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In Vivo Assay of Wound Healing Activities of Silymarin Extract on Cutaneous Wounds Caused by *Leishmania major*

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Abstract

Background: Cutaneous leishmaniasis (CL), caused by the intracellular protozoa *Leishmania*, causes full-thickness skin wounds and scar formation afterwards, which may bring about some complications such as scar formation, secondary infection, and wound expansion.
Objectives: This investigation was conducted to determine the effect of hydroalcoholic extract of silymarin, as an anti-oxidant, anti-inflammatory, and anti-scar, for treatment of CL caused by *Leishmania major*.
Methods: A total of 28 female BALB/C mice were injected with amastigotes of *Leishmania major* and after the wounds came up on their tail-base they were randomly divided into four groups (n = 7): E1 and E2, which received silymarin 5% and 10% gel, respectively, and C1 and C2 were treated with normal saline and vehicle gel, respectively, every 24 hours for 20 days. Wound size was measured every three days. Finally, the mice were sacrificed with high dose of ether, and full-thickness skin samples from the wound site was obtained for stereological estimation of collagen and vessel volume densities, fibroblasts population, vascular length density, and mean diameter of the vessels.
Results: Silymarin gel in either concentration of 5% and 10% accelerates wound closure ($P < 0.05$) and also improves collagen synthesis and revascularization by means of increasing length density, volume density, and mean diameter of blood vessels ($P < 0.05$).
Conclusions: Topical administration of silymarin extract in both doses of 5% and 10% showed promising effects on the healing process of CL-induced wounds.

Keywords: Silymarin, Leishmaniasis, Cutaneous Leishmaniasis, Wound, Stereology

1. Background

The term "Leishmaniasis" is referred to a group of diseases, namely including cutaneous leishmaniasis (CL), visceral leishmaniasis (VL), and mucocutaneous leishmaniasis (MCL), caused by *Leishmania*, a protozoan parasite that exists in either forms of a flagellated promastigote or amastigote (1). According to recent assessments, about 12 million patients with CL are present in different regions of the world while about 350 million humans are at risk of being infected (2, 3). Alongside with many countries such as Brazil, Colombia, Algeria, and Afghanistan, CL is a common disease in Iran being endemically present in several provinces of the country especially the south and south west regions (2, 4). Having several side effects and a relatively high chance of recurrence, the common therapies for CL are still pentavalent antimonial derivatives such as sodium stibogluconate (Pentosam) and meglumine antimoniate (Glucantime) (5, 6). Moreover, drug failure and resistance to these treatments are increasing in different parts of the world (5, 7).

Nowadays, the use of herbal medicines has attracted many researchers' attention as a novel method of treatment. Silymarin, also known as silybin, is a complex of flavonolignans, which can be obtained from the plant *Silybum marianum* (8). It is declared that silymarin possesses strong anti-inflammatory, anti-oxidant, and anti-cancer properties (9); in addition, up to now, no certain side-effect was reported for this herbal medicine (10). The antioxidative ability is shown by means of a number of endogenous antioxidants such as superoxide dismutase (SOD), Catalase, Glutathione peroxidase (GPx), and etc. (10, 11); IL-10 and IL-12 produced by silymarin are also known as anti-inflammatory cytokines (12). The potential to hasten the tissue regeneration process and production of fi-

broblasts and collagen bundles in the skin has also been reported for silymarin (13, 14).

2. Objectives

Regarding the aforementioned points and the necessity for searching for new treatments for leishmaniasis and also the reported effects of silymarin on the skin tissue, in this study we examined the hypothesis that silymarin can improve the wound healing process in *Leishmania major*-induced CL in BALB/c mice models.

3. Methods

3.1. Plant Extract Preparation and Gel Provision

Silymarin plant was provided by Medipress™, Shiraz, Iran, with herbarium voucher number of 0301. Dried silymarin plant, including aerial parts, leaf, and root were used to produce the hydro-alcoholic extract. The plant was powdered and then extracted with an ethanol (70%)-water mixture (1:1, v/v) for 72 hours. Then, the produced material was filtered and evaporated in order to provide the hydro-alcoholic extract with the yield of 17.75%. In order to facilitate the topical application of the extract, we prepared carboxymethylcellulose (CMC) gels that consisted of a vehicle gel (2% CMC concentration gel) and a specific concentration of plant's extract, 5% and 10% concentrations (14).

