ISSN: 0974-2115
www.jchps.com Journal of Chemical and Pharmaceutical Sciences

Effect of Peel and Leaf Extract of Walnut (Juglans Regia L.) on Cutaneous Leishmaniasis Caused by *Leishmania major* in BALB/c Mice

Ali Sharafi Chegeni[1,2], Behrouz Ezatpour[1*], Mehdi Mohebali[3], Hossein Mahmoudvand[1], Mohammad Zibaei[4], Farzad Ebrahimzadeh[5], Marzieh Rashidipour[6], Naser Babaei[7], Samaneh Chaharmiri Dokhaharani[8]

[1]Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences, Khorramabad, Iran.
[2]Department of Communicable Disease Control and Prevention, Deputy of Health, Lorestan University of Medical Sciences, Khorramabad, Iran
[3]School of Public Health and Institute of Public Health Research, Tehran University of Medical Sciences, Tehran, Iran
[4]Department of Parasitology and Mycology, School of Medicine, Alborz University of Medical Sciences, Karaj, Iran.
[5]Department of Biostatistics, School of Health and Nutrition, Lorestan University of Medical Sciences, Khorramabad, Iran
[6]Young Researchers Club, Khorramabad Branch, Islamic Azad University, Khorramabad, Iran
[7]Isfahan University of Medical Sciences, Isfahan, Iran.
[8]Young Researchers and Elite Club, Falavarjan Branch, Islamic Azad University, Isfahan, Iran
***Corresponding author: E-Mail: bezatpour@gmail.com, Tel. /fax: +98-661-3204005**

ABSTRACT

Introduction: Cutaneous leishmaniasis (CL) is an endemic disease in many countries, including Iran. Walnut (*Juglans Regia L*.) has been intensively used in traditional medicine for treatment of many diseases. We demonstrated in this study for first time the effects of topical application of the ointment-based extract of *Juglans regia in vivo*.
Materials and methods: BALB/c mice were infected at the base of the tail with *Leishmania major* (MRHO/IR/75/ER) subcutaneously. Mice were assigned to 4 groups as treatment and control groups.
Results: The results showed generally significant differences between the experimental groups in terms of mortality (P=0.063).
Conclusion: This study indicated a significant post-treatment decrease in the lesion size and parasite count in infected animals, compared to control groups. The results of this study establish the efficacy of Walnut-based topical ointment, and pave the way for further research in its therapeutic applications.

KEY WORDS: Cutaneous leishmaniasis, *Leishmania major*, mice BALB/c, *Juglans Regia L*, scar.

1. INTRODUCTION

Despite considerable progress in the control of infectious diseases, some parasitic diseases such as leishmaniasis are still a health concern. WHO has estimated that 350 million people are exposed to leishmaniasis worldwide and its development is 12 million people per year in more than 98 countries (WHO; Ponte-Sucre, 2012). Cutaneous leishmaniasis (CL) is the most common form of leishmaniasis that infects 1-1/5 million people a year (Alvar, 2012). This infection has been endemic in Iran with reports from at least 17 provinces (Zoonoses Control Office, 2010). The cause of CL is *Leishmania major* or *Leishmania tropica* in Iran and *Phlebotoms sergenti* and *Phlebotoms papatasi* are the most common carriers in the human (Kheirandish, 2011; Ghorbanzadeh, 2014). Considering that 10 percent of cases become chronic CL treatment is necessary (Tabatabaie, 2004).

No pharmacologic prevention is available for CL and to date,date; there are no vaccines against leishmaniasis (Esmaeili, 2008). Treatment of CL is carried out by injecting Penta valent antimonial compounds, Glucantime ® (meglumine antimonate) into the wound with cryotherapy for 12 weeks (Momeni, 2003). However, some factors such as elevated liver enzymes, changes in electrocardiogram, long time injection, drug resistance and low efficiency have prompted researchers to look for a suitable alternative treatment (Hadighi, 2007). On the other hand, international traveling, global warming, climate change, lack of vaccines, increasing drug-resistance and rising cases of *Leishmania*/HIV have more highlighted risk of leishmaniasis (Ali, 2012).

