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Antileishmanial effect of zinc sulphate on promastigotes viability of *Leishmania (L) major [MRHO/IR/75/ER]*

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ABSTRACT

Cutaneous Leishmaniasis (CL) is an endemic disease in developing countries. Glucantime has been recommended for CLtreatment by world health organization, there are some restrictions in this case including high expense, side effects, frequent injections need, and incomplete efficacy. Considering different methods which have been used for disease treatment thus far; in present study, the zinc sulphate (ZS) effect on rural strain CL' Viability of old world in vitro is under investigation. Iranian endemic species including Leishmania (L) major [MRHO/IR/75/ER] was appropriately provided, in different intervals, parasite numbers were counted in mentioned culture through two methods including the slide and cell proliferation ELISA. Finally, the considered counting was compared with the controlling group. Parasite species showed sensitivity to ZS. In vitro and in comparison with the controlling group, their numbers were reduced. ZS with 0.5%, 1%, 2%, and 3 % of density was added to the culture containing parasite and through the first to fifth days, the live parasite numbers in aforesaid culture was counted through the slide method by Neubar slide. The findings were analyzed statistically and level revealed P < 0.05. The findings revealed that all the cultures containing ZS (3%, 4%, 5%, and 6 %) in comparison with the controlling group showed a slower growth. The mentioned difference was statistically analyzed and revealed P < 0.05. Although ZS In vitro was affected, but probes are mandatory in the cases of animal and sick persons, figure out its daily dosage, concentration, time and duration.

Keywords: Leishmania (L) major, Zinc sulphate, Promastigotes, Viability.

INTRODUCTION

Leishmaniasis is a spectrum of diseases caused by infection with different species of the protozoan parasite Leishmania .These diseases range from self-limiting cutaneous leishmaniasis (CL) to visceral leishmaniasis (VL), also known as Kala-azar, which is a fatal infection if not treated successfully. Leishmaniasis effects over 12 million people in 88 countries, over 350 million are at risk and over 2 million new cases emerge every year. Different species of sandy transmit Leishmania and reservoirs include canine, wild rodents, and human. Within the insect host, Leishmania is present as flagellated promastigotes form and upon infecting the mammalian host it differentiates into the smaller aflagellated round amastigotes stage and multiplies in the phagolysosome vacuole of macrophages. Leishmaniasis is difficult to treat and there is increasing

Resistance developing against the currently available drugs (1).

From the morphological view, different species of parasite are identical and their difference was recognized by isoenzyme Petron and DNA analysis or monoclonal antibody**.** The clinical aspects cannot solely be the factor of different species. However, a special kind of disease or a special feature of a distinct kind is possible to exist. Leishmania parasite including an expanding cycle is in the female sand fly intestine. When there is a severe derma

