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A NEW APPROACH TO THE THERAPEUTIC EFFICIENCY OF NOVEL SCROPHULARIA BAVANATIA EXTRACT AGAINST *LEISHMANIA MAJOR*

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Running title: Scrophularia bavanatia extract against *Leishmania major*.

Abstract

Background & Objectives: Developing countries have been suffering from Cutaneous Leishmaniasis as an endemic disease. WHO has recommended Glucantime as a cure for it; yet, there are some constraints in the use of this remedy such as high costs, unexpected complications, the need for frequent injections, and unsatisfactory efficacy. Accordingly, this study introduced a new therapy with novel Scrophularia bavanatia extract (SBE) against *Leishmania major* compared to Glucantime and investigated the *in vitro* anti-Leishmania activities of the Local SBE against Leishmania tropica Promastigotes.

Materials & Methods: This experimental laboratory trial investigated the efficacy of *in vitro* anti-Leishmania activities of Scrophularia bavanatia extract against Leishmania tropica promastigotes compared to Glucantime. The Iranian endemic species including Leishmania (L) major [MRHO/IR/75/ER] was proliferated and maintained in the standard culture. Then, the proper densities of SBE were provided, sterilized, and added to cultures containing the parasites. The parasites were counted and divided among micro plates for cell proliferation and then read by ELISA reader. The data were analyzed and compared to the control group.

Results: The mean survival percentage of the parasite in various concentrations of SBE and the 25 µg Glucantime concentration in the logarithmic phase at 24 h indicated a statistically significant difference with the control group (P=0.000); yet, there was no such a significant difference in the stationary phase (P=0.855). Similar to 24 h, there was a significant difference at 48 h in the logarithmic phase (P=0.007) though there was no such a significant difference in the stationary phase (P=0.460). There was no significant difference in the mean survival percentage of the parasite at various SBE concentrations and at 25 µg Glucantime concentration in either the stationary or logarithmic phases at 72 h compared to the control group (P>0.05).

Conclusion: The results suggested that prevalent SBE had a medical potential similar to Glucantime and induced a better and more tangible effect on promastigotes survival without parasite resistance against it, without any complications compared to Glucantime, and with greater availability. However, further studies are necessary to evaluate the effect in cell culture and *in vivo* conditions to confirm this.

Keywords: Leishmania major, *Cutaneous Leishmaniasis*, Scrophularia bavanatia extract, in vitro.

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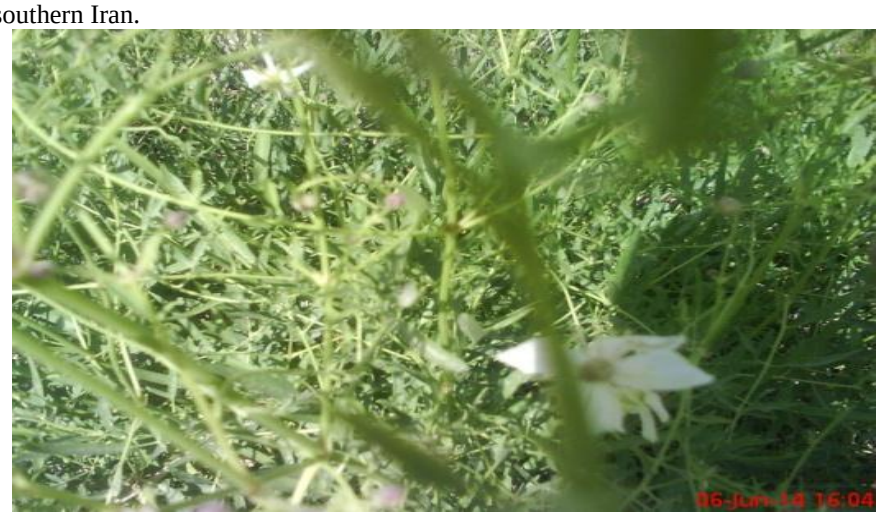
1. Introduction

