

**EVALUATION OF ANTI-LEISHMANIAL EFFICACY BY IN VIVO
ADMINISTRATION OF HERBAL EXTRACT ARTEMISIA AUCHERY ON
LEISHMANIA MAJOR IN BALB/C MICE**

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Summary

Cutaneous infections caused by protozoa the genus *Leishmania* are a major worldwide health problem, with high endemicity in developing countries including the Middle East, Africa and Latin America. In the absence of a vaccine, there is an urgent need for effective drugs to replace and supplement those in current use. We report in this study the *In vivo* efficacy of *Artemisia auchery* Herbal extract on the *Leishmania major* cutaneous infection in susceptible *Balb/c* mice. To carry out this investigation, mice were assigned to 8 groups (each with 3 mice) as healthy negative control, non-treated control, Glucantime-treated and Herbal extract treated-groups. Experimental Leishmaniasis was initiated by the subcutaneous (s.c) application of the 2×10^6 promastigotes of *L. major* (MRHO/IR/75/ER) into the basal tail of the mice of all groups except the healthy negative control group. The development of lesions was determined weekly by measuring the diameters. Inoculations of both Glucantime and Herbal extract were carried out by subcutaneous injections once daily for 15 days. At the end of the treatment course, all mice were killed humanely by terminal anaesthesia and target tissues including lymph nodes, spleen and liver from each mouse were removed and weighted and their impression smears were also prepared. The results indicated that the Herbal extract was effective on lesion size, its performance and to prevent visceralization of the parasite. This is the first report indicating visceralization caused by the cutaneous form of *L. major* in the *Balb/c* mice. During this experiment no side effects were observed in mice treated with Herbal extract. The impression smears showed a reduction of parasite burdens in spleen, liver and lymph nodes. In comparison with Glucantime, the present Herbal combination was more effective on this murine Leishmaniasis, therefore it could be suggested as a substitute for Glucantime in the treatment of Leishmaniasis for human and animal purposes.

Keywords: *Leishmania major*, *artemisia aucheri*, *balb/c* mice, herbal extract, glucantime

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Introduction

Leishmaniasis, a vector-borne disease, caused by a kinetoplastid protozoan parasite of the genus *leishmania*¹⁻³. The parasite is transmitted from one host to another through the bites of female sandfly, or occasionally through non-vector routes including blood transfusion, congenital, sexual, laboratory acquired and person to person³⁻⁵.

There are three main clinical manifestations of leishmaniasis, caused by various species of *leishmania*^{1,3,5} cutaneous leishmaniasis (oriental sore), mucocutaneous leishmaniasis and visceral leishmaniasis (Kala-azar)^{1,6}. Parasites are inoculated by the vector as the flagellated and mobile promastigotes enter the mammalian blood stream, where they are phagocytosed by macrophages, differentiating into nonmotile amastigotes and multiplying as such^{1,7,8}. Immune responses during leishmaniasis include antibodies, cytokines, immune cells, mediators and acute phase proteins^{2,9,10}. Victims of this illness present an immune deficiency and are not able to eliminate the parasites through a natural mechanism of defense^{11,12}. In the absence of a vaccine, there is an urgent need for effective drugs to replace/supplement those in current use^{1,11,13}. The clinically used drugs, many of which are based on pentavalent antimony compounds, were developed before 1959. The toxicity of these agents and the persistence of side-effects even after modification of the dose level and duration of treatment are, however, severe drawbacks, the search for antileishmanial agents has been exhaustive. Alternative drugs, such as amphotericin B and pentamidine, also have unpleasant side effects^{7,11,14,15}. On the other hand, plant extracts or plant-derived compounds are likely to provide a valuable source of new medicinal agents^{1,11,16} and the urgent need for alternative treatments has led to a program to screen natural products for potential use in the therapy of leishmaniasis^{17,18}. In the present study, species of *Artemisia aucheri* was screened in vivo for antileishmanial activity.

Material and Methodes

Animals

Female inbred Balb/c mice (supplied by Karaj Laboratory Animal Unit, Pasteur Institute of Iran) were used in this study. The initial body weight was 18 ± 1.3g (mean ± standard error of mean, SEM) and mice were housed at room temperature (20-23 °C) on a 12 h light and 12 h dark cycle, with unlimited access of food and tap water. Experiments with animals were done according to the ethical standards formulated in the Declaration of Helsinki, and measures taken to protect animals from pain or discomfort. It has been approved by institutional ethical review board (Ethical Committee of the Pasteur Institute of Iran), in which the work was done².