infection in vertebrate host, amastigotes form of the parasite exists in reticulocyes cells and leads to necrosis of mononuclear ells. The parasite shape is cyclical or orbital and its length without flagellate is 2-3 micrometer. In Romanofsky staining, Kineoplast cell is to be deeply colorful and results in a particular view of organism. Leishmaniasis kind exists in sand fly body and culture in the form of promastigotes that is able to move and has a anterior flagellate. Sand flies live in hot and wet areas which are close to costs in rodents nest and trees holes. The infection transference is started with sand fly bite typically during the night and in open area. The parasite is zoophilic, and usually one type of parasite lives in the body of one or some kind of a vertebrate animal which is named as a reservoir host. Human is called as an accidental host; however, he can be a reservoir host in facing with anthroponotic kind. Germane to the mentioned evidence, the geographical expansion of Leishmaniasis occurs in special climates (2, 3, and 4). Although infection is in the forms of CL and visceral Leishmaniasis in human, some visceral Leishmaniasis infection making can lead to skin complications. In south and center of the America, skin complications of Leishmaniasis Braziliensis parasite are probable to suffering and they can result in Mucal or mucocutaneous Leishmaniasis. Mucal suffering in other Leishmaniasis species is relatively rare (5). Up to now, various treatments have been done that none of them has absolute outcomes. Owing to the restrictive nature of the disease, in final step the completion of cell immune system leads to the sore recovery. Incubation and clinical period are too unstable, and from one to some month changes. The evaluation of used healing drugs' results is too difficult and it is in need of scientific and standard methods in which are rarely used and most of the time sore recovery is reported as a result of drug effect. Antileishmanial activities of ZS have been reported recently. ZS has been investigated many times in the forms of oral and local injection through sore related to human and it shows the relatively positive results but the results have been too various. Owing to the mentioned factors, in the first phase, the following were investigated: the effect of drug on different species of parasite in in vitro with different dosages and different methods and intervals, and then the drug effect in vivo and finally the mentioned effect on human. And in the final step, there was a hope to find a more influential treatment for the disease. It is necessary to mention that immunotherapy methods and newer probes to prepare the vaccine are in the process, but the acceptable results have not been reported yet. Antileishmanial As a result of an attempt to establish a link between CL and different gradients ZS cultured and their antimicrobial properties have also been examined in vitro. The aim of the study was the efficacy of ZS on Urban and rural strains CL' viability of old world in vitro (6, 7, 8, 9).

EXPERIMENTAL SECTION

The present study is experimental (laboratory-trail) which it was conducted in several phases.

A. The preparation of parasite

Leishmania (L) major strains promastigotes (PMs) were obtained from the medical parasitology department, School of medical sciences, Tehran Tarbiat Modares University. And it was proliferated in NNN and $RPMI_{1640}$ with high density as follows:

In short, the mentioned parasite in accordance with Fattahi et al was proliferated (Fattahi et al, 2003)**.** Leishmania (L) major strain (MRHO/IR/75/ER) was maintained in BALB/c mice. Amastigotes were isolated from mice spleens, and then transformed to PM in Novy-Nicolle-Mac Neal (NNN) medium. Subsequently then supplemented with, the third passage PM from NNN medium were progressively adopted to $RPMI_{1640}$ media(gibco)were increasingly implemented to $RPMI_{1640}$ supplemented with antibiotics, glutamine and FCS(complete medium) penicillin(100 U/ml), streptomycin(100 µg/ml) and 20% heat-inactivated fetal calf serum(FCS)at 25°C (10).

B. Zinc Sulphate preparation

ZS heptahydrate ZnSO4. 7H2O (BDH) was dissolved in dis- tilled water in known concentration and autoclaved at 121oC for 20 min before use. Melamine antimonite: ampoules of melamine antimonite (Glucantime®) purchased from Spica, France were used.

C. Parasite counting

1. The slide method

A fixed initial density of the parasites was transferred to screw-capped vials containing 5 ml of $RPMI_{1640}$ with different densities of 0.5%, 1%, 2% and 3% of ZS and melamine antimonite added and for each density three vials were chosen; in addition, each run also included a control vial. The vials were then incubated at $26^{o}C$ for PM respectively. On the next four days, the cultures were counted. A 1:10 dilution in saline along with the appropriate dye was prepared. The PM permeable to the blue dye died while viable ones exclude the dye (11). The chamber of a Neubauer slide was charged and number of organisms in 16 small corner squares was counted. The total number per ml =N (counted) x 10 (number in 1 mm3 x 103 number 1 ml) x 10 (dilution factor). The LD50 was calculated according to the method of Hearly (12). This method was first described by Sharquie (13). A modified method was used in the present study and results obtained were compared with gained ones by the previous method. Of L major

were used. The appropriate dye was added to a dilution of the parasites growing on liquid medium. Trypan blue 0.4% was used for PM. One drop containing the parasites was put on a slide along with a drop of a drug solution and covered by a cover slip. The slides were examined under the light microscope and the percentage of stained parasites was noted. Normal saline was used as a control (14, 15, 16, and 17).