3.2. Animals, Parasite Injection, Drug Administration, and Sampling

In this study, twenty eight 5 ± 1 week old female BALB/c mice (weight: 16 ± 3 g) were provided from the Pasteur Institute, Tehran, Iran. The mice were kept in standard cages and condition (humidity 35% - 45%, temperature 23 ± 3°C, and 12-hour dark-light cycle). The mice had free access to water *ad libitum* and were on a standard diet. Amastigotes of *Leishmania major* (MRHO/IR/75/ER) was used for parasite injection. Regarding the previous experiences in which available parasites in culture medium lose their pathogenesis due to various passages, we preferred to use the direct parasite transmission method from mouse to mouse; thus, 0.2 mL of a suspension consisted of 4×10^5 parasites was injected subcutaneously in the area on the top of the tail-base by an insulin syringe. The lesions were observed after 22 days of inoculation in all mice. The animals were then randomly divided into four groups (n = 7): The control group, which only received topical normal saline, the first (E1) and the second (E2) experimental groups, which received the 5% and 10% silymarin gels, respectively, and the gel base group, which were treated with the vehicle CMC gel. Treatments were performed every 24 hours and continued for 20 days from the day in which an open wound on the mouse tail was observed. At the end, the mice were sacrificed with a high dose of ether and full thickness skin specimens from the wound sites, fixed in buffered formaldehyde (pH = 7.2), and were sent for stereological examinations.

3.3. Measurements and the Stereological Study

Measurement of the wound was done in both vertical and horizontal diameters every three days by using a standard Vernier Caliper and the area was calculated based on the ellipsoid surface formula.

Nine pieces were cut from the skin samples, each about 1 mm^2, and were randomly embedded in a cylindrical paraffin block. Isotropic uniformly random (IUR) cuts from the blocks with 15 μm thickness were performed and the slices were stained with Haematoxylin and Eosin (H and E) as well as Heidenhain's azan-trichrome stains. The volume densities of the collagen bundles, vessels, and hair follicles were estimated by using the stereological point counting method (14). The vascular length density (Lv), the mean diameter of the vessels, and the fibroblasts numerical density (Nv) (number of the cells per unit volume of the dermis) were estimated by using previously occupied methods (15). For determining the numerical density of the fibroblasts, the "Optical dissector" device was used and 5 μm distance from the top and the bottom of the tissue was set as the "area of safety" (16).

3.4. Flow Cytometry

The effect of silymarin on the parasite was investigated *in vitro* prior to the main study. Silymarin was dissolved in dimethyl sulfoxide (DMSO) and after that in phosphate buffered saline (PBS) to produce different concentrations of the compound (0.125 - 8 mM). Promastigotes of *Leishmania major* were with different concentrations of the compound for two hours at 4°C. Then, they were collected in Eppendorf tubes and were incubated for 30 minutes at 4°C with 50 g/mL propidium iodide (Sigma™, USA) in a dark condition. Thereafter, the parasites were maintained on ice until analysis. Alcohol (70% ethanol) solution was used for positive control group of parasites. After transferring the suspension into polystyrene flow cytometry tubes (BD Falcon™, USA), data acquisition and analysis were performed by a FACSCalibur flow cytometer (Becton-Dickinson™, USA). A total of 10000 events were obtained in the region, which had already been established as corresponding to the *Leishmania* parasites.

3.5. Statistical Analysis of the Data

Statistical analyses of the outcome were performed by SPSS software (version 19.0. Chicago: SPSS Inc. IBM

Corp.) by using the Kruskal-Wallis and Mann-Whitney U tests. Data were reported as mean and standard deviation (mean ± SD). $P < 0.05$ was considered as statistically considerable.

4. Results

4.1. In vitro Parasite Load Evaluation

Our data showed that silymarin had no significant effect on the parasite load at any concentration comparing to the positive control group, which had a mortality rate of about 88% (Figure 1).