Medicinal plants have always been a source of drugs for the treatment of diseases and disorders. One of its variants is the walnut plant. *Juglans regia* is one of the most common nuts in the world. This plant has been used for nutritional purposes and in traditional medicine since ancient times. In Chinese medicine, leaves of *Juglans regia* are used for diarrhea and vascular deficiencies in traditional practices (Noumi, 2011). In some regions, walnut tree's bark and hulls as an astringent substance are known and it used for constipation cases, washing and healing injuries, skin inflations, tuberculosis and killing parasites (Noumi, 2011). Its antiparasitic effects have been the subject of many studies (Zhai, 2006; Urban, 2008; Kale, 2011). As herbal healing beliefs among some tribal areas in Zagros regions (Poldokhtar City, Southwest of Iran), walnut extract can serve as a suitable agent for treatment of skin ulcers and *Leishmaniasis*. Walnut leaf, because of cheapness and availability without damaging the tree, can be a proper substitute of synthetic and non-synthetic medicines (Valnet, 1992). The leaves are full of antioxidants like phenolic

acids (caffeoylquinic and coumaroylquinic) and flavonoids (Juglan, quercetin 3-pentoside and kaempferol 3-pentoside derivatives) (Solar, 2006).

Effect of *Juglans regia* on CL have not been experienced yet; therefore we planned to determine effect of peel and leaf extracts on the scars caused by *Leishmania major* in BALB/C mice.

2. MATERIALS AND METHODS

Preparation of hydroethanolic plant extract: The leaf and green peel of *Juglans regia L.* collected from gardens outside Khorramabad City, Lorestan Province, Iran. The plants were dried in open air, shady conditions until completely dried and then ground to a powder. A 35 g based on dry weight powdered leaves and green peel added to the cartouche and the extraction was carried out using 50% ethanol and soxhlet apparatus for 24 h. The obtained extract was not filtered and it was changed into a viscous liquid using the rotary (Heidolph, Germany). The procedure of extraction and filtration were operated at room temperature. The extract was stored at 4°C until use (Simsek, 2011). The extract weighed 3.5 gr and the extraction efficiency (yield) reported to be 10%. The obtained extract (Eucerin as ointment base) was prepared at concentrations of 2% and 4%.

Preparing the Preparation of the parasites: The strain of *Leishmania major* promastigotes, MHROM/IR/75/ER, was isolated from CL cases from Faculty of Health Sciences, Tehran University of Medical Sciences. Having prepared thin smear and stained with Giemsa and direct visualization of the amastigotes, some of the secretion was removed and cultured in the RPMI 1640 medium (Gibco, UK) with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, UK) in the aseptic condition. After 5 days, the obtained promastigotes, in stationary growth phase, were harvested and the numbers of parasites were counted in a neubauer chamber. In this phase, the numbers of promastigotes fixed at 2.5×10^6 /mL (Esmaeili, 2008).

Experimental animals: BALB/c mice (6 to 8-week-old males) were purchased from Razi Vaccine and Serum Research Center, Karaj, Iran. The animals were maintained under standard conditions, in an environment (Animal house in Razi Herbal Medicines Research Center) with controlled temperature, humidity, and 12/12 h light and dark cycles as well. After mass proliferation of the parasites and reaching count of 25×10^6 promastigotes/ml in one ml of RPMI 1640 medium, 0.1 ml was subcutaneous injected into the tails of the mice. Then, the mice were kept in a proper condition (a 24-h light-dark cycle). After 32-35 days, a scar appeared on the tail. To confirm the existence of the parasites, smear was prepared from the scar. Following methanol fixation, Giemsa staining was performed and all the smears were evaluated for the existence of parasites using the light microscope. The animals were divided into four groups, one of which had 5 mice as control (non-infected and non-treated) (Mohebali, 2004).

Group (1): 13 mice were numbered and treated with 2% peel and leaf extract of walnut twice a day for 8 weeks.