2. The cell proliferation ELISA, Nrdu (Chemiluminescent) Method
The cell proliferation ELISA was performed as elucidated by Roche Diagnostics GmbH Roche Applied Science 68298 Mannheim Germany (Version march 2005, Cat. No. 11 669 915 001) that in brief is:

1- A fixed initial density of the parasites was transferred to screw-capped vials containing 5 ml from liquid medium with different densities of 0.5%, 1%, 2%, 3%,4%,5% and 6% ZS and melamine antimonite were added and each density was done in triplicates. Each run also included control.
2- The cell proliferation ELISA, Nrdu (Chemiluminescent) method was conducted in accordance with Kit instruction (18). And then, all the data were collected to be analyzed through statistical procedures.

D. Data Analyses
Data are expressed as Mean ± SEM of lesion's diameter and animal's weight, and were statistically analyzed by multivariate tests for analyzing the effect of time within-subjects and multiple comparisons test for comparing the variables between groups. In this study, $p < .05$ was considered as the significant level.

RESULTS

Lethal Dose 50(LD_{50}) of ZS and Glucantime studied on L(L).major PMs and identified it.

The LD_{50} for ZS was 221.9 mg/ml or 44.4 mg/ml of Zn++ if calculated for zinc (Table1). Comparing the effect of zinc to that of the pentavalent antimony com- pounds the LD_{50} for antimony is 334.7 mg/ml Sbv (Table1).

Table 1. The LD_{50} of zinc and pentavalent antimony against of Leishmania (L) major [MRHO/IR/75/ER] PMs

Organism	Zn^{++}(mg/ml)	Glucantime (mg/ml)
Leishmania (L).major PM	44.4	334.7

Repeat Measure Test P=0.0001

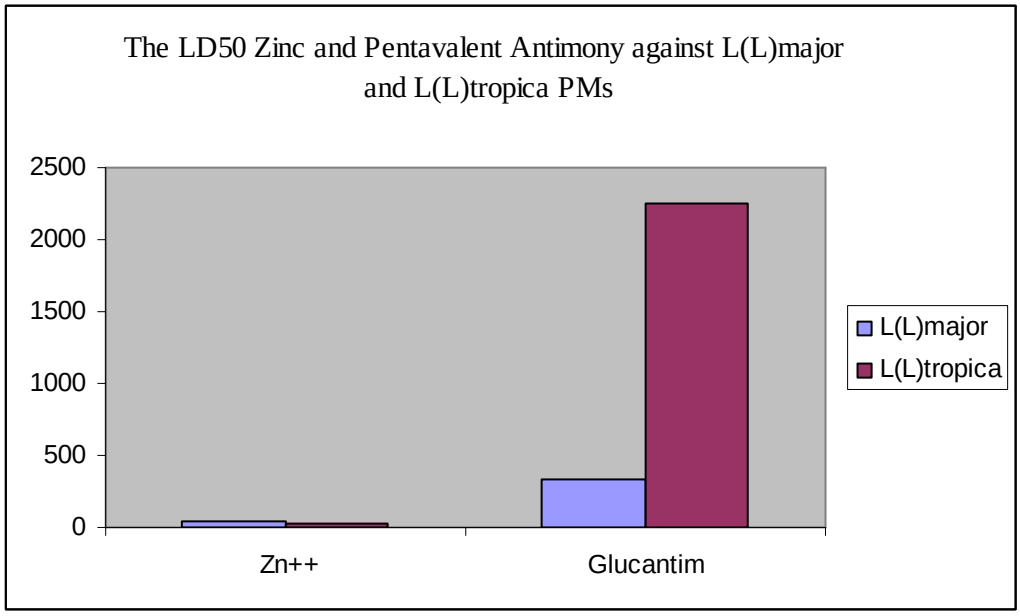

Fig.1 The LD50 of Zinc and Glucantime against Leishmania (L) major [MRHO/IR/75/ER] & tropica PMs

Effect of ZS against Leishmania (L) major [MRHO/IR/75/ER] of PMs –Table and Fig. 2 shows the effect of different concentrations of (ZS) on PMs of Leishmania (L) major [MRHO/IR/75/ER]. Zinc, in geometrically increasing concentrations, dose dependently inhibited the growth of Leishmania (L) major PMs according of days.