Leishmaniasis, an intracellular protozoan-parasitic disease, is transmitted by sand fly as the common vector of transmission. Endemics of both zoonotic and Anthroponotic Cutaneous Leishmaniasis (CL) have been observed in different parts of the world. There are about 1–1.5 million cases of the cutaneous form reported per year while 90% of the cases occur in just eight countries including Afghanistan, Algeria, Iran, Iraq, Saudi Arabia, Syria, Brazil and Peru [1]. This refractory condition is rendered as a major problem for WHO which considers it as one of the most stubborn afflictions. This disorder is difficult to manage due to intra-macrophagic location of the infectious form [2]. An effective vaccine is presently lacking, so effective drugs are urgently required to replace or supplement the cures and remedies currently used. The drugs used in clinical practice today are mainly based on pentavalent antimony compounds which were developed before 1959. Moreover, Amphotericin B and Pentamidine are among the other medicines used commonly today [3]. In this disease, an ulcer forms when the crust covering the nodules of CL lesions sheds off; then, this ulcer is frequently infected with other microbial pathogens. These secondary infections diminish the number of Amastigotes to submicroscopic levels so that they may surpass the original Leishmania infection. On the contrary, in the regions in which CL is an endemic condition, such bacterial or fungal cutaneous infections may be detected on the basis of their clinical characteristics. In that case, they may be cured using drugs which frequently induce unexpected after-effects. In this study, a great number of clinically suspected CL lesions in individuals presenting to us for parasitological testing were examined. Hence, we performed bacteriological tests, too, to determine the role of bacterial infections in suspected CL lesions [7, 8]. Bacterial colonies form mostly by staphylococci and streptococci in more than% 60 of patients who manifest open CL ulcers. As stated previously, secondary bacterial infections are among the complications of CL and are often seen in Leishmaniasis patients, especially on the CL ulcer. Of course, bacterial colonization did not disturb the recovery course in these patients [4-7]. No appropriate guidelines are presently available about the when and how of the use of antibiotics as an adjunct treatment for CL. This adjunct therapy may administer antibiotics for gram-positive bacteria as an aid to the specific Antileishmanial therapy just in situations in which there is pain and erythematic conditions. The presence of these signs in chronic leg ulcers demonstrates that the superficial bacterial colonization results in dermal infection while the latter demands systemic antibiotics for treatment. Nonetheless, our case reported here goes beyond the mere control of dermal bacterial infection [8-9].

Image1. The local herbal plant, Schrophularia bavanatia, grows naturally in Bavanat District in the northern part of Fars Province in southern Iran.



The raw poultice of this herb is very caustic with a sharp flavor.(Image1.). It is applied topically by Bavanati aboriginals to fight CL ulcers and is very effective in quick healing of the ulcer. However, it is very caustic and should not be left on the ulcer for more than 15 min, otherwise, it will burn the tissue. Consequently, this laboratory trial explored the *in vitro* anti-Leishmania activities of Scrophularia bavanatia extract (SBE) using specified concentrations against Leishmania tropica promastigotes. In the next phase, this study surveyed a new approach to the therapeutic efficiency of novel Scrophularia bavanatia extract against *Leishmania major*.

2. Material sand Methods

2.1. Preparation of Plant Extract

The Scrophularia bavanatia leaves and stems were dried in an oven using a ventilation system at 30 °C. Then, it was macerated for seven days to obtain the fluid extract using 80% ethanol as a solvent and 20% water, according to the Regulation Norm 309 (Regulation Norm, 1992). Subsequently, the solvent was evaporated, the extract was lyophilized and dissolved in Dimethyl-Sulfoxide (DMSO, BDH, England) at 20 mg/ml and stored at 4°C [10].

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2.2. Parasite Culture

Promastigotes of Leishmania major [MRHO/IR/ER/75] were maintained by $RPMI_{1640}$ medium which was supplemented with 10% fecal calf serum (FCS), 100 µg of Streptomycin/mL, and 100 IU of Penicillin/mL. This was passaged every 3 or 4 days at 26 ºC. Promastigotes of L. major (1×10^6 parasites/mL) were incubated at 26 ºC for 24, 48, and 72 h in fresh medium in the absence or presence of different concentrations (0.01, 0.1, 1, 10, 100 mg/mL) of the methanol extract of SBE. The parasites were discarded after 10 *in vitro* passages. Inactive Leishmania major [MRHO/IR/ER/75] promastigotes in the stationary growth phase were added to the plate wells. We had a negative control and a positive control (Glucantime: 25 and 125 µg/mL [11-12]. To determine anti-Leishmania activity of the SBE, the experimental groups were designed as follows:

Group 1:100 µl of $RPMI_{1640}$ $+1\times10^6$ cells/ml PMs
Group 2: 100 µl of $RPMI_{1640}$ $+1\times10^6$ cells/ml PMs + 10 µl of 0.01 hydro alcoholic *SBE*
Group 3: 100 µl of $RPMI_{1640}$ + 1×10^6 cells/ml PMs+ 10 µl of 0.1 hydro alcoholic *SBE*
Group 4: 100 µl of $RPMI_{1640}$ + 1×10^6 cells/ml PMs+ 10 µl of 1 hydro alcoholic *SBE*
Group 5: 100 µl of $RPMI_{1640}$ + 1×10^6 cells/ml PMs + 10 µl of 10 hydro alcoholic *SBE*
Group 6: 100 µl of $RPMI_{1640}$ + 1×10^6 cells/ml PMs+ 10 µl of 100 Hydro alcoholic *SBE*
Group 7: 100 µl of $RPMI_{1640}$ + 1×10^6 cells/ml PMs+ 10 µl of 25 µg Glucantime

