



**ANTISCHISTOSOMAL AND IMMUNO-BIOCHEMICAL EFFECT OF
MILTEFOSINE AND PLANT EXTRACT ON INFECTED ALBINO MICE WITH
*SCHISTOSOMA MANSONI***

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ABSTRACT

This study aims at assessing the impact of miltefosine and *Euphorbia splendens* extract on mice infected with *Schistosoma mansoni*. Then, the parasitological parameters, worm burden/mouse, number of ova/g tissue in the liver and intestine and the developmental stages of ova in the small intestinal wall (Oogram) of infected treated mice were determined. In addition, certain biochemical parameters, e.g., total protein, albumin, ALT, AsT, AkP and AcPin mouse serum were recorded and cytokines were assessed in an infected hamster model. The results showed that treatment of *S. mansoni* infected mice with miltefosine and *Euphorbia splendens* extract reduced the worm burden/ mouse and the few worms recovered from sacrificed mice in this treatment failed to lay ova (100% reduction of ova/g tissue). The results, also, revealed that treatment of infected mice with miltefosine followed by *Euphorbia splendens* extract ameliorated the activities of the serum enzymes ALT, AsT, AkP and AcP in comparison with those of an infected untreated group. In addition, immunization caused an increase in the immunoglobulins and cytokines.

Keywords: *Schistosomamansoni*, miltefosine, *Euphorbia splendens* extract, liver function enzymes, Albino mice

INTRODUCTION

Schistosomiasis is one of the tropical diseases most widely, which is focused mainly in sub-Saharan Africa's burden and impact on approximately 207 million people[1-4]. Therapy of established infections relies almost exclusively upon praziquantel, which affects schistosome voltage-gated calcium channels[5] that make treatment with praziquantel is the core component of schistosomiasis control programs [6-8].

A combination of Artemether and PZQ in humans and animals showed that combination therapy resulted in a significantly higher reduction in worm burden than administration of either drug alone[9]. Acute bilharzias have a major impact on the functions of the liver and specific alterations in defining protein isoforms and up regulation of the unique proteins that may be of value as markers of a new disease [10]. It may be of value as new signs of disease[10]. Bilharzias disease is a direct result of the immune response to lay eggs in the host tissues, especially the liver. The associated liver injury usually with the infiltration of inflammatory cells, lead to cirrhosis [11].

Investigators have concentrated on various preventive methods against schistosomiasis using several fractions soluble egg antigen (CEA) which have been identified and tested in the

experimental models with induces at various levels of protection against infection[12]. Immunisation of mice stimulates specific immunity which causes reduction in worm burden, intestinal egg load and liver pathology[13]. Until recently, none of immunization fractions were able to induce more than 67% protection, partially protective immunity would make a logical complement to drug therapy[14].

Miltefosine (hexadecylphosphocholine), an alkylphosphocholine ester of hexadecanol, a membrane-active, drug with demonstrated activity against various parasite species and cancer cells

This work aims to assess the impact of miltefosine and *Euphorbia splendens* on infection of mice with *S. mansoni* cercariae and liver enzymes in some *S. mansoni* infected mice. Moreover, parasitological parameters and the dynamics of serum-specific immunoglobulins and splenic cytokines associated with changes in hepatic pathogenesis were assessed in an attempt to study the effect of treatment with miltefosine and *Euphorbia splendens* on infected hamster model.

MATERIALS AND METHODS:

Experimental Animals

Male Swiss albino mice (weight, 20 ± 2 g), bred and maintained at

Schistosomias are Biological Supply Centre (SBSC) Theodor Bilharz Research Institute (Giza, Egypt), were used. They were fed a standard commercial pellet diet and were kept in an air-conditioned room at 21°C. All animal experiments were conducted in accordance with valid guidelines for the animal ethics committee.

Mice Infection

S. mansonicercariae were provided by SBSC, Theodor Bilharz Research Institute (TBRI). Infection was subcutaneously performed using freshly shed 60±10 *S. mansonicercariae* per mouse.

Drugs

Miltefosine was manufactured and provided by [Sigma-Aldrich Chemie GmbH, CA 58066-85-6, MW 407.57, Germany]. The administration doses of the drug started after ten days post infection (PI).