Table 2. Mean Frequency of Viability PM of L (L) major[MRHO/IR/75/ER] in culture according to ZS gradients in due times comparing with control group by the slide

Densities Days	Control	0.5%	1%	2%	3%
1st	78	61±2.82	46±2.82	44±1.41	20±0.70
2nd	247	76±2.12	64±1.41	54±2.82	33±0.707
3rd	294	94±1.41	91±5.65	62±1.41	41±0.707
4th	**387**	**101±2.82**	**89±5.65**	**68±1.41**	**48±1.41**

Repeated measure Test P-value =0.001

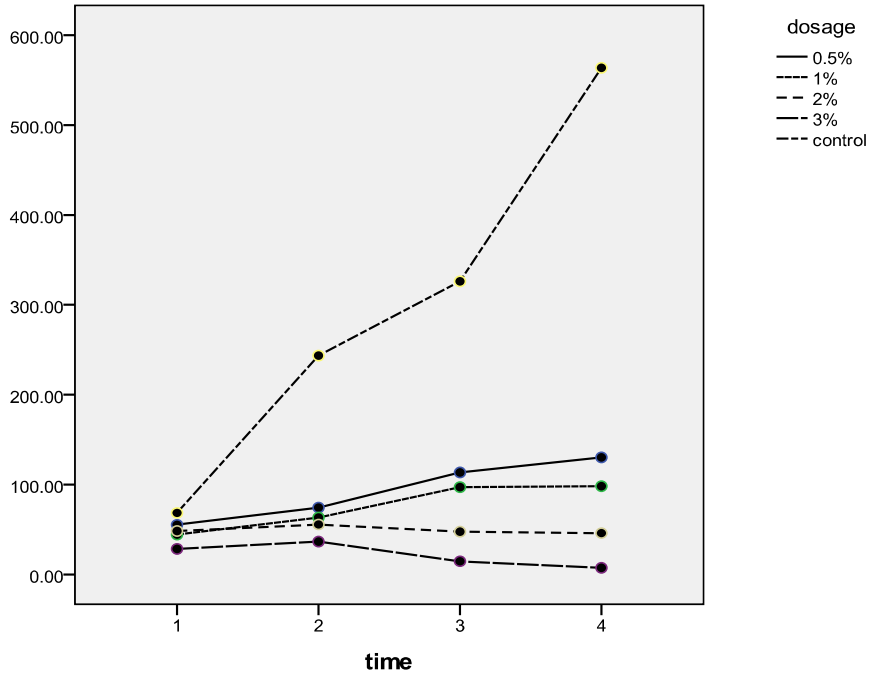

Fig.2 Mean Frequency of Viability PM of L (L) major[MRHO/IR/75/ER] in culture according to ZS gradients in due times comparing with control group by the slide

Table 3. The effect of ZS on Rural Strain of Cutaneous Leishmaniasis, Viability of Old World In Vitro

Gradients Species	0.5	1	2	3	Control
L(L)major	**0.14067**	**0.12367**	**0.1173**	**0.092**	**0.16733**

Repeat measure P-value <0.003

The results were statistically analyzed Repeat measure test showed that ZS significantly reduced the growth of Leishmania (L) major [MRHO/IR/75/ER] in comparison with the control group. To study the effect of various concentrations of ZS on viability Leishmania major [MRHO/IR/75/ER] results with four different concentrations of the test without a control group (repeated measure) were treated with (P-value = 0.003) showed a significant increase in the of the drug (ZS) parasite development is decreased and the concentration is greater inhibitory effect on parasite growth is greater (Table no.3).