In vitro cultivation of *L.major* IR/75

The *Leishmania major* IR/75 used in this study was the standard strain MRHO/IR/75/ER(IR/75). The infectivity of the parasites was maintained by regular passage in susceptible Balb/c mice. The parasites were cultured in the RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 292 µg L-glutamine and 4.5 µg glucose (all supplied by sigma). Under these culture conditions, the stationary phase of parasite growth was obtained in 6 days as determined by Kavooosi et al¹⁹.

Infection of Balb/c mice with *L.major* IR/75

Promastigotes of *L.major* IR/75 strain were harvested from culture media, counted and used to infect Balb/c mice. The base of the tail was injected intradermally with inoculation of 1 × 10⁶ promastigotes. The animal experiments were performed once in eight groups (n=3 mice/group) considering time, budget and long-period monitoring of animals according to the ethical issues for sample size and replication in order to protect animals from further pain or discomfort. It has been approved by institutional ethical review board in which the work was done. The leishmania infection was carried out in experimental animals and terminated at week 6 after injection².

Experiments and groups

Total number of animals used in this experiment were 24 Balb/c mice, divided into 8 groups (n=3 mice/group) including group 1 (nave), group 2 (nave +0.09 extract), group 3 (nave +0.36 extract), group 4 (nave +1.44 extract), group 5 (*L.major* +1.44 mg/kg extract), group 6 (*L.major* + salin), group 7 (*L.major* + 6 mg/kg extract), group 8 (*L.major* +Glucantime)².

Assessment of pathology

Measurement of lesion size

Lesion size was measured at every week after inoculation in millimeters(mm) by a digital caliper(Chuan Brand, China)in two diameters (D and d) at right angles to each other, and the size (mm) was determined according to the formula: $S=(D+d) \div 2$ ^{2,20}.

Microscopical examinations and smear preparation

The clinical diagnosis was confirmed by laboratory demonstration of the parasite in the lesion by making stained smears at the end of the experimental period. Lesions were cleaned with ethanol and punctured at the margins with a sterile lancet and exudation material was smeared. Impression smears were prepared from liver, spleen and lymph nodes by placing a small piece of tissue between two glass slides and pushing them in different directions. The smears were dried in air, fixed by methanol and stained with Giemsa for detection of amastigotes by light microscopy^{2,9}.

Measurement of proliferation in amastigotes

The proliferation of parasite was evaluated by counting of amastigotes inside macrophages on Geimsa stained lesion smears at the end of the experimental period. Ten random macrophages were selected; counted and mean presentages were calculated as indicators for the degree of proliferation in amastigotes inside each macrophage.

Assessment of degree of hepato/splenomegaly

Entire liver and spleen were removed post mortem at the end of the experimental period from mice after induction of terminal general anaesthesia by inhalation of diethyl ether (Sigma).Organ wet weights were measured and compared with controls as indices for degree of hepatomegaly and splenomegaly.

Body weight

Body weight was measured initially and at different times of experimental using a top pan balance (OHAUS Scale Crop;USA).

Plant materials

Aerial parts of *A.aucheri* were collected from the north state of Iran in the month of May 2006. A voucher specimen was made for sample and was taxonomically identified at the Botany Department, Mazandaran University, Sari, Iran.

Preparation of methanolic extraction

Shade dried and powdered whole plant (100g) was placed in glass percolator with ethanol and was allowed to stand at room temperature for about 24 h (over night). The percolate was collected and combined extract was filtered, concentrated under vacuum using rotavapor at 40 °C and dissolved in sterile 0.9% saline and injected at doses 1.44 mg/kg and 6 mg/kg once daily. Control animals were injected with 0.9% pyrogen-free saline (Pasteur Institute of Iran) and Glucantim (28 mg/kg) as a vehicle once daily at same weeks. In this study, subcutaneous (s.c) route was selected for injections among test and control mices^{20,21,22}.

Statistical analysis

Values for concentrations are presented as the mean \pm SEM for groups of samples. The significance of differences was determined by Analysis of Variances (ANOVA) and student t-test. The upper level of significance was chosen as $p < 0.001$

Results

Healing of cutaneous lesions was studied by measurement of lesion size in both control and test group. Salin had no effects on lesion size, however herbal extract (6 mg/kg and 1.44 mg/kg) showed anti-leishmanial activity, as these compounds reduced the lesion size ($p < 0.05$) after week 6 (fig 1-3).