Group (2): 14 mice were numbered and treated with 4% peel and leaf extract twice a day for 8 weeks.

Group (3): 14 mice were treated with standard Glucantime ®, 60 mg/kg intraperitoneally injected once a day for 20 days.

Group (4): 11 mice were numbered and treated with Eucerin ointment twice a day for 8 weeks.

Before the treatment and weekly, lesion size recorded by measuring the tail thickness in two dimensions (D+d) at right angles to each other by a caliper. Size of the lesion (S) estimated by calculating mean diameter of the tail using the formula:

$$S = (D+d)/2$$

To measure load of parasites in each mouse, every 15 days, a smear of the scar was taken to evaluate the promastigotes. After methanol fixation and Giemsa staining, the samples were graded by light microscope (×1000) based on the parasite counts as follows:

If one parasite was detected in ten microscope fields, the given point was (+l). If 1-10 parasites were detected in ten microscope fields, the give point was (+2). If 1-100 parasites were detected in ten microscope fields, the given point was (+3). If more than 101-1000 parasites were detected in ten microscope fields, the given point was (+4), and, if no parasite was detected, it was considered negative. To treat the mice after their control, the scars were treated using medicinal substance. The treatment lasted for 8 weeks twice a day. Ethics Review Committee for Animal Experimentation of Lorestan University of Medical Sciences approved the experimental protocol.

Statistical analysis: The results were analyzed using t-test, Fisher's exact probability test, one-way Analysis of Variance (ANOVA) along with Tukey's post-hoc test and Kaplan-Meier graphs with the log rank test.

3. RESULTS

Effect of the extract on the scar size: Repeated measures test showed a significant difference in the mean size of the lesions at various times during the study (P=0.0004) and time factor leading to changes was determined as linear one (Figure 1).

Effect of the extract on mortality among the mice: Results of Chi-square test showed generally significant differences between the experimental groups in terms of mortality (P=0.063) but non-significant difference at P

= 0.05. However, control group 2 placed in a distinct category of the best group with the lowest mortality rate and other groups fell within a common class with higher mortality (Figurer 2).

Effect of the extract on survival time of the mice: Comparison of the experimental groups in terms of median survival time based on the test log-rank indicated that overall median survival time of the experimental groups was significantly different between the four groups (*p=0.01*).

Comparing the two groups based on log-rank test showed the lowest and highest survival time in control group 1 with concentrations of 4% and 2% and control groups 2, respectively (Figure 3).

Effect of the extract on the parasite counts in the mice: According to repeated measure test, significant difference was observed between sampling the first and fourth lesions in terms of the mean number of parasites in the prepared smears (df =3, f=43.972, *P*<0.001).

Effect of time lapse on the average number of parasites was predominantly linear (df=1, f=66.398, *P*<0.001). In other words, the mean number of parasites in all the experiment groups was constantly increasing or decreasing (Figure 4) (Table 2).

Results of co-variance analysis showed that the interaction effect between time and type of experimental group was significantly different (dF=21, f=2, *P*=0.021). Findings revealed that linear effect of time lapse on the mean number of parasites was not similar in all the groups; in some groups, it was absolutely increasing and, in some others, it was decreasing. Tukey's test showed the least reduction in the number of parasites during the treatment in control group 2 (treated with Eucerin) and the highest decrease observed in the number of parasites at concentration of 2% (Figure 4). Based on the statistical tests, no significant difference was observed between the mean number of parasites during biopsies of the first and second wounds (on days 0 and 15); however, after the second biopsy (on days 15, 30 and 45), a significant decrease was observed in the mean number of parasites.

Table 1: Recovery rates in the studied groups after the treatment period.