Table 4.Staining mean of Urban PMs in Culture on Cell Proliferation, ELISA Method

Species Zinc Sulphate gradiants	L(L)major Mean±SD
Control	0.167±0.005
0.5%	0.140±0.003
1%	0.123±0.006
2%	0.117±0.004
3%	0.092±0.006
Kruskal Wallis Test	*P-value = 0.009*

The comparison of optical density mean in the case of parasite in the culture among different densities was conducted by Kruskal-Wallis Test and it showed p-value =0.009 in Leishmania(L) major[MRHO/IR/75/ER] in which it was a statistically significance among different densities and controlling group. The ELISA, findings with different densities of ZS without considering the controlling group was investigated. It means with the increase in density, the inhibition effect on the parasite was faced an increase (Table and Fig. no 4).

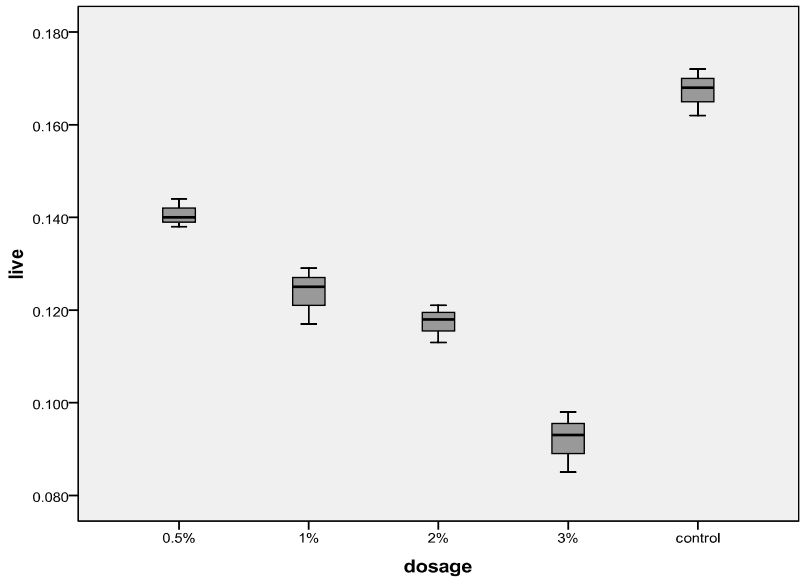

Fig 3. Staining mean of Rural PMs in Culture on Cell Proliferation, ELISA Method

The ZS Effect on Urban strain CL' Viability of old world in vitro was under investigation, and then in different intervals, parasite numbers were counted in mentioned culture through two methods including the slide and cell proliferation ELISA, NRDU (Chemiluminescent).

Results of the present study clearly indicate that ZS inhibits the growth of Promastigotes Leishmania (L) tropica in vitro. Not many report about the effect of Glucantime, expect of (19). That agree with the result of present study and however the other pentavalent antimony compounds was reported to have low activity against Leishmania (L) major PMs in vitro (20). Since parasites infect in the form of intracellular amastigotes, it might be argued that promastigotes are of limited value to study drugs in vitro. About mechanism of the antileishmanial effect of ZS, I am well known that ZS plays an important role in specific metaloenzye systems concerned with nucleic acid and protein synthesis (21). Furthermore zinc has been reported to inhibit DNA polymerase of herpes simplex virus (22). Therefore, we may speculate that a very probable site of action of zinc is on some enzymes concerned with nucleic acid metabolism (23).

