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Comparative *In vivo* Antioxidant Levels in *Schistosoma mansoni* Infected mice Treated with Praziquantel or the Essential Oil of *Melaleuca armillaris* Leaves

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Abstract: Plant extracts are continuously investigated for their extensive inclusion of biologically active constituents that exert therapeutic activities against many diseases. The aim of this study was to assess the antioxidant/anti-schistosomal activities of the essential oil of the fresh leaves of *Melaleuca armillaris* (*M. armillaris*) compared to Praziquantel (PZQ) on normal and *Schistosoma mansoni*-infected mice. The oil was isolated by hydrodistillation and analyzed by gas chromatography/mass spectrometry (GC/MS). The oil was rich in 1,8-cineole (33.93%), terpinen-4-ol (18.79%), limonene (10.37%) and B-pinene (6.59%). *M. armillaris* oil (150 mg kg^{-1}, orally) was administered from the second week post infection twice per week for six weeks. PZQ (500 mg kg^{-1}, orally) was administered for two successive days 8 weeks post infection and mice sacrificed one week later. Total protein, Malondialdehyde (MDA), Glutathione (GSH), vitamins C and E, the antioxidant enzymes catalase and superoxide dismutase, as well as liver weights and liver/body weight were determined in the liver tissues. Results showed that, both treatments significantly ameliorated the disturbed levels of GSH and MDA in infected mice. Both vitamins were significantly elevated after treatment with the oil while a significant increase in catalase accompanied by a pronounced decrease in SOD were obtained after treatment with PZQ. Both treatments markedly improved liver and body weights in infected mice compared to the infected-untreated ones. In conclusion, natural plant sources may be used as promising alternative agents to chemical drugs for schistosomiasis treatment, since the latter may result in drug-induced resistance arising from repeated use.

Key words: Schistosomiasis, *M. armillaris* leaves, essential oil, praziquantel, antioxidant

INTRODUCTION

Plants of the genus *Melaleuca* are rich in volatile oils. They are mainly used in the manufacture of cosmetics as germicides and as antiseptic agents. They are also used as carminatives and in the treatment of several ailments. In Egypt; few investigations have been carried out on *Melaleuca* species (Aboutabl *et al*., 1991; Farag *et al*., 1998).

Schistosomiasis is a water-borne tropical disease that is caused by trematode worms infecting humans and some other mammals (Li *et al*., 2011). The World Health Organization (WHO) indicated that more than 200 million people are infected worldwide (Wu *et al*., 2010). When infecting their mammalian hosts, schistosomes are encountered by the release of reactive oxygen species generated by their own aerobic respiration as well as by the host's immune assault (Sayed *et al*., 2008). In this atmosphere, these worms possess active mechanisms to maintain cellular redox balance correlated with their survival in the host tissue. Due to the high rate of oxidative processes and the peroxidative damage to the liver microsomal membrane lipid, an excessive formation of hepatic malondialdehyde (MDA) results with impairment of the endogenous antioxidant defence (Othman *et al*., 2008).

Most pathology in *Schistosoma*-infected animals is attributed to the host's reaction to the eggs which is maximal by the 8th week of infection. The toxic egg material destroys the host tissue cells and the antigenic material stimulates the development of large inflammatory reactions (granuloma) around the egg (El-Shenawy and Soliman, 2003). This granuloma is considered to serve as a protective barrier by sequestering the toxic and antigenic substances secreted continuously from *Schistosoma* eggs.