Plants

The tested plant was *Euphorbia splendens* (Euphorbiaceae) were collected from the fields of the Giza governorate (May 2005) and were kindly identified via a specialist in the Botany Department, Faculty of Science, Cairo University. Shade dried, powdered and stored in a clean, dry dark glass bottle.

Plant's Extract

The dry powder of the plant was extracted by soaking with 95% methanol

(0.5 kg/l) for seven days. Then the solvent was filtered and distilled off under vacuum and the crude extract residues were stored in a clean, dry dark vessel till use [15].

Toxicity of The Tested Plant Extracts in Albino Mice

The acute toxic effect of the plant methanol extract to albino mice (20–25 g) was previously recorded by Kamelet al. [16].

Experimental Design

Three main groups: group (I) is the normal control (ten mice) which received only the vehicle., group (II) was orally administered with miltefosine in a dose of 20 mg /kg /day, group (III) orally administered with the plant extract dose of 20 mg /kg /day and group (IV) infected with *schistosomamansoni* which was further subdivided into three equal subgroups (ten mice each).

Group (IVa) infected untreated mice which received only the vehicle., group (IVb) infected orally administered with miltefosine in a dose of 20 mg /kg /day for 2 consecutive days 7 weeks post infection, Group (IVc) orally administered with plant extract dose of 20 mg /kg /day for 2 consecutive days 7 weeks post infection and Group (IVd) orally administered with miltefosine in a dose of 20 mg /kg /day followed by plant extract after an hour for 2 successive days.

Forty-five days after exposure to cercariae, 3 mice from each infected group of the experiments were sacrificed individually and dissected. The worm load in each hamster was carried out by perfusion according to the method of Kloetzel, [17].

The different developmental stages of *S. mansoni* (the Oogram) are determined to follow the method of Pellegrino *et al.* [18]. The ova count/g tissue (Digestion of liver) was calculated according to Cheever, [19] and Kamelet *al.* [20] biochemical and immunological studies were also done. Another eight group was immunized with SEA (10 µg x3) 6 wks before infection and treated with miltefosine and plant extract (used for the immunological study).

Perfusion of Infected Mice:

Two weeks post treatment; mice were euthanized by decapitation and perfused. The mean number of worms/mouse was determined in each experiment [21].

Egg Developmental Stages (Oogram)

The percentages of immature, mature and dead eggs from the small intestinal wall of infected mice were computed from a total of hundred eggs per intestinal segment. Immature eggs are characterized by partially developed embryos with clear transparent parts within the eggshell. The mature ones contain fully developed meracidium. Dead eggs exhibited

dark, retraction and irregular outline of dead embryos. Three segments per animal were examined [22].

Tissue Egg Load

The number of eggs per gram tissue (liver and intestine) of infected mice was determined [20].

Biochemical Parameters in Serum of Infected Mice:

The serum of sacrificed mice was collected for spectrophotometrically evaluation of total protein [23], albumin [24] and the activities of transaminases (AST & ALT) [23] and phosphatases (ACP and AkP) [25] enzymes.

Immunological Study (12 Weeks Post Infection)

Serum-Specific Immunoglobulin Isotypes

Determination of anti-SEA immunoglobulin subclasses IgG1, IgG2 and IgG4 were measured using indirect enzyme linked immunosorbent assay (ELISA), based on the method of Engvall and Perlman [26]. ELISA Microtiter plates were coated with 100 µl/well of 30 µg/ml of SEA. Sera were diluted 1:20 and anti-mouse IgG subclasses (Binding site, Birmingham, UK) were used at a dilution of 1:500. Absorbance at 492 nm was measured.

Cytokine Assay:

Serum IFN-γ, IL-4 and IL-10 levels were measured by a sandwich ELISA

technique. Briefly, plates were coated with capture antibodies and 100 µl of serum samples. Following the addition of the biotinylated detection antibody and streptavidin-alkaline phosphatase conjugate, the reaction was developed with paranitrophenyl phosphate (Sigma) and absorbance was measured at 405 NM.

Statistical Analysis:

The data are presented as mean \pm standard deviation. The main groups were compared by analysis of variance. Comparison of means was done by 2-tailed unpaired t-test [27] SPSS computer program (version 13.0 windows) was used. The lethal dose (LD₁₀₀) of the tested plants and its 95% confidence limits were calculated [28].