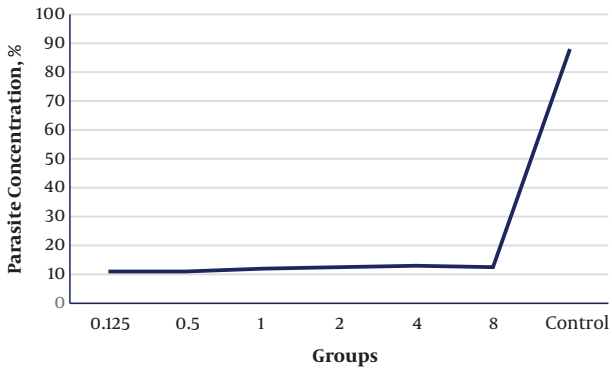

Figure 1. Impact of various concentrations of silymarin (mM) and alcohol (70% Ethanol, Control) on parasites *in vitro*. No significant effect was seen in silymarin mediums.

4.2. Wound Healing Rate

As it is shown in Figure 2, the groups treated with silymarin gels have had a higher closure rate, which presents a significant contrast with the control and gel-base treated groups from day six to the end of the study.

4.3. Stereological Study

As seen in Table 1, silymarin treatments in E1 and E2 groups have led to higher collagen bundle synthesis ($P = 0.037$ and $P = 0.031$, respectively), and also higher revascularization considering the volume density ($P = 0.022$ and $P = 0.018$, respectively), length density ($P = 0.048$ and $P = 0.011$, respectively), and mean diameter of the vessels ($P = 0.019$ and $P = 0.029$, respectively) in comparison to the untreated control group. Fibroblast population and hair follicles showed no considerable contrasts with the control and gel base groups. Moreover, the control group and the gel-base treated group depicted no significant different regarding the stereological parameters.

5. Discussion

Many investigations have taken place in order to find a better cure for the wounds caused by CL with the main goals of improving the closure rate and reducing the scar formation (5). In this study, we evaluated the effects of topical administration of silymarin extract on the process of wound healing in CL. Our results indicated that this herbal medicine can noticeably hasten the rate of wound healing in both 5% and 10% concentrations. Although the population of the fibroblasts presented no considerable increase regarding the stereological study, measurements of collagen bundles and vessels in the silymarin treated groups showed significantly higher values with no remarkable difference between the two concentrations.

Many studies have declared effective potentials for silymarin having positive influences on the process of wound healing. Our previous study on the healing impacts of this agent on normal incisional wounds showed its efficacy in increasing the fibroblast production, reducing scar formation, and improving collagen synthesis (14). A number of previous studies have been done to evaluate the anti-inflammatory and anti-oxidant properties of silymarin, which plays a role in the process of wound healing. Regarding the anti-inflammatory effects, being assessed in several *in vivo* and *in vitro* models, it was reported that silymarin inhibited both lipoxygenase and cyclooxygenase activities, which made it capable of acting as an anti-inflammatory agent, most probably by inhibition of cyclooxygenase-2 expression (17, 18). Silymarin also plays an anti-oxidant role through blocking the protein kinase-mediated IκB degradation, which then leads to NF-κB activation (19, 20). It has also been demonstrated that silymarin decreases nitric oxide production and inducible nitric oxide synthase (iNOS) gene expression in macrophages by inhibiting the activation of NFκB/Rel, which ends up with reducing reactive oxygen species (ROS) (18, 20). Topical administration of this agent also showed anti-inflammatory, anti-oxidant, and anti-apoptotic activities on the skin in different conditions such as UV irradiation, ulcers caused by herpes labialis infection, atopic dermatitis, burns, and even skin cancers (13, 21-25). Moreover, Jabini et al., reported a significant increase in wound healing rate and leishmanicidal activity of the low-dose glucantime when in it was used along with silymarin, however, they did not purely attributed this effect to silymarin (26). None of these papers and any other papers that we have skimmed have reported any certain side effect for silymarin and its ingredients.