In the last two decades ambitious efforts have been made to develop an effective vaccine against schistosomes, but without resounding success (Quack *et al*., 2006) and praziquantel remains the mainstay of disease control. PZQ has high cure and egg-reduction rates with only mild side effects, but suffers from two

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serious drawbacks. First, it is active only against the adult stages of the worm which means that it exerts its effect after sexual maturation and oviposition. Despite parasitological cure, the patient may still suffer from the consequences of the progress of disease pathology. Second, a series of laboratory studies and clinical trials have raised concerns about the possible development of tolerance and/or resistance to praziquantel (Doenhoff *et al*., 2008). This urges the need to develop complementary and alternative new antihelminthic agents that are both effective and with minimal side effects (El-Banhawey *et al*., 2007). In this concern, several recent research studies have shown that certain plant extracts such as *Zingiber officinale* (Mostafa *et al*., 2011), *Allium sativum* and *Allium cepa* (Mantawy *et al*., 2011), *Artemisia annua* and *Frumaria officinalis* (Ferreira *et al*., 2011) possess potent antischistosomal activity. Also, Ali (2007) and Allam (2009) reported the efficiency of *Citrus reticulata* and curcumin as antioxidant and immunomodulatory agents against schistosoma infection. Furthermore, Bakkali *et al*. (2008) reported that the essential oils contained in different plant species exert a wide range of biological activities.

In the present study, the chemical composition of the essential oil from *M. Armillaris* leaves was investigated and its antioxidant effect on normal and *Schistosoma mansoni* infected mice was studied and compared to the antischistosomal drug, praziquantel.

MATERIALS AND METHODS

Plant materials: The experiment started in January and specimens were collected over a period of ten months. *M. armillaris* leaves were collected from Anshas, Sharkia governorate. Seasonal variation in the oil content of the fresh samples during February, May and November was considered to assess fluctuations in the level of the essential oil in order to determine the best time to harvest the leaves.

Voucher specimen of *M. armillaris* is kept in Orman garden, Giza, Egypt under store code 6-03-61 number of sheet 4.

All chemicals used were of high grade, products of Sigma (USA), Merck (Germany) and Fluka (Switzerland). PZQ was a product of the Egyptian International Pharmaceutical Industries Company (EIPICo).

Extraction of the essential oil of *Melaleuca armillaris*: Specimens were collected in February, May and November. Fresh leaves (200 g) of *Melaleuca armillaris* from each specimen were hydrodistilled for 3 h in a Clevenger's apparatus and extraction was repeated in triplicate. The oil was collected and dried over anhydrous sodium to remove water and the mean weight value was recorded. The percentage of oil in February was 0.39% while it increased in May and November to 0.92%. A mean sample of the three replicate oil specimens (0.74%) was used for the GC/MS investigation using Hewlett Packard spectrometry. Qualitative and quantitative identification of the oil constituents were carried out by comparing the retention times and the mass fragmentation patterns with those of the available authentic, in the database of Kato Aromatic Co, as well as in the previously published data (Jennings and Shibamoto, 1980; Adams, 1989).

Experimental design: Sixty mice were divided into six groups (ten mice each) as follows: Group I: normal healthy control group. Group 2: Normal healthy control group treated orally with praziquantel (500 mg kg^{-1} b.wt. for two successive days) (Shaheen *et al*., 1994). Group 3: normal healthy control group administered the essential oil of the fresh leaves of *M. armillaris* (150 mg kg^{-1} b.wt. twice weekly for six weeks) (Pasquele *et al*., 1991). Group 4: infected group (100 cercariae/mouse subcutaneously) (Peters and Warren, 1969). Group 5: infected and treated with PZQ 500 mg kg^{-1} b.wt. for two successive days eight weeks post infection and then sacrificed after one week. Group 6: infected and treated with essential oil of the fresh leaves of *M. armillaris* twice weekly for six weeks two weeks post infection and then sacrificed . All mice were weighed before scarification. Appropriate anaesthetic and sacrifice procedures were followed ensuring that animals did not suffer at any stage of the experiments and are complied according to legal ethical guidelines of the Ethical Committee of the Federal Legislation and National Institute of Health Guidelines in USA and approved by the Ethics Committee of the National Research Centre in Egypt. Livers were separated from each mouse, weighed and homogenized in PBS buffer, then subjected to the different biochemical analyses. Protein was estimated using the method of Bradford (1976) using bovine serum albumin as standard. Hepatic Lipid Peroxidation (LPOX) level was measured by a colorimetric reaction with Thiobarbituric Acid-positive Reactant Substances (TBARS) and was expressed in terms of the malondialdehyde (MDA) concentration as described by Ohkawa *et al*. (1979). The levels of TBARS were expressed as micromoles of MDA per mg of tissue (mmol mg^{-1}). The hepatic reduced glutathione (GSH) level was determined by the method of Moron *et al*. (1979) using 5,5'-dithiobis-2-nitrobenzoic acid (DTNB). GSH levels were calculated using standard curve prepared by known amounts of GSH. The concentration of GSH was expressed as mg g^{-1} tissue. Vitamin C was estimated by