RESULTS

Parasitological studies (Table 1) show that administering miltefosine to *S. mansoni* infected mice in oral dose 20 mg/kg significantly ($p < 0.001$) reduced the number of *S. mansoni* worms recovered from infected mice by 68.6% compared to untreated infected mice. Treatment of infected mice with 20 mg/kg methanol extract of *Euphorbia splendens*. A significantly reduced the number of *S. mansoni* worms recovered from infected mice by 66.1% ($p < 0.05$). miltefosine administration with a plant methanol extract after 7 weeks of infection exhibited a highly antischistosomal effect as the

reduced rate of worm burden/mouse were 97.5%.

Moreover, the ova in the liver and intestine of mice treated with miltefosine alone or after 7 weeks (pi) were diminished 83.9% and 90.8% respectively. Treatment of infected mice with methanol extract of *Euphorbia splendens*. A significantly reduced the number of the ova in the liver and intestine of infected mice by 60.5% and 76.2%, respectively. Therefore, no developmental stages of ova were detected in the liver and the intestinal wall of these mice groups treated mefloquine and plant extract.

Biochemical Studies:

Infection of mice with *S. mansoni* reduced the serum total protein and albumin levels, while the activities of ALT, AsT, AkP and AcP enzymes were increased compared to those of uninfected control group (Table 3).

From table (3), treatment of infected mice groups with miltefosine alone or miltefosine followed by plant extract *Euphorbia splendens* increased the levels of total protein and albumin compared to those of an infected untreated control group. The levels of total protein in the group treated miltefosine followed by plant extract *Euphorbia splendens* was $6.2 \pm 0.4^*$ g/dl compared to $4.8 \pm 0.2^{**}$ g/dl for infected untreated control group ($P < 0.01$). Meanwhile, the activities of the enzymes

AIT, AsT, AkP and AcP in the treated infected groups were reduced compared to those of an infected untreated control group. Thus, the values of AIT and AsT for infected untreated control group were reduced from 78.2 ± 1.2 and 112.2 ± 6.7 U/L to 49.7 ± 1.2 and 62.1 ± 2.1 U/L after treatment of infected mice with miltefosine followed by plant extract *Euphorbia splendens*, respectively. A Similar trend was recorded for infected groups treated with the plant's extract,

Although the serum biochemical parameters of infected mice treated with plant's extract or with miltefosine were ameliorated in comparison with those of infected untreated control group yet, they were still higher than those of uninfected control mice.

Immunological Parameters :

Serum-Specific Immunoglobulin

Isotypes:

There was no significant change in IgG isotypes in the infected control group when compared to normal control. Nevertheless, there is a significant increase in IgG isotypes in immunized infected control and miltefosine, *Euphorbia splendens* and miltefosine with *Euphorbia splendens* extract treated groups when compared to normal control.

Serum-specific immunoglobulin isotypes showed no significant change in the level of IgG isotypes in the treated

groups as compared to immunized infected control. Contrary, there was a highly significant increase in IgG2 level in the group treated with miltefosine with *Euphorbia splendens* (Table 4).

Serum Cytokines Level:

There is a significant increase in the profile of Th-1 related cytokine IFN- γ in the infected ($p < 0.001$) compared to the control. From another side, Cytokine IFN- γ showed a slight increase in the immunized infected control compared to infected control. The group treated with miltefosine, *Euphorbia splendens* and miltefosine with *Euphorbia splendens* indicated a significant decrease in the treated group compared to the immunized infected control ($p < 0.01$).

There is a highly significant increase in the cytokines IL-4 in the infected control as compared to the control ($p < 0.001$). Serum cytokines level for cytokines IL-4 demonstrated a significant reduction in the immunized infected control, group treated with miltefosine, *Euphorbia splendens* and miltefosine with *Euphorbia splendens* ($p < 0.01$) as compared to infected control.