Experimental groups	Total	Complete recovery	
		No.	%
Group 1 (Concentration 2%)	13	2	15/4
Group 2 (Concentration 4%)	14	4	28/6
Control Group 1	14	0	0
Control Group 2	11	0	0
Total	52	6	11/5

Table.2.Comparision of the Mean and Standard deviation (SD) of the parasites number in smears at different stages of sampling

Experimental groups		1st stage of sampling	2nd stage of sampling	3rd stage of sampling	4th stage of sampling	Total
Group1 (concentration 2%)	Mean	2.2000	1.6000	0.3750	0.0625	1.4750
	SD	0.86189	0.69921	0.44320	0.17678	0.74282
Group2 (concentration 4%)	Mean	2.2667	1.8333	1.1000	0.3000	1.8167
	SD	1.09978	0.98319	0.82158	0.44721	0.91352
Control Group1	Mean	2.5385	2.2500	2.2500	1.3750	2.3558
	SD	0.96742	.50000	0.50000	1.10868	0.97608
Control Group2	Mean	2.3182	2.7500	2.2500	1.7500	2.2159
	SD	1.48783	0.50000	0.95743	0.50000	1.21461
Total	Mean	2.3241	1.9583	1.2619	0.6905	1.9329
	SD	1.07790	0.80645	1.04426	0.88708	0.99192

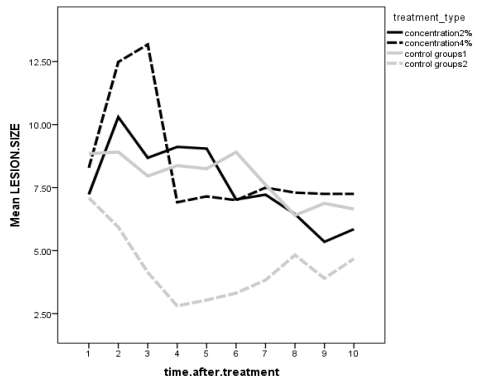

Figure.1. Liner Figure of mean size of ulcer in millimeters, separated on each observation time in the intervention and control groups BALB/c mice

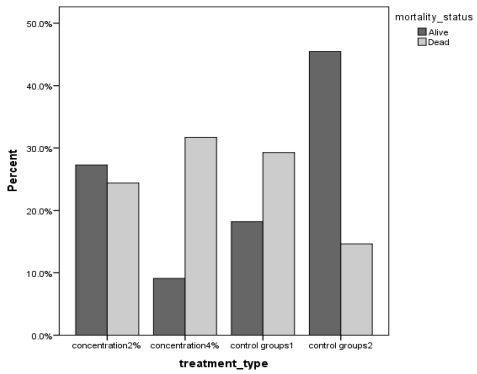

Figure.2.Mortality rates in the intervention and control groups in BALB/c mice

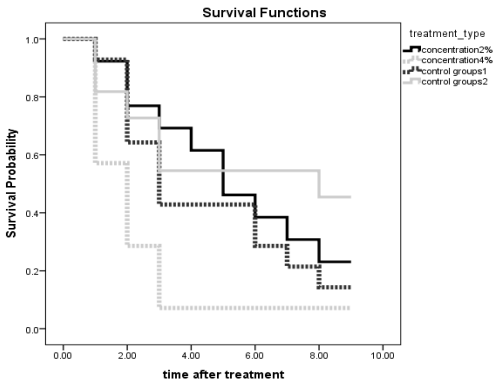

Figure.3. Estimated survival time in Experimental groups based on the Kaplan-Meier curve

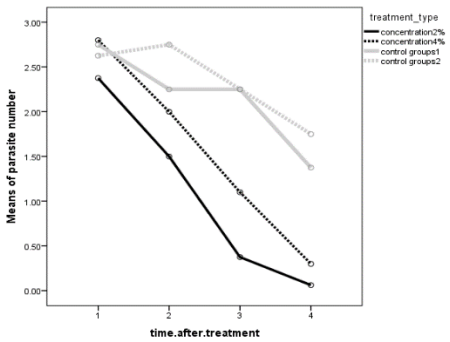

Figure.4.Linear plot number of parasite in the smears, separated on the stages of observation and type of experimental group

DISCUSSION

For treatment of CL, investigators are seeking the compounds that could heal wound faster and reduce depth and breadth of wound scar with the least side effects and minimal systemic absorption (Nilforoushzadeh, 2011). The effects of treatment and prevention of various medicinal plants are due to flavonoid and phenolic bioactive materials, antioxidants, anthocyanins, tannins and etc (Mahmoudvand, 2014; 2015; 2016; Ezatpour,2015; Motamedi, 2016; Ahmadvand, 2015; Azadpour, 2015). The ideal drug for the treatment of CL should also reduce size of wound with complete abolition of leishman bodies because the remaining parasite is able to reproduce and relapse.