the method of Jagota and Dani (1982) using ascorbic acid as standard. Vitamin E was determined using α-tocopherol as standard according to Baker and Frank (1968). The activity of superoxide dismutase was assayed as described by Nishikimi *et al.* (1972) using NADH and measuring the change in absorbance with time. Catalase activity was measured according to the method of Lubinsky and Bewley (1979) based on the disappearance of H_2O_2 using molar extinction coefficient of 62.4.

Statistical analysis: The data of the present study were statistically evaluated using SPSS computer program and comparison between groups was analyzed using the student t-test. The level of significance was determined at p-level ≤ 0.001.

RESULTS

The oil content *M. armillaris*, increased from 0.39% in February to 0.92% in May and remained at the same level during November.

Twenty five compounds have been identified in the *Melaleuca armillaris* oil, amounting to 93.66% of the oil. GC/MS investigations of these constitiuents are presented in Table 1. It could be noticed from the table, that 1,8-cineole is the major constituent in this oil (33.93%), followed by terpinene-4- ol (18.79%) and γ-terpinene (10.37%). Sabinene and α-terpineol constituted 6.59% and 5.80% of the oil, respectively beside much lower concentrations of α-thujene, β-pinene, β-myrcene, limonene, p-cymene, terpinolene, cispinene hydrate, linalool, trans- pinene hydrate, β-germacrene, trans-alpha-dehydroterpineol, terpinyl acetate, α-terpineol, germacrene-D, delta-elemene, cis-piperitol, α-cadinene, geraniol and eugenol.

The data obtained show that total protein content of uninfected mice that were treated with *M. armillaris* oil was found to be higher , while total protein content of infected untreated and treated mice were found to be significantly lower than that of control untreated group. In addition, the level of the disulphide antioxidant GSH declined significantly in the infected group as compared to the control group, while both treatments significantly reversed this reduction, with nearly similar effects. On the other hand, liver MDA increased significantly in the infected group than that measured in the control group and treatment with PZQ or *M. armillaris* oil decreased the elevated MDA level significantly the latter showing a more pronounced amelioration (Table 2). In infected mice, the levels of vitamin C, vitamin E and catalase were reduced while that of SOD was increased. The levels of both vitamins were significantly elevated after treatment with the oil while treatment with PZQ was less effective. In contrast, PZQ resulted in a significant increase in catalase activity accompanied by a pronounced decrease in SOD, while *M. armillaris* oil did not show a positive improving impact on those enzymes (Table 3). It should be pointed out that both treatments on the uninfected control mice caused non-significant alterations in the levels of the studied parameters.

Table 1: Chemical constituents of the essential oil of *M. armilaris*

	M^+ m/e	Oil percentage	Identified constituents
93	136	0.02	α-Pinene
91	136	1.52	α-Thujene
93	136	1.35	β-Pinene
93	136	6.59	Sabinene
41	136	2.28	β-Myrcene
41	136	5.8	α-Terpinene
43	136	2.61	Limonene
43	154	33.93	1,8-Cineole
93	136	10.37	γ-Terpinene
119	134	0.59	ρ-Cymene
93	136	2.03	Terpinolene
71	223	0.04	Cis-pinene hydrate
43	154	0.67	Linalool
			Tans-pinene hydrate
69	219	0.43	Terpinen-4-ol
71	154	18.79	β-germacrene
93	204	0.29	T. α- dehydroterpineol
59	136	0.28	Terpinyl acetate
196	196	3.09	α-Terpineol
			D-Germacrene
59	154	3.49	D-elemene
121	204	0.24	Cis-Piperitol
93	204	0.21	α-Cadinene
84	154	0.14	Geraniol
161	204	0.29	Eugenol
69	154	0.06	
164	164	0.09	