The cytokine IL-10 level showed a slight increase in the infected control compared to the normal control and a highly significant increase in the immunized infected control and group treated with miltefosine, *Euphorbia splendens* and miltefosine with *Euphorbia splendens* ($p < 0.001$) compared to the infected control. It also showed that a

slightly significant increase in the groups splendens compared to immunized infected treated with miltefosine, *Euphorbia* control (Table 5).

splendens and miltefosine with *Euphorbia*

Table 1: Mean worm burden after treatment of *s. Mansoni* infected mice with miltefosine and plant extract of *euphorbia splendens*. After 7 weeks (pi) as a successive treatment with an hour interval, plant's extract doses were for 2 consecutive days:

Animal groups	Mean Worm burden \pm SD (liver and porto-mesentric)				reduction of % total worm %
	Male	Female	Couple	Total	
Control infected	3.4 \pm 1.2	2.6 \pm 1.6	5.2 \pm 0.8	12.1 \pm 0.6	
miltefosine	0.4 \pm 0.3***	0.6 \pm 0.6	2.1 \pm 0.5***	3.8 \pm 0.3***	68.6%
<i>Euphorbia splendens</i>	0.5 \pm 0.5**	0.8 \pm 0.5	2.8 \pm 1.6**	4.1 \pm 0.1***	66.1%
miltefosine followed by plant extract <i>Euphorbia splendens</i> after an hour	0***	0***	0.2 \pm 0.7***	0.3 \pm 0.1***	97.5%

***P < 0.001, **P < 0.01 and * P < 0.05

Table 2: Egg developmental stages after treatment of *s. Mansoni* infected mice with miltefosine and *euphorbia splendens* extract after 3 and 7 weeks (pi) as a successive treatment with an hour interval, plant's extract doses were for 2 consecutive days

Animal groups	Egg developmental stages \pm SD			Number of ova / g tissue (% reduction)	
	Dead	Mature	Immature	Liver (% reduction)	Intestine (% reduction)
Control infected	7.4 \pm 1.2	55 \pm 1.6	21.2 \pm 1.2	1324.2 \pm 243.9	1354.2 \pm 125.3
miltefosine	24.3 \pm 1.5	12.2 \pm 1.1	45 \pm 1.2	213 \pm 3.21 (83.9%)	124.2 \pm 5.3 (90.8%)
<i>Euphorbia splendens</i>	10.2 \pm 4.2	15.2 \pm 1.4	38.8 \pm 2.3	523 \pm 1.3 (60.5%)	322 \pm 7.4 (76.2%)
miltefosine followed by plant extract <i>Euphorbia splendens</i> after an hour	8.1 \pm 2.1	0***	0***	0***	0***

***P < 0.001, **P < 0.01 and * P < 0.05

Table 3: Serum biochemical of mice infected with *s. Mansoni* and treated with miltefosine and *euphorbia splendens* extract after 7 weeks (pi) as a successive treatment with an hour interval, (total protein, albumin, alt, ast, akp and acp), (Mean \pm SD)

Animal groups (doses)	Total Protein g/dl	Albumin g/dl	ALT U/L	AST U/L	AKP U/L	ACP U/L
Control uninfected	7.5 \pm 0.3	5.4 \pm 1.2	42.1 \pm 1.5	39.1 \pm 1.2	49.2 \pm 5.1	9.6 \pm 0.4
Control infected	4.8 \pm 0.2**	3.2 \pm 0.2**	91.2 \pm 5.2***	78.2 \pm 1.2***	112.2 \pm 6.7***	45.1 \pm 1.5***
miltefosine	5.6 \pm 0.4*	4.5 \pm 0.5**	72 \pm 4.1**	56.2 \pm 4.3**	88.2 \pm 11.2***	34.2 \pm 1.1***
<i>Euphorbia splendens</i> extract	4.3 \pm 0.5**	3.1 \pm 0.4*	73 \pm 3.2**	59.2 \pm 2.5**	92.1 \pm 3.2***	35.2 \pm 1.2***
miltefosine and <i>Euphorbia splendens</i> extract	6.2 \pm 0.4*	5.9 \pm 0.2*	59 \pm 2.2**	49.7 \pm 1.2**	62.1 \pm 2.1**	26.2 \pm 1.2***

***P < 0.001, ** P < 0.01 and * P < 0.05

Table 4: Serum anti-sea igg subclasses levels in mice immunized with sea (10 μ g x3). Before infection and treated with miltefosine and *euphorbia splendens* extract then sacrificed 12 wks post infection