DISCUSSION

Different methods to cure Leishmaniasis have been recommended that most of them have been topical and systemic treatments(24, 25, 26). The pentavalent Antimony compounds have been posed since 1940 for systemic treatment and the pentavalent Antimony compounds have been used as the first priority in treatment since 1984. The mentioned drug is relatively expensive and leads in severe side effects. Ignoring the considered issues, there are many cases of inefficacy in wound recovery. Owing to the reason, various drugs have been used so far including sodium colored and ZS. The first report of 2% ZS efficacy is related to the study of Sharquie and al azzawi 1996 (27) A study in 1998, in which Leishmania (L) major and Leishmania (L) tropica was proliferated in vitro and then the effect of different ZS from 0.02 to 2 % in comparison with the control group revealed a decrease in parasite proliferation**.** After that ZS with densities of 100 to 400 ml in kg were given to the mice. The results showed that the drug-taker group in comparison with nondrug-taker one suffered less and the disease deterioration was less, too. The anti-leishmaniasis of ZS is not completely clear. The manifest point is the role of ZS in different enzymes which interfere in DNA and proteins synthesis. 32 Furthermore, it was reported that zinc can deactivate DNA enzyme of Herpes virus. 33 Thus it is more probable that the zinc effect domain is in enzymes which play role in Nucleic acids metabolism. Due to the mentioned fact they cause the parasite proliferation stop in vitro.The present study showed the direct effect of drug on parasite. The conduct of more precise studies for exact effect mechanism of drug is mandatory, though. Following the study Sharque et al who reported the drug effect in animal model, a probe in 33

mice were affected by Leishmania (L) major and then they were divided into three groups by random. First group was received 0.35 ml/kg selonid sodium for 30 days, second group received 2 mg/kg zinc ZS for 30 days, and third group received 10 ml/kg distilled water (control group). All the groups received 60 mg/kg standard Glucantime injection for 14 days. The size of wound in Selonid sodium was bigger, in second group it stopped but any significant difference between ZS group and distilled water one was not observed. The findings of the study showed that ZS has not significant effect on disease control in laboratory animals. Owing to the said issue, the reduplication of experiments in the case of drug effect on parasite in vitro was needed. Many researchers have focused on the drug effect on human beings. A Study used ZS through injection in animate wound and reported the positive results. 63 leishmaniasis-suffered persons were divided into 4 groups by random. Three groups received 2% ZS, 7% colored sodium, and antimony injection into the wound, respectively. Fourth group who was the control one received no treatment. At the end of 45-day treatment, the results revealed that the first three groups obtained comparable treatment outputs and 94.8 % ZS showed the best recovery. In the other study, 104 patients diagnosed with Leishmania (L) major were divided into four groups. Three groups received oral dosages of 2.5, 5, 10 ml/kg, and fourth group which was the control one did not receive any treatment. All the patients were observed for 45 days, and finally the results showed that clinical and laboratory recovery in the groups of 2.5, 5, and 10 was 83.9, 93.1, and 96.9, respectively. None of patients in control group showed any healing. The obtained result emphasized on the efficacy of oral ZS as an appropriate and harmless in Leishmaniasis treatment. The findings of mentioned studies which were conducted in Iraq showed the positive effect of the drug; in addition, the studies of drug effect on parasite in vitro and animal model were conducted in this country; in contrast, many probes were conducted in Iran which did not report the drug efficacy. A study was conducted by [Yazdan panah et al 2007] in Mashhad to observe the efficacy of oral zinc sulphate on Leishmaniasis. 31 patients were cured by 10 mg/kg oral ZS during 45 days. 22 patients completed the treatment and only two of them showed perfect recovery. Healing worth of oral ZS in Cutaneous Leishmaniasis treatment was assessed as insignificance by conductors (28). Other study was conducted [Iraji et al 2004] in Isfahan, Iran. In this study 104 patients suffered from severe Leishmania (L) tropica participated and were divided into two groups by random. One group received 2% ZS and the other received antimony during 6 weeks. 66 patients (35 persons form antimony group and 31 persons from ZS completed the treatment. The recovery percent in antimony group was 60% and in ZS group was 83.8%. In the end, the conductors came to this conclusion that the injection of 2% ZS into wound can be the alternative treatment for Cutaneous Leishmaniasis (29). Other study was conducted [Firooz et al 2005]. 72 patients suffered from severe Urban CLwere selected and divided into two groups. One group received ZS and the other one received Glucantime injection weekly. 35 patients (13 persons from zinc sulphate group and 22 ones from Glucantime) completed the treatment. The perfect recovery was observed one week after the treatment completion in 2 and 19 patients in two groups, respectively (30 The findings revealed that the ZS injection in comparison with Glucantime had fewer effects (31). Various studies emphasized on the effect mechanism of ZS on laboratory animal and human being in the case of its role in the change of immune response. The shortage of this component changes the immune response of a cell from Th1 toward Th2 i.e. humeral immune response. Considering the fact that the Th1 immune response leads in the production of say to such as IL2 and gamma-INF which in the control of virus infections and other inside-cell pathogens in comparison with Th2 response is more effective (32). Also, the ZS role in determining molecule construction of Leishmaniolysin in which in joining and entering the Leishmaniasis parasite in white cells interfere is important 35. In myriad probes, the realities were reported in the case of the low level of zinc serum, selenium and iron accompanying with a decrease in related enzymes activity such as glutathione peroxides and katalaz (33, 34).Two other studies focusing on the dogs suffered from Leishmaniasis and human beings suffered from Leishmaniasis showed the low level of zinc and iron and other cytokines related to these components (35). The mentioned difference was statistically analyzed and revealed $P < 0.05$. In the present study, zinc sulphate with high density (3-6 %) showed a high capability in inhibition of parasite proliferation in vitro and with density increase the mentioned effect was escalated regularly.