As a limitation of the present study, not performing a pathological study on the skin specimens for evaluating the scar formation and inflammation of the tissue can be mentioned. We could not perform such investigations

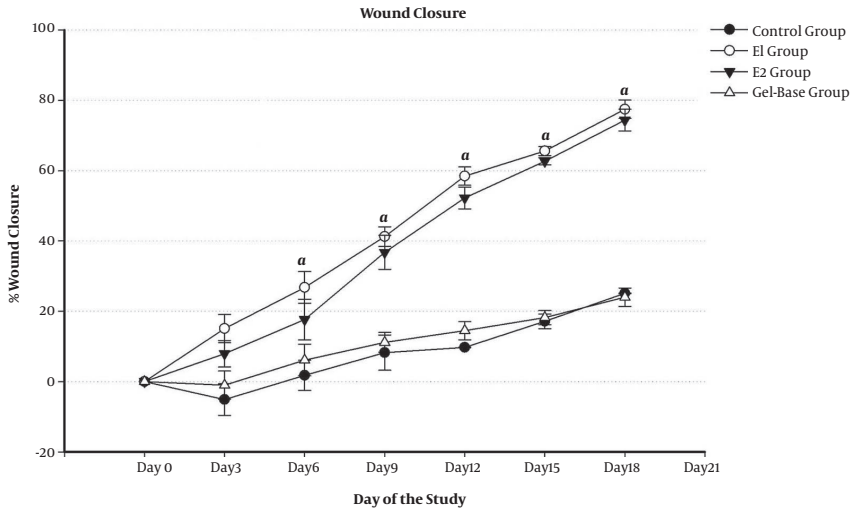

Figure 2. The effect of topical silymarin extract on the closure rate of leishmaniasis induced wounds in BALB/c mice of the control group, gel base treated group, silymarin 5% (E1) and 10% (E2) gels treated groups. Each point exhibits the mean closure percentage of the group in the specific day of the study. The "a" letter depicts P value < 0.5 regarding the comparisons between the silymarin treated groups and the control group.

Table 1. The Population of the Fibroblasts (Nv; $\times 10^3$ per mm^3), Volume Density (Vv) of the Collagen Bundles, the Vessels, the Hair Follicles, and the Length Density (mm/mm^3) and Mean Diameter (μm) of the Vessels in the Dermis Specimen of the Leishmaniasis Induced Skin Wounds of Rats Treated with Topical Silymarin Gel 5% (E1) and 10% (E2) Compared to the Untreated Control Group and the Gel-Base Treated Group

Variables	Groups			
	Control	Gel base	E1	E2
Fibroblasts				
Numerical Density	149.4 (11.5)	152.5 (12.5)	163.9 (102.2)	161.5 (22.78)
Collagen Bundles				
Volume Density	56.4% (5.8%)	60.6% (7.8%)	86.8% (6.5%)[a]	86.2% (4.6%)[a]
Vessels				
Volume Density	1.1% (0.5%)	1.2% (0.9%)	2.2% (0.8%)[a]	2.8% (0.4%)[a]
Length Density	11.2 (9.9)	13.1 (9.6)	16.4 (11.5)[a]	21.9 (12.8)[a]
Mean Diameter	21.3 (11.4)	22.9 (13.7)	40.1 (26.1)[a]	35.2 (18.9)[a]
Hair Follicles				
Volume Density	2.6% (2.5%)	3.1% (1.4%)	2.1% (2.9%)	2.7% (1.1%)

[a] $P < 0.05$, Experimental groups vs. control group.

since most of the specimens were used for making stereology slides. Another limitation was not estimating the parasite load from the wound site. Since the skin lesions are a desirable environment for the growth of opportunist bacteria such as bacillus and other bacteria, providing cultures from the wound sites or an antibiogram with silymarin could be beneficial as well.

5.1. Conclusion

Our study showed that silymarin, as a traditional medicine, in both 5% and 10% concentrations were effective in improving the tissue regeneration and epithelization in skin wounds caused by CL in BALB/c mice models. Despite having no effect in an *in vitro* evaluation in our research, silymarin can be considered as an additional therapy to today's treatments for cutaneous leishmaniasis. Moreover, further investigations, specifically clinical trials, are still required to see its efficacy in human and to realize if any possible side effects may present.

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Footnotes

Authors' Contribution: Mohammad Rastegarian, Zahra Ghanbarinasab, Roham Borazjani, Mahnaz Hosseini, and Shiva Aminnia conducted the experiments, gathered data, and helped in writing the draft. Soheil Ashkani-Esfahani designed the study, analyzed data, provided the charts and figures, and wrote the final draft. Qasem Asgari and Bahador Sarkari helped in study design, leishmaniasis induction, and writing the final draft.

Conflicts of Interests: There are no conflicts of interest.

Ethical Considerations: The study protocol was reviewed and accepted by the Medical Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran.

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