Percentages of positive Geimsa – stained smears were counted from lesion, liver, spleen and lymph nodes of mice infected with *L.major*. extract (6 mg/kg) had anti – leishmanial activity by reduction positive smears from lesion, liver, spleen and lymph nodes. This extract could be reduced percentage of positive smears in spleen from 66/6% (group 8) to 0% (group 7). They also reduced positive lymph nodes smears from 100% (group 8) to 0% (group 7).

Comparative proliferation of amastigotes inside macrophages was made by observation of Geimsa – stained smears of cutaneous lesions in leishmania groups (fig 4). Efficacy of extract was evidenced to reduce parasite proliferation.

Pathophysiological signs including hepatomegaly, splenomegaly and body weight all were evaluated in groups of nave and *L.major* infected Balb/c mice with or without treatment with extract (fig 5). Leishmaniasis in association with saline and Glucantim showed its pathological effects by increasing degree of hepatomegaly and splenomegaly (fig 6 and 7) as a pathophysiological consequences of disease in infected mice (group 7). Although, extract indicated a reduction in degree of hepato- splenomegaly by comparing with leishmania in group 6 and 8 there are pathologic effects by reducing body weight after treatment with saline.

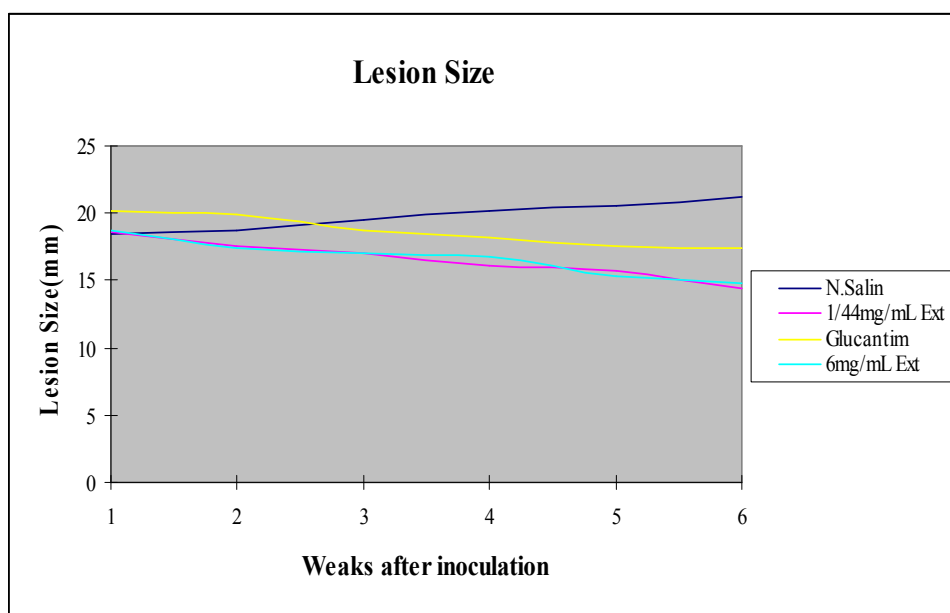


Fig 1. Lesion size of mice after inoculation

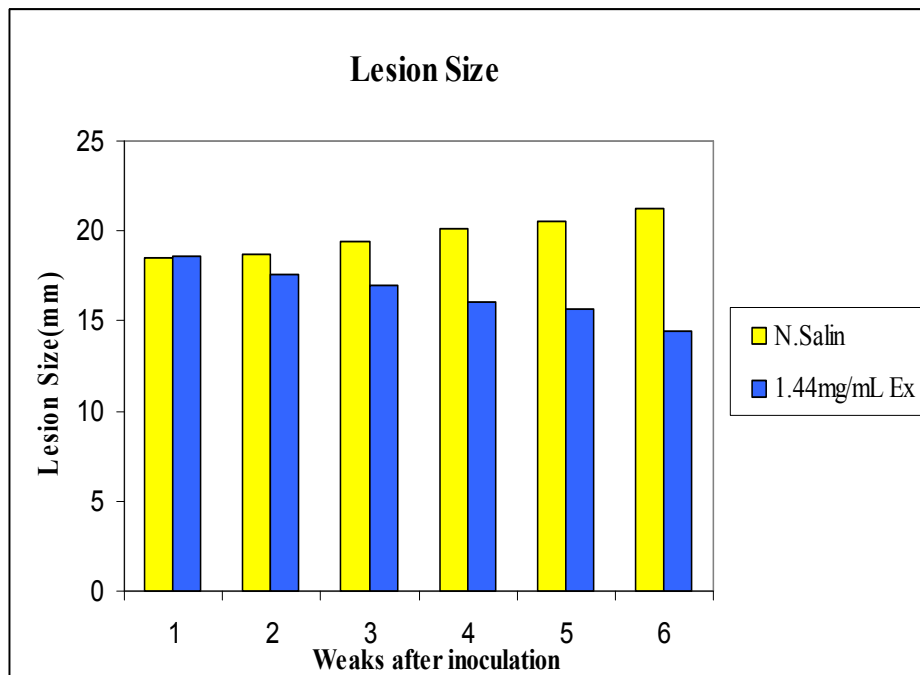


Fig 2. Comparison of lesion size in treated and control groups

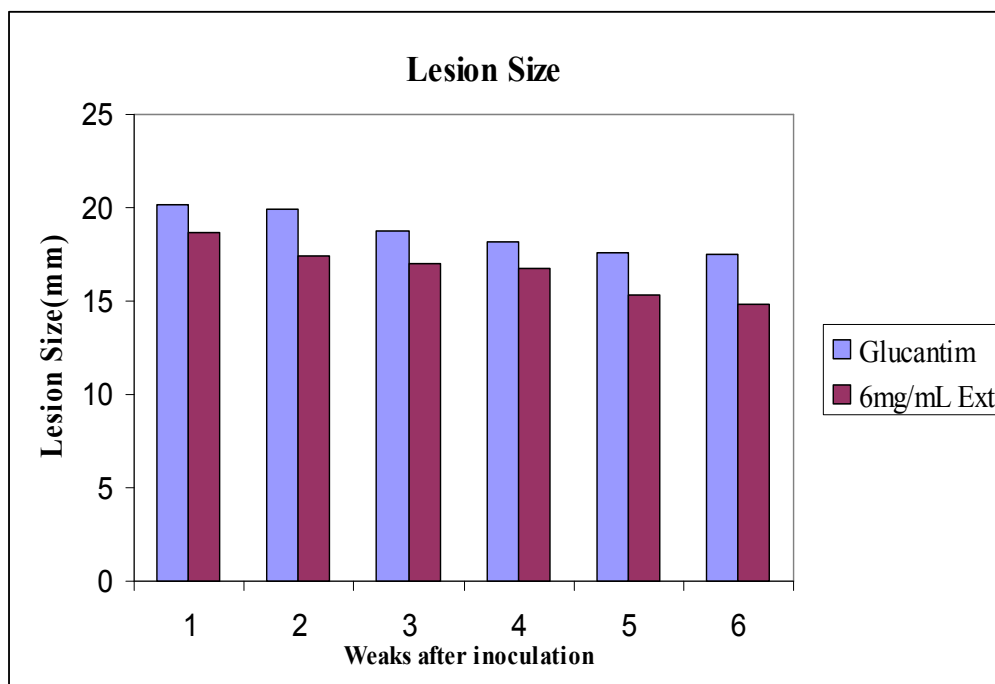


Fig 3. Comparison of lesion size in treated and control groups