CONCLUSION

Considering the made compound safeness in comparison with Glucantime, its use possibility in the treatment of species, Leishmania(L)major, is not far to reach**.** Although zinc sulphate in vitro was affected, but probes are mandatory in the cases of animal and sick persons, figure out its daily dosage, concentration, time and duration.

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REFERENCES

[1] A. Ghosh, W. W. Zhang, G. Matlashewski. *Vaccine*, **2002**, 20, 59–66.
[2] V, López F, Hay RJ. Parasitic Worms and Protozoa. In: T. Burns, S. Breathnach, N.Cox, C. Griffiths, editors, *Rook's Textbook of Dermatology*, **2010**, 8 th ed. Wiley-Blackwell, 37.33-39.
[3] Bryceson A.Therapy in man. In: Peters W, Killick-Kendrick R, eds, *The Leishmaniases in Biology and Medicine*, **1987**, Vol. 2. London: Academic Press, 56–61.
[4] Weatherall DJ, Ledingham JGG, Warrell DA, eds, *Oxford Textbook of Medicine*, **1987**, 2nd edn. Oxford: Oxford University Press.
[5] Rioux JA, Groubert JR, Lanotte G et al, *Les Cahiers D'orl*, **1980**, 15: 423–5.
[6] Bellazoug S.*Bull SocPatholExot*; **1983**, 75: 497–504.
[7] A.K.Arezou, A. Z. Hoseini, A. F. *Journal of Shahid Sadoughi University of Medical Sciences and* Health Services, **2005**, Vol.11,No.2,PP.61-66.
[8] Bryceson ADM, *Trans R Soc Trop Med Hyg*, **1987**, 64: 369–79.
[9] Neva FA, Petersen EA, Corsey R et al, *Am J Trop Med Hyg*, **1984**, 33: 800–4.
[10] F. B. Ali et al, *African Journal of Microbiology Research*, **2011**, Vol. 5(12), pp. 1504-1510.
[11] Jaffe CL, Grimaldi G, McMahon-Pratt D, The cul- tivation and cloning of Leishmania, p. 47-90. In CM Morel, *Genes and Antigens of Parasites: A Laboratory Manual*, **1987**, Fundação Oswaldo Cruz, Rio de Janeiro.
[12] Hearly MJP. Estimation of LD50 without a com- puter, *Parasitol Today*, **1988**, 4: 25-27.
[13] Sharquie KE, *J Dermatol (Japan)* , **1995**, 22: 732-737.
[14] El-On J, Weinrauch L, Livshin R et al. *BMJ* ; **1985**, 291:704–5.
[15] WHO, The Leishmaniases. Report of WHO Expert Committee. *Technical Report Series*, 701. Geneva. **1985,** World Health Organization.
[16] Ballou WR, McClain JB, Gordon DM et al. *Lancet*; **1982**, ii: 12–6.