On the other hand, the body weight, liver weight and percentage of liver weight to body weight were also unaffected by both treatments to control mice although infection caused a high significant body weight loss accompanied with an increase in liver weight and in liver to body weight ratio. Treatment of infected mice revealed a pronounced improvement in these parameters (Table 4).

DISCUSSION

Previous animal studies have shown that the acute phase of schistosomiasis occurs roughly five to six weeks post-infection resulting in liver dysfunction and disturbances of metabolic processes (Manivannan *et al.*, 2010).

An early event following infection with *S. mansoni* is the production of excessive Reactive Oxygen Species (ROS) which induces hepatic oxidative stress (Mohamed *et al.*, 2008). In the present work, infection with *S. mansoni* impaired the antioxidant system since the levels of the measured endogenous antioxidants were

Table 2: Levels of total protein, lipid peroxides and glutathione in different groups

	Control(C)	C+PZQ	C+MA	Infected	Inf.+PZQ	Inf.+MA
Total Protein (mg g^{-1}) ±S.D.	148.60 ±1.55	146.53± 1.15	158.39±1.46	124.80± 1.10	120.13 ±0.42	125.56 ±1.50
p≤		n.s	0.001	0.001	0.001	0.001
% change		-1.39	+15.78	-16.00	-19.12	-10.95
Lipid Peroxides (µmol/mg)	2.83± 0.06	2.20 ±0.07	2.75± 0.12	4.72± 0.095	3.37± 0.19	2.9± 0.18
p≤		0.001	0.001	0.001	0.001	0.001
% change		-22.0	-18.7	+66.78	+18.90	+32.50
GSH (mg g^{-1})	1.662± 0.03	1.46± 0.02	1.64± 0.022	0.997± 0.025	1.15± 0.035	1.33± 0.02
p≤		0.001	0.05	0.001	0.001	0.001
% change		-12.0	-4.5	-40.0	-32.0	-20.0

C: Control, PZQ: Praziquantel, Inf: Infection, MA: *Melaleuca armillaris*, GSH: Reduced glutathione, Data are the mean (S.D.) of determinations of ten mice in each group, the significance level as compared to control (p 0.001: highly significant), n.s: No significant

Table 3: Levels of vitamin C (µg g^{-1}), vitamin E (mg g^{-1}), superoxide dismutase (units/mg protein) and catalase (µmol/mg protein) in different mice groups

	C	C+PZQ	C+MA	Inf.	Inf.+PZQ	Inf.+MA
Vit.C	186.3±2.0	193.2±2.3	202.25±7.5	173.6±4.3	175.7 ±5	206.8± 13.0
p≤		0.01	n.s.	0.001	0.01	0.01
%change		3.65	-3.65	-6.8	-5.7	-5.8
Vit.E	2.96±0.44	2.89±0.21	3.38± 0.23	1.87±0.26	1.98 ±.14	6.42± 0.76
p≤		n.s.	n.s.	0.001	0.01	0.01
%change		-2.4	-7.1	37	-33	-32.56
SOD	156.3±5.7	162.4±6.0	146.54±6.08	183.1±6.9	168.6±6.19	187.3± 9.7
p≤		n.s.	n.s.	0.001	0.05	0.01
%change		3.9	3.8	17.14	7.9	9.7
Cat.	85.54±4.6	89.91±3.6	80.7± 1.40	64.08±2.6	74.94± 2.9	56.9± 2.67
p≤		n.s.	n.s.	0.001	0.01	0.001
%change		5.12	-2.22	-25	12.4	-18.5

