garlic and Mirazid administration showed marked anti-inflammatory activity where it significantly reduced the volume of granuloma size by 38, 46 and 67% in groups treated with garlic, mirazid and mixture of both them respectively when compared with the infected untreated group. Also, the hepatic lobular architecture restored its normal organization and most hepatocytes showed normal appearance, even in the vicinity of the granuloma. However, some sinusoids were still dilated and infiltrated with lymphocytes and some Kupffer cells were still hypertrophied, where the more hepatic lobular architecture restored its normal organization increasingly in treatment with garlic, mirazid and combination of them.

To our knowledge, this study is the first in using the combination of garlic and Mirazid in schistosomal treatment in mice. Marked treatment results were in infected mice treated with the combination of garlic and Mirazid than mice treated with either mirazid or garlic alone.

In conclusion, garlic, mirazid and a combination of both have the anti-schistosomal activity in mice. It significantly reduced the production of eggs output in *S. mansoni* infected mice. In addition this study showed that garlic and mirazid clearly reduced liver granuloma size and this modulation of the liver granulomas was reflected on some degree of improvement in the status of the liver.

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