2.3. Cell proliferation ELISA, Brdu (Chemiluminescent) Method

After 24 h, the anti-Leishmania bioassay was done by XTT (Sigma, St. Louis, MO, USA) detecting kit using chemiluminescent assay. The Nucleon bases are nitrogen-containing biological compounds including adenine, cytosine, guanine, and thymine. Briefly, in this technique, the detector substance acts on the thymine base. XTT solution was prepared as 5 mg/mL in $RPMI_{1640}$ without phenol red and filtered through a 0.2 µm filter. Then, 20 µL of this concentration was added to each well and incubated at 25°C for 24 h. After this incubation and in order to solve the formazan crystals, 150 µL of acidic isopropanol was added to each well. The plate was read on an ELISA reader using 450 nm as test wavelength and 630 nm as the reference wavelength [13].

2.4. Statistical Analysis

All experiments were performed in triplicate. The mean and standard error of at least three experiments were determined. Statistical analysis of the differences between mean values obtained from experimental groups was done using student's t-test ($P\leq0.05$).

3. Results

3.1. Results of challenging parasite promastigotes with SBE and 25 µg Concentration of Glucantime in Terms of Time Compared to the Control Group

The survival percentages of rural Leishmaniasis promastigotes at various concentrations of SBE and 25 µg concentration of Glucantime in the stationary and logarithmic phases at 24 h indicated a significant difference in the survival percentages of promastigotes in the logarithmic phase in 0.01, 0.1, 1, and 10 µg/mL concentrations of SBE and 25 µg Glucantime concentration compared to the control group (P=0.000); yet, there was no such as significant difference in the stationary phase in 0.01, 0.1, 1, and 10 µg/mL concentrations of SBE compared to the control group (P=0.855).

Also, similar to 24 h, the survival percentages of rural leishmaniasis promastigotes at different concentrations of SBE and 25 µg concentration of Glucantime in the stationary and logarithmic phases at 48 h demonstrated a significant difference in the survival percentages of promastigotes in the logarithmic phase in 0.01, 0.1, 1, 10, and 100 µg/mL concentrations of SBE compared to the control group (P=0.007); yet, there was no such as significant difference in the stationary phase in 0.01, 0.1, 1, and 10 µg/mL concentrations of SBE and 25 µg concentration of Glucantime compared to the control group (P=0.460).

Moreover, the survival percentages of rural leishmaniasis promastigotes at different concentrations of SBE and 25 µg concentration of Glucantime in the stationary and logarithmic phases at 72 h suggested a significant difference in the survival percentages of promastigotes in the logarithmic phase in 0.01, 0.1, 1, 10, and 100 µg/mL concentrations of SBE compared to the control group (P=0.868); nonetheless, there was no such as significant difference in the stationary phase in 0.01, 0.1, 1, and 10 µg/mL concentrations of SBE and 25 µg concentration of Glucantime compared to the control group (P=0.277) (Figure 1 and Figure 2).

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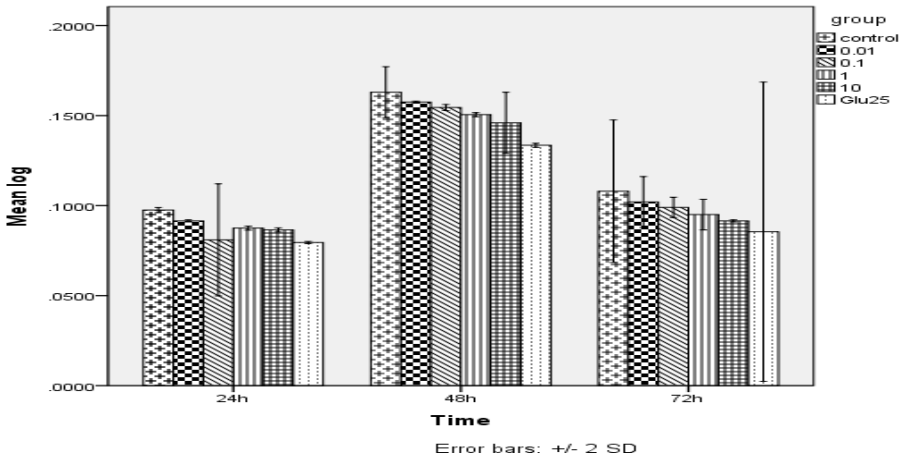

Fig1: The effect of SBE on survival of Leishmania major in the Logarithmic phase in terms of time.