Vit.C: vitamin C, Vit E: Vitamin E, SOD: Superoxide dismutase, Cat.: Catalase, C: Control, PZQ: Praziquantel, Inf: Infection, MA: *Melaleuca armillaris*, Data are the mean (S.D.) of determinations of ten mice in each group, the significance level as compared to control (p 0.001: highly significant), n.s: No significant

Table 4: Body weight, liver weight and liver weight/body weight percent in different infected and treated groups

	C	C+PZQ	C+MA	Inf.	Inf.+PZQ	Inf.+MA
b.wt.(g)	31.7±0.66	30.7± 0.33	31.25± 0.47	26.6± 0.55	27.5 ± 0.50	28.5± 0.86
p≤		0.05	n.s.	0.001	0.001	0.001
%change		-3.1	-1.57	-13.8	-13	-11.56
L.wt.(g)	2.0±0.049	1.98± 0.08	1.98± 0.05	2.44± 0.04	2.33± 0.05	2.45±0.05
p≤		n.s.	n.s.	0.001	0.01	0.01
%change		-1	-5	22	16.5	12.5
L.wt./b.wt. %	6.40 ±.07	6.46 ±.20	6.31± 0.14	8.86± 0.29	8.48± 0.10	8.67±0.08
p≤		n.s.	n.s.	0.001	0.001	0.001
%change		0.9	-3.4	38.39	32.45	25

b.wt.: body weight; L.wt.: liver weight, Data are the mean (S.D.) of determinations of ten mice in each group, The significance level as compared to control (p≤0.001: highly significant), n.s: Nonsignificant

depleted associated with an overproduction of malondialdehyde, one of the final products of lipid peroxides in host cells. Lipid peroxides serve as a marker of cellular oxidative stress and have long been recognized as a major consecutive factor of oxidative damage in different diseases (Son *et al*., 2007).

The present results indicate that GSH, a key antioxidant , is markedly depleted in infected mice groups, a sign that hepatic cells are utilizing more antioxidant defences. Gharib *et al*. (1999) attributed the decreased level of glutathione to the increased oxidative stress and the cytoxicity with H_2O_2 which is produced as a result of inhibition of glutathione reductase that keeps glutathione in its reduced form. Since administrations of PZQ or *M. armillaris* oil ameliorated the levels of GSH and lipid peroxides, it appears that the protective effect of these treatments involves the maintenance of antioxidant capacity in protecting the hepatic tissue against oxidative stress by scavenging the very reactive hydroxyl and peroxyl radicals.

In addition the data obtained revealed that infection resulted in a decline in protein content which may be due to protein shift towards catabolism and disabling of the infected liver to synthesize proteins (El-Fakahani *et al*., 1993). Aly and Aly (2006) attributed the decrease in total protein to increase in messanger RNA degradation. Also, Moawad (2007) reported that liver dysfunction and tissue damage of *S. mansoni* infected mice induced by oxidative stress result in damage in cell membrane structure and intracellular organelles, thus increasing protein permeability and its loss in urine. These effects were only reversed by using *M. armillaris* which may possess

potent antioxidant activity that enhances the host immune system and retain the liver functions. Also both vitamins C and E decreased by infection due to the impaired antioxidant system and the scavenging activity of these vitamins to the generated free radicals. In accordance with these data, Rizk *et al.* (2006) and Maghraby *et al.* (2010a) reported that the free peroxyl radicals produced during infection are effectively trapped by ascorbate and that vitamin E protects biological membranes, hence maintenance of cell function against oxidative stress. Both treatments, however had a slight impact on vitamin E but did not affect the level of vitamin C.