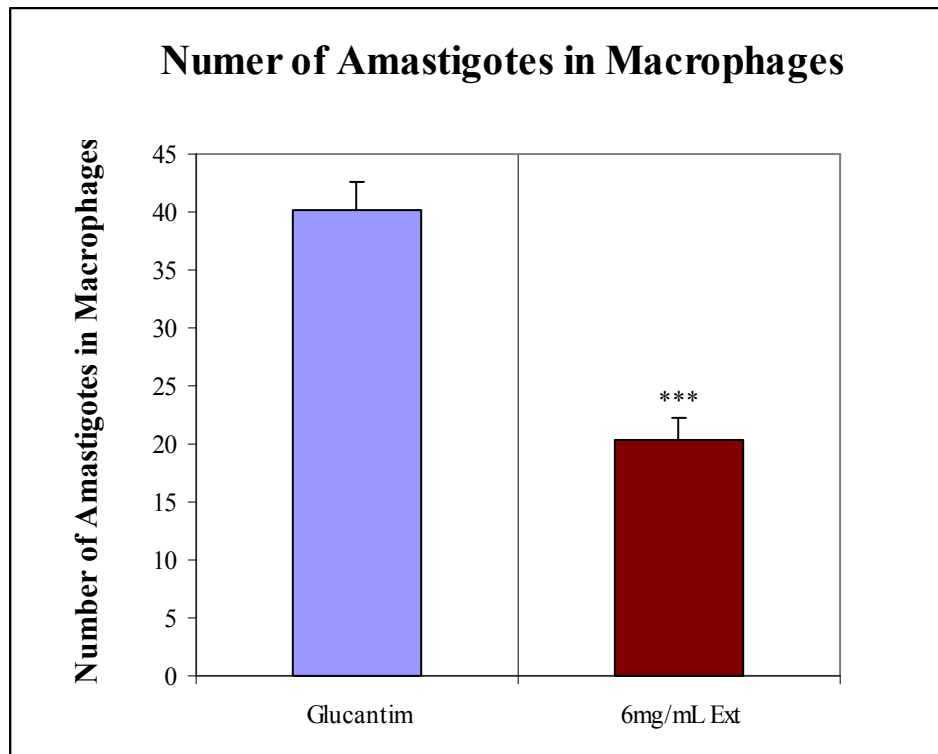


Fig 4. Number of amastigotes in macrophages

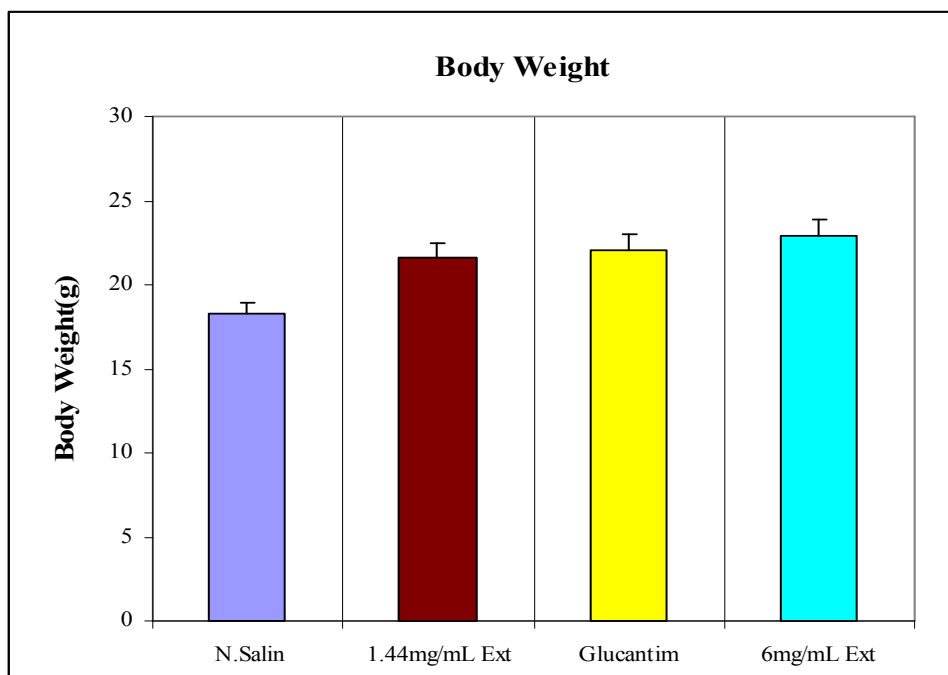


Fig 5. Body weight in response to treatment

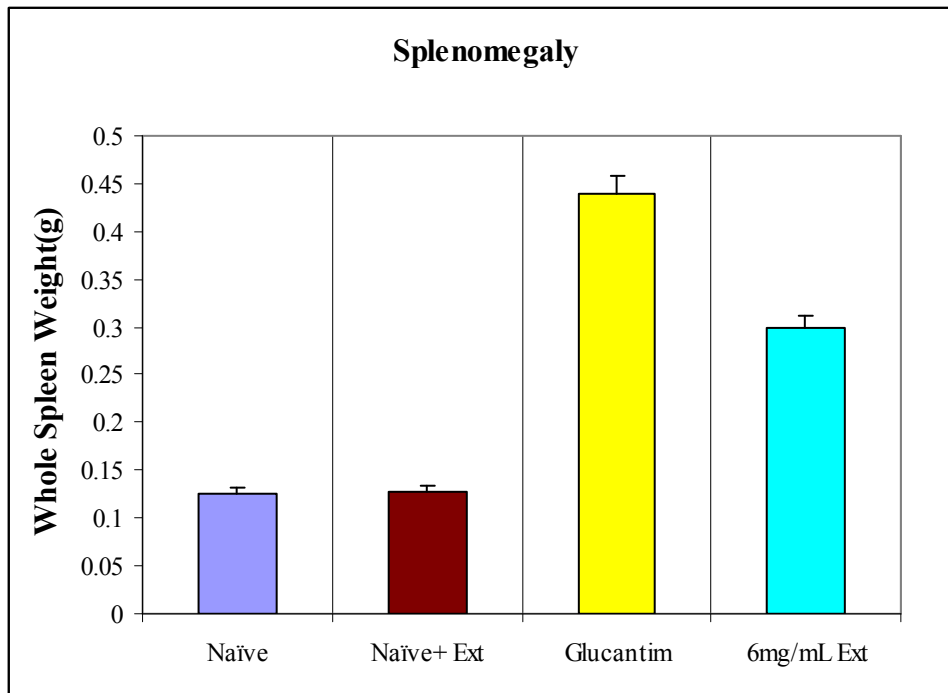


Fig 6. Pathological effects (splénomegaly) after treatment

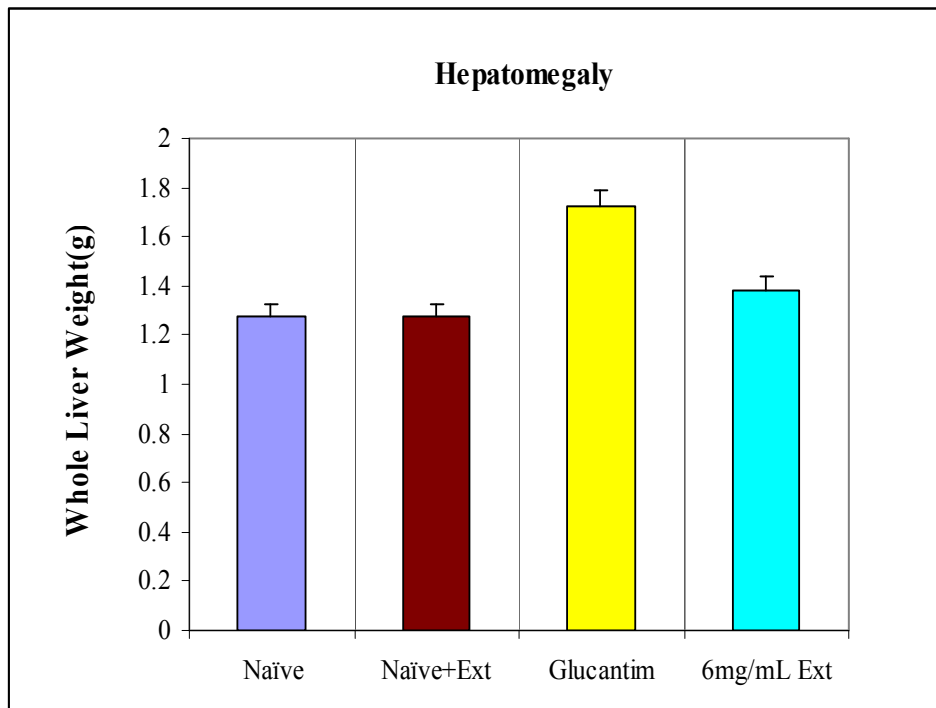


Fig 7. Pathological effects (hepatomegaly) after treatment

Discussion

There are general lack of effective and inexpensive chemotherapeutic agents for treating parasitic protozoan diseases that occur mainly in the developing world^{1,5,11}. One such disease is leishmaniasis. Pentavalent anti monial drugs are the first – line treatment for leishmaniasis in most affected areas, with amphotericin B and pentamidine being used as alternative drugs¹⁵. These agents are not active orally and require long-term parenteral administration .they also have serious side effects and are expensive^{1,5,11}. In addition, resistance to these compounds has become a severe problem. Therefore, new drugs are urgently required. The effectiveness and low toxicity of qinghaosu against malaria are well established^{23,24}. Clinical trials of QHS and its derivatives against malaria are presently being conducted by the World Health Organization and the U.S. Department of Defense²⁵. In addition to the inhibitory effects of QHS on the asexual erythrocytic stage of malaria, QHS and its derivatives have been reported to be partially effective against *Schistosoma mansoni*, *S.japonica* and an unspecified *Schistosoma* also *Toxoplasma gondii*^{26,27,28}.