[17] Bryceson ADM. Clinical variations associated with various taxa of Leishmania. In: *CollInt CNRS/INSERM*, 1986, Montpelier: IMEEE, 221–8.
[18] Sharquie KE, Najim RA, Farjou IB. *Clin Exp Dermatol*, **1997**; 22(4):169-73.
[19] Rafid A Najim, Khalifa E Sharquie, Imad B Farjou. *Mem Inst Oswaldo Cruz, Rio de Janeiro*, **1988**, Vol. 93(6): 831-837.
[20] Neal RA. Experimental chemotherapy, p. 793-837. In W Peters, R Killick-Kendrik (eds), *The Leishmaniasis in Biology and Medicine*.**1987**, Academic Press, London.
[21] Cousins RJ. *Physiol Rev*. **1985**, Apr; 65(2):238-309
[22] Fridlender B, Chejanovsky N, Becker Y. **1987**, *Virology*. Feb;84(2):551-4.
[23] Najim RA, Sharquie KE, Farjou IB. *Mem Inst Oswaldo Cruz*; **1988**, 93(6):831-7.
[24] Mahesh Verma, Anil Bhandari, and Rajesh Kumar Nema, *J. Chem. Pharm. Res*., **2010**, 2(3):244-250.
[25] N. Raghav, M. Singh, S. Jangra, A. Rohilla, R. Kaur and P. Malik, *J. Chem. Pharm. Res*., **2010**, 2(4):801-807.
[26] Sachin Malik, , Sachin Kumar, , Ashish Choudhary, Arun Kumar, Avrendra singh and Garima Avasthi, *J. Chem. Pharm. Res*., **2010**, 2(3):70-91.
[27] Sharquie KE, Al-Azzawi KE, Intralesional therapy of cutaneous leishmaniasis with 2% zinc sulphate solution. Iraqi Central Organization for Specification & Quality Control/Patent section, **1997**, Patent number 2583, Baghdad, Iraq.
[28] Sharquie KE, Najim RA, Farjou IB, Al-Timimi DJ. *Clin Exp Dermatol*; **2001**, 26(1):2.
[29] Iraji F, Vali A, Asilian A, Shahtalebi MA, Momeni AZ, *Dermatology*, **2004**, 209(1):46-9.
[30] Alireza Firooz , Mehdi R Firoozabadi, Behnaz Ghazisaidi and Yahya Dowlati, *BMC Dermatology*, **2005**, **5**:1 doi:10.1.
[31] Sprietsma JE. *Med Hypotheses*, **1997**, 49:1-14.
[32] Hejazi H, et al. Identification of Leishmania species using monoclonal antibodies in Isfahan, *Iranian Journal of Dermatology*; **2000**, 1:7-11.(In Persian).
[33] Puentes F, et al. *Exp Parasitol*, **1999**, 93:7-22.
[31] Kocyigit A, et al. *J Egypt Soc Parasitol*, **1998**, 28:133-142.
[34] Kocyigit A, et al. *Biol Trace Elem Res*,**1998**, 5:271-281.
[35] Kocyigit A, et al. *Biol Trace Elem Res*, **2002**, 90:47-55.