Animal Group	X' O.D \pm SEM Ig G1	X' O.D \pm SEM Ig G2	X' O.D \pm SEM Ig G4
Normal control	0.36 \pm 0.4	0.54 \pm 0.46	0.46 \pm 0.5
Infected control	0.34 \pm 0.3	0.52 \pm 0.2	0.45 \pm 0.6
Immunized infected	0.62 \pm 0.5**	0.73 \pm 0.2**	0.82 \pm 0.8**
Treated with miltefosine	0.64 \pm 0.5**	0.75 \pm 0.6**	0.81 \pm 0.5**
Treated with <i>Euphorbia splendens</i>	0.61 \pm 0.5**	0.71 \pm 0.3**	0.81 \pm 0.4**
Treated with miltefosine and <i>Euphorbia splendens</i>	0.65 \pm 0.2**	0.82 \pm 0.6***	0.92 \pm 0.6***

*** P < 0.001, ** P < 0.01, * P < 0.05 relative to infected control.

Table 5: Serum Cytokine Level In Mice Immunized With Sea (10 Mg X3) Before Infection And Treated With Treated With With Miltefosine And Euphorbia Splendens Extract Then Sacrificed 12 Wks Post Infection

Animal Group	IFN – γ Pg/ml \pm SEM	IL – 4 Pg/ml \pm SEM	IL – 10 Pg/ml \pm SEM
Normal control	234 \pm 24.16	42 \pm 0.16	112 \pm 3.4
Infected control	575 \pm 22***	88 \pm 4***	383 \pm 16.6**
Immunized infected	298 \pm 14**	52 \pm 1.4**	586 \pm 18.2***
Treated with miltefosine	165 \pm 26**	59 \pm 5.2**	632 \pm 111***
Treated with <i>Euphorbia splendens</i>	182 \pm 25**	56 \pm 4.1**	623 \pm 114***
Treated with miltefosine and <i>Euphorbia splendens</i>	160 \pm 22**	50 \pm 3.2**	645 \pm 132***

*** P < 0.001, ** P < 0.01, * P < 0.05

DISCUSSION

In the present study, treatment of *S. mansoni* infected mice with miltefosine alone or *Euphorbia splendens* reduced the rates of worm burden/mouse by 68.6% & 66.1% respectively. Moreover, the rates of worm highly reduced from mice Treated with miltefosine and *Euphorbia splendens* (97.5% reduction). This may indicates that development and activities of the alive worms in infected mice treated with miltefosine alone still suffered from this treatment; therefore they died after treatment with plant's extract. The same observation (100% reduction) was recorded in the number of ova in the intestine and liver of infected mice treated with miltefosine alone or followed by plant's extract. This could be due to that treatment of mice with miltefosine alone or followed by plant's extract may deteriorate the reproductive system of the few worms recorded from these mice. Nassauw et al. [29] reported that mefloquine treatment of *S. mansoni* infected mice have a significant effect in reducing egg burden, while it

appeared not to be a useful agent against adult *S. mansoni* worms at the dose 150mg/kg.

This efficacy was also reported by Nwaka and Hudson [30], more than 80% reduction in worm burden was seen by using five consecutive mefloquine doses given interperitoneally or subcutaneously to *S. mansoni* infected mice. Yang et al. [31] reported that the invivo killing effect of mefloquine against juvenile *S. japonicum* is faster than that of other kinds of antishistosomal drugs, such as artemether, niridazole and fuvinazole, and consistent to the mefloquine invitro has fast killing effect against schistosomula [32, 33]. Ingram et al. [34] represent that mefloquine possesses a compelling antischistosomal prototype and might therefore serve as a starting point to identify one or more related compounds with high antischistosomal efficacy invitro and invivo. Also, Kamel et al. [16] recorded 54.4%, 53.7% and 76.9% reduction in worm load/mouse post treatment with these plants. In the same year, Mostafa et al. [35] reported that worm burden and egg density

in liver and faeces of *S. mansoni* infected mice treated with ginger were fewer than in non-treated ones. Parallel findings on the antischistosomal activity of Calotropis procera, Ficus elastic and Zingibarofficinale in mice infected with *S. mansoni* were found by Seif-el-Din et al [36].