The antioxidant enzymes superoxide dismutase and catalase keep homeostasis and protect against oxidative damage by removing toxic free radicals in vivo (El Shenawy *et al.*, 2008; Jia *et al.*, 2009). The results obtained showed that the activity of SOD increased while that of catalase decreased by infection and both treatments under study restored the activities of these enzymes. The decrease in catalase activity could be attributed to its utilization in scavenging the free redicals overload generated during schistosomiasis. Enzymatic scavengers like catalase and SOD protect the system against the deleterious effects of reactive oxygen species (Aslan and Karafakioglu, 2010; Maghraby *et al.*, 2010b). These data coincide with the data of El-Rigal *et al.* (2006) who found that catalase, vitamin C and vitamin E were reduced by Schistosoma infection and were elevated by administration of *Ailanthus altissima* and *Ziziphus spina* extracts. Furthermore, Rizk (1998) and Allam (2009) reported that catalase activity was enhanced in infected mice using curcumin.

The present study also revealed that infection caused a decrease in body weight accompanied by an increase in liver weight resulting in higher values of liver to body weight ratios and treatment with PZQ or M.armillaris partially ameliorated these effects, the latter showing more pronounced improvement. According to these data, Fiore *et al.* (1996) found a reduction in body weight and an increase in liver weight starting from the sixth week of infection and attributed this to the eggs and other metabolites produced by worms which affect the host tissue. These dramatic changes in infectious state can be explained on the basis of *S.mansoni* eggs trapped in the host live which elicit a chain of oxidative processes that may be, at least in part, responsible for the pathology and progression of fibrosis associated with schistosomal infection. In a parallel study, Hamed (2011) attributed the increase in liver weight to egg deposition by worms and release of other metabolites that affect the hepatic tissue. The significant improvement in the previously mentioned parameters after treatment of infected mice with praziquantel result from the effect of PZQ on worm burdens since PZQ causes high degree of worm tegumenal damage (that is accompanied by a large influx of calcium into worms caused muscular contraction, surface disruption and eventual death of the parasite) that consequently limit higher immune response so enhance immune response of the host and generate a reversal of the level of fibrosis (Botros *et al.*, 2006; Pica-Mattoccia *et al.*, 2008). Thereby as evidenced by several studies the significant reduction in oxidative stress initiate a positive impact on the preservation of liver integrity, function and antioxidant systems (Martins-Leite *et al.*, 2008).

Generally, the chemotherapeutic control against an important disease such as schistosomiasis relies on just one drug, praziquantel (Cioli *et al.*, 2008). Consequently, many herbal formulae or plants have been traditionally prescribed to be potential therapeutic candidates for hepatic diseases (Shin *et al.*, 2010).

The mechanisms whereby the consumption of certain plants and plant extract can affect parasite viability, mobility and fecundity both *in vivo* and *in vitro* are largely unknown. For some plants, it has been suggested that their consumption could be associated with an enhanced immune response of the host towards the parasites, as result of nutrient supplementation and thus improved nutrition. It is known that high dietary protein intake in animals can enhance the immune response of ruminants towards parasites. However, it appears that many plants that have been reported to have anthelmintic properties actually contain compounds that are directly active against parasites. In many cases these active compounds are secondary metabolites, i.e., plant products that have been associated with defensive mechanisms. Saponin, alkaloids, non- protein amino acids, tannins and other polyphenols, liginin , glycolides are all secondary metabolites and some of them have been considered responsible for the anti-parasitic effect of plants (Domo *et al.*, 2009; Jatsa *et al.*, 2009; Chabir *et al.*, 2011). Brophy and Lassak (1983) previously reported that the oil of *M. armillaris* contained 1, 8-cineole as a main constituent and this was emphasized in the present study, since a high content of 1,8-cineole and terpinene-4-ol were found in M.armillaris oil which may be responsible for the anthelmintic effect detected in the present study. In good correlation with the present data, Parreira *et al.* (2010) and Magalhaes *et al.* (2012) confirmed the potent antischistosomal activity of the essential oil from *Baccharis dracunculifolia* leaves and *Piper cubeba* L, respectively.

CONCLUSION

This study provides findings on the antioxidant effect of *M. armillaris* oil and hence may be helpful for future application as a traditional plant which can be safely used to enhance the antioxidant defence system, reduce disease complications and compensate for the probable occurrence of drug resistance.

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