The trematode *Clonorchis sinensis*^{26,29} and the *Naegleria fowleri*^{26,30} were like wise susceptible. Gram-positive and gram-negative bacteria tested were not inhibited by QHS^{26,31}. Analysis of data resulted from this study revealed an association between extract concentration with the evolution of disease, which had an effect on pathological signs of *L.major* infected Balb/c mice and could affect the proliferation of amastigotes, lesion size, degree of splenomegaly / hepatomegaly and presence of amastigotes in smears of lesion, liver, spleen and lymph nodes. The alternations may depend on extracts dose, number and route of inoculation and innate tolerance variability of hosts³² and needs to be clarified more in murine intracellular leishmania parasites as well. In this study, liver, spleen and lymph nodes were studied as target organs to detect amastigotes and to evaluate visceralisation of this cutaneous form of leishmania parasite. This extract had anti- leishmanial activity by reduction positive smears from lesion, Liver, Spleen and lymph nodes. This could emphasize the role at extract to inhibit visceralisation of *L.major* in target organs of infected susceptible BALB/c mice (Fig 4,5) our results reveal a novel pharmacological activity against *L.major* and suggest that this methanolic extract may be useful for topical application in wound healing, in addition to its non-allergenic properties.

References

- 1-Carvalho PB, Ferreira EI. Leishmaniasis phytotherapy. Nature's leadership against an ancient disease review. *Fitoterapia*. 2001; 72: 599-618.
- 2-Nahrevanian H, Farhmand M, Aghighi Z, Assmar M, Amirkhani A. Pharmacological evaluation of anti-leishmanial activity by in vivo nitric oxide modulation in BALB/c mice infected with *Leishmania major* MRHO/IR/75/ER; An Iranian strain of cutaneous leishmaniasis. *Experimental Parasitology*. 2007; (in press)
- 3-Singh S. New developments in diagnosis of leishmaniasis. *Indian J Med Res*. 2006: 311-330.
- 4-Dey A, Singh S. Transfusion transmitted leishmaniasis: A case report and review of literature. *Medical Sciences*. 2006; 24: 165-170.
- 5-Hepburn NC. Cutaneous leishmaniasis: an overview. *SYMPOSIUM*. 2003; 49: 50-4.
- 6-Ashford RW. The leishmaniases as emerging and reemerging Zoonoses. *Int J Parasitol*. 2000; 30: 1269-1281.
- 7-Carvalho PB, Arribas MAG, Ferreira EI. Leishmaniasis: What do we know about its chemo therapy? *Rev Bras Ci Farm*. 2000; 36: 69-96.
- 8-Araujo CAC, Alegrio LV, Leon LL. Antileishmanial from *Centrolobium Sclerophyllum*. *Phytochemistry*. 1998; 49: 751-754.
- 9-Erel O, Kocyigit A, Bulut V, Salin Gurel M. Reactive nitrogen and oxygen intermediates in patients with cutaneous leishmaniasis. *Mem Inst Osm Cruz*. 1999; 94: 179-183.
- 10-Jaim M, Soto Julia T, Toledo Patricia Gutierrez Margarita Arboleda Ruben S, et al. Treatment of Cutaneous leishmaniasis with a topical antileishmanial drug (WR279396): Phase 2 pilot study. *Am J Trop Med Hyg*. 2002; 66: 147-151.