In this study, 2% and 4% concentrations of leaf and fruit peel extracts of *Juglans regia* were evaluated in vivo conditions on the *Leishmania major* as topical. Some studies have shown that walnut extract has a remarkable effect on the number of pathogens such as bacteria, yeast, helminthes, amastigotes and promastigotes of *Leishmania* genus (Alkhawajah, 1997; Pereira, 2007; Noumi, 2011) and the extract characteristics have been attributed to Juglone. Juglone (5-hydroxy-1, 4-naphthoquinone) is only found in fresh and green portion of walnuts and its effectiveness disappears in dried leaves (Solar, 2006). Probably, the absence of desirable result in this study could due to use of dried leaves of walnut, which the juglone was polymerized.

In this study, among the experimental groups, the mean number of parasites in the respective smear was significantly different. As observed, 2% and 4% concentrations could completely eliminate parasites in scar in 2 (15.4%) and 4 (28.6%) mice, respectively; however, complete eradication of leishman bodies in control groups, compared to the treated groups, was not observed in prepared smears.

The results of Tukey's test also showed that the smallest reduction in the number of parasites during the treatment was observed in control group 2 (the group treated with Eucerin) and control group 1 (Glucantime treatment group). The highest reduction in the number of parasites was seen in groups 2 (4% drug concentration) and 1 (2% drug concentration). In addition, based on the statistical tests in the first and second sampling, a significant difference observed in the mean number of parasites; but, after the second sampling, there was a considerable decrease in the mean number of parasites. The present study lent support to the reports evaluating effects of different concentrations of walnut extract on *Leishmania major* in vitro (Ali, 2012; Yektaian, 2012) and show agreement with El-on, finding (El-On, 1984). Although many plants have been studied or tried to treat leishmaniasis, only a few have been found to be effective. As revealed in the present study, wound clot was not formed in the treated group compared with the control. Kheirandish using the *Satureja khuzestanica* essential oil (SKEO) on cutaneous leishmaniasis in BALB/c mice showed that SKEO had no effect on diameter of the lesions. However, survival rates of mice in SKEO treated groups were higher than that in the control groups (Kheirandish, 2011). In Mohebali survey, 2.5 and 5% concentrations of hydro-alcoholic lotion cassia had no therapeutic effect on the wound caused by *Leishmania* in mice, while 25 and 40% concentrations caused significant reduction in wound size compared with the control populations. In addition, lotion with 75% cassia with concentration of 2% DMSO increased permeability of cassia in the inner wounds and completed recovery of some mice (Mohebali, 1999).

4. CONCLUSION

Notwithstanding some studies carried out with walnut fruits, as far as we know, this is the first report considering the in vivo antileishmania potential of peel and leaf of walnut. Comparing and evaluating lesions of cutaneous leishmaniasis in this study indicated that walnut extract could prevent secondary bacterial infection and eliminate clot on the lesions in the treated group. It could be recommended that higher concentration of this herbal extract could give better therapeutic results. We hope results of this study will help to treatment of cutaneous leishmaniasis.

5. ACKNOWLEDGEMENTS

This study was financially supported by Deputy of Research and Technology Affairs, Lorestan University of Medical Sciences, with scientific cooperation of Faculty of Health, Tehran University of Medical Sciences. We express our deep thanks to Dr Bahram Rasulian, Chair of Razi Herbal Medicines Research Center, and appreciate our co-workers Mr. Reza Rostami and Ms. Fatemeh Zeidali Beyranvand, who helped us during the study.

Conflicts of interest: The authors declare that there is no conflict of interest in the current study.

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