Administration of miltefosine and plant's extract alone or combined with each other resulted in a significant improve in serum levels of total protein, albumin, ALT, AsT, AkP and AcP. This was associated with greater granuloma circumscription, more ova degeneration and fewer inflammatory cells, which met with the observations of Nassauw et al. [29]. This was, also, confirmed by EL-Lakkany et al [37] who recorded the levels of total protein, albumin and ALT in serum of infected treated mice with mefloquine were improved, in addition to a great reduction in granuloma diameter with few inflammatory cells. The present results on improvement levels of biochemical parameters of infected treated mice were comparable with those of kamel at al. [16] using *C. ambrosioides* and *S. sesban* alone or combined with each other. And also agree with that of mice groups infected with *S. mansoni* and treated with either thymoquinone [38] or artemether [39]. The combination between miltefosine and *Euphorbia splendens* extract revealed an improved

effect on parasitological and biochemical parameters by enhancing in the histopathological changes.

The percentage decrease in the number of eggs in both SEA infected and treated groups with miltefosine and *Euphorbia splendens* extract was found to be higher in the intestinal tissue than in hepatic tissue. This difference can be attributed to excretion of some ova from the intestine prior to digestion and to hepatic shift of worms after treatment [40]. In SEA experiment, treatment with miltefosine and *Euphorbia splendens* extract caused a drop in immature egg stages and the number of mature eggs with the large increase in the number of dead eggs compared to the findings of Botros et al. [41]. The parasitological improvement may be due to the effects of miltefosine and *Euphorbia splendens* extract that causes a direct or indirect toxic effect in combination with the effect of immunization of SEA that to reduction in tissue egg quantity. The combined effects may have attributed to the significant decrease in the number of worm fertility in the disability of the egg-laying process^[42] According to Abath et al. [43] the manifestations of schistosomiasis are primarily due to granulomatous inflammation from the parasite eggs.

The increase in the production of an immune response an important role in improving liver pathology may play a role

in reduction of the number of Schistosome cercariae eggs found, but also in the worm burden [44-46].

In the present study, also indicated increased production of IgG1 and IgG4 levels. All the treated groups increased in IgG2 levels, This increase in the immune production of renders an important role in improving the pathology and at the same time, then at the same time, the reduction of the number of eggs and the worm burden [44-46].

Cytokines are of particular interest because of their role in the immune responses [47]. Cheever and Anderson, [48] indicated cytokine responses, During schistosomal infection, both Th1 and Th2 responses interferon [IFN- γ] responses to soluble egg antigens and the IL-13, IL-10, and IL-5 response to adult worm antigen [48, 49].

In this study, it may be involved in the production of Th1-cytokine IFN- γ and Th2-cytokine IL-4 in the group immunized

Groups treated with miltefosine and *Euphorbia splendens* extract showed significant decrease in IFN- γ and IL-4. Recent studies indicate that Treg cells play a key in suppressing Th1 cell development as well as limiting the magnitude of Th2 response against egg antigens dependent upon IL-10 [48]. The increasing level of IL-10 is probably implicated in the down regulation of granuloma as it reduces the

inflammatory response in the liver, and therefore an antifibrotic effect [50]. These results indicated the importance of the effect of miltefosine and *Euphorbia splendens* extract having a potent antifibrogenic role.

In conclusion, treatment of *S. mansoni* infected mice with miltefosine and *Euphorbia splendens* extract significantly reduced both worm burden and egg production and ameliorate liver function to seminormal levels. Moreover, normal control mice treated with miltefosine and *Euphorbia splendens* extract did not show any side effects, for most of the parameters, compared to the normal healthy control group. This may give additional support for the protective role of plant extract against schistosomiasis. Treatment with miltefosine and *Euphorbia splendens* extract in conjunction with immunization resulted in a significant decline in the parasitological parameters; and a rise of specific immunoglobulins. In addition, miltefosine and *Euphorbia splendens* extract has antifibrotic and antipathology effects, minimizing and ameliorated liver fibrosis by inhibiting the activation of HSC and the reduction of Treg cells effects. Albeit more research are required, miltefosine and *Euphorbia splendens* extract can possibly be applied, clinically, or in preventive therapy against schistosomiasis, enhancing the positive effects of praziquantel as anti-schistosomiasis drug.

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