- 11-Rocha LG, Almeida JRGS, Macedo RO, Barbosa-Filho JM. A review of natural Products with anti leishmanial activity. *Phytomedicine*. 2005; 12: 514-535.
- 12-Torres-Santos ECS, Moreira DL, Kaplan AMC, Meirelles MN, Rossi-Bergmann B. Selective effect of 2,6-dihydroxi-4-methoxychalcone isolated from *Piper aduncum* on *Leishmania amazonensis*. *Antimicrob Agents Chemother*. 1999; 43:1234-1241.
- 13-Nakayama H, Philippe M Loiseau, Christian Bories, et al. Efficacy of orally Administered 2-Substituted Quinolines in experimental murine cutaneous and visceral Leishmaniasis. 2005; 49: 4950-4956.
- 14-Balana FR, Reguera R, Cubria JC, Ordonez D. The pharmacology of leishmaniasis. *Gen Pharmacol*. 1998; 30: 435-443.
- 15-Hadighi R, Mohebbali M, Boucher P, et al. Unresponsiveness to Glucantime treatment in Iranian Cutaneous leishmaniasis due to drug-resistant *Leishmania tropica* Parasites. *PLOS Medicine*. 2006; 3: 0659-0667.
- 16-Akendengye B, Ngou-Milama E, Laurens A, Hocquemiller R. Recent advance in the fight against leishmaniasis with natural Products. *Parasite*. 1999; 6: 3-8.
- 17-Ali H, Konig GM, Khalid SA, Wright AD, Kaminsky R. Evaluation of selected Sudanese medicinal plants for Their in vitro activity against hemoflagellates, selected bacteria, HIV-1-RT and tyrosine Kinase inhibitory, and for cytotoxicity. *Ethnopharmacology*. 2002; 83: 219-228.
- 18-Weniger B, Robledo S, Arango GJ, et al. Antiprotozoal activities of Colombian Plants. *J Ethnopharmacol*. 2001; 78: 193-200.
- 19-Kavoosi G, Ardestani SK, Kariminia A, Tavakoli Z. Production of nitric oxide by murine Macrophages in duced by lipophosphoglycan of *Leishmania major*. *Korean J Parasitol*. 2006; 44: 35-41.
- 20-EI-on J, Witztum A, Schnur LF. Protection of Guinea Pigs against Cutaneous leishmaniasis by combined infection and chemo therapy. *Infect Immunit*. 1986; 51: 704-706.
- 21-Chen M, Christensen SB, Thor G, Theander., Kharazmi A. Antileishmanial activity of Licochalcone A in mice infected with *Leishmania major* and in Hamsters infected with *Leishmania donovani*. *Antimicrobial Agent and Chemotherapy*. 1994. 1339-1344.
- 22-Ricardo R, Mendonca-Filho, Igor A, et al. Leishmanicidal activity of polyphenolic-rich extract from husk fiber of *Cocos nucifera* Linn (Palmae). *Microbiology*. 2004; 155: 136-143.
- 23-Rath K, Taxis K, Walz G, et al. Pharmacokinetic Study of artemisinin after Oral intake of a traditional Preparation of *Artemisia annua* L. (Annual wormwood) *Am J Trop Med Hyg*. 2004; 70: 128-132.
- 24-Valecha N, Biswas S, Badoni V, Bhandari KS, Sati OP. Antimalarial activity of *Artemisia Japonica*, *Artemisia maritime* and *Artemisia nilegarica*. *Indian Journal of Pharmacology*. 1994; 26: 144-146.
- 25-Gordi T, Lepist EI. Artemisinin derivatives: toxic for laboratory animals, safe for humans? *Toxicology Letters*. 2004; 147: 99-107.
- 26-Li Y, Yu P, Chen Y, et al. Studies on artemisinin analogs. I synthesis of ethers, carboxylates and carbonates of dihydroartemisinin. *Chem Abstr*. 1982; 97: 92245.
- 27-Yang K, Krug EC, Marr JJ, Berens RL. Inhibition of growth of *Toxoplasma gondii* by Qinghaosu and derivatives. *Antimicrobial Agents And Chemo therapy*. 1990; 1961-1965.
- 28-Ying MA, Diam-Mei LU, Xiao-Jun LU, Lin L, Xiao-su H. Activity of dihydroartemisinin against *Leishmania donovani* both in vitro and vivo. *Chinese Medical Journal*. 2005; 117: 1271-1273.
- 29-Chen R, QU Z, Zeng M, Li J. Effect of qinghao SU and its derivatives on *Clonorchis sinensis* in rats. *Chem Abstr*. 1984; 100: 17251.
- 30-Cooke DW, Lallinger GJ, Durack DT. In vitro sensitivity of *Naegleria fowleri* to qinghaosu and dihydroqinghaosu. *Parasitol*. 1987; 73: 411-413.
- 31-Klayman DL, Lin AJ, Action N, et al. Isolation of artemisinin (qinghaosu) from *Artemisia annua* growing in the unites states. *J Nat Prod*. 1984; 47: 715-717.
- 32-Dascombe MJ, Nahrevanian H. Pharmacological assessment of the role of nitric oxide in mice infected with lethal and non-lethal species of malaria, *Parasite Immunol*. 2003; 25: 149-159.