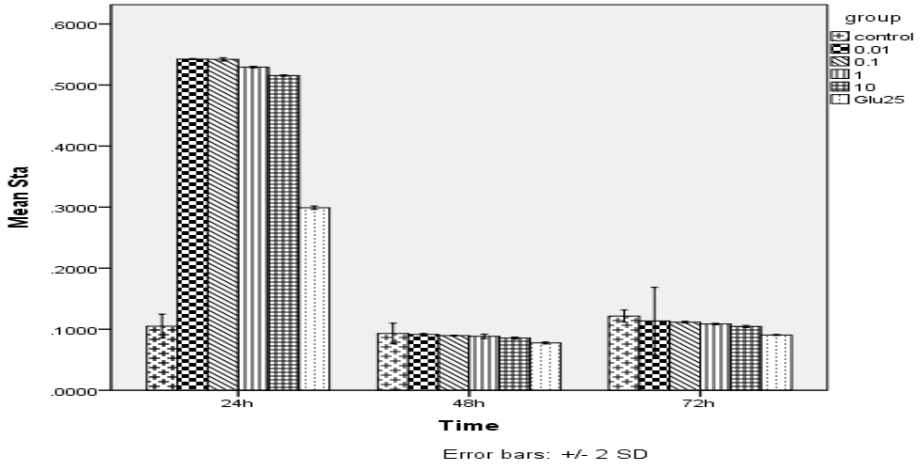

Fig2: The effect of SBE on survival of Leishmania major in the Stationary phase in terms of time.

3.1. Results of challenging parasite promastigotes with SBE and 25 µg Concentration of Glucantime in Terms of Time Compared to the Control Group

3.2. Survival Percentages of rural Leishmaniasis Promastigotes at 0.01, 0.1, 1, and 10 µg/mL Concentrations of SBE and 25 µg Concentration of Glucantime in the Stationary and Logarithmic Phases at 24, 48, and 72 h in Terms of Groups Compared to the Control Group

The survival percentages of rural leishmaniasis promastigotes at various concentrations of SBE and 25 µg concentration of Glucantime in the stationary and logarithmic phases at 24 h indicated a significant difference in the survival percentages of promastigotes in the logarithmic phase in 0.01, 0.1, 1, and 10 µg/mL concentrations of SBE and 25 µg Glucantime concentration compared to the control group (P=0.000); additionally, there was such as significant difference in the stationary phase at 24 h in 0.01, 0.1, 1, and 10 µg/mL concentrations of SBE and 25 µg Glucantime concentration compared to the control group (P=0.000). However, there was no significant difference between various concentrations of SBE and Glucantime (P>0.05).

Moreover, the survival percentages of rural leishmaniasis promastigotes at different concentrations of SBE and 25 µg concentration of Glucantime in the stationary and logarithmic phases at 48 and 72 h revealed no significant difference in the survival percentages of promastigotes in the logarithmic phase in 0.01, and 0.1 µg/mL concentrations of SBE compared to the control group (P=0.271 and P=0.110); nonetheless, there was a significant difference in 1 and 10 µg/mL concentrations of SBE and 25 µg concentration of Glucantime compared to the control group (P=0.033, P=0.010, and P=0.001). There was no perceptible effect on reducing the number of parasite promastigotes with increasing concentrations of SBE at any time and in any group. In other words, increased concentration of SBE had no significant effect on decreasing parasite promastigotes (Figure 3 and Figure 4).

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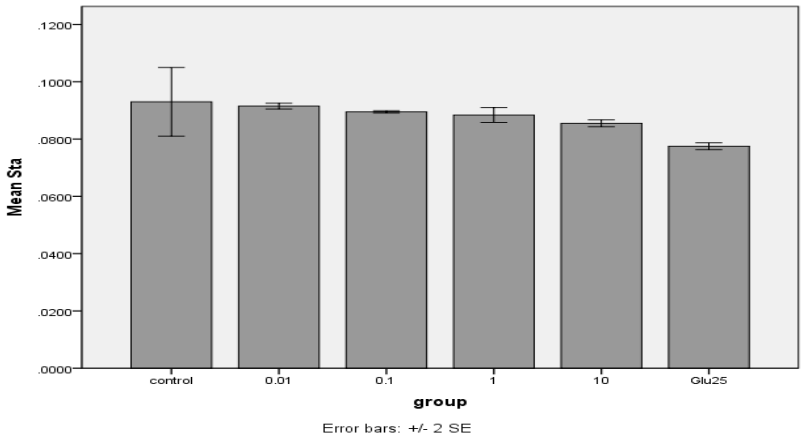

Figure 3: The effect of SBE on survival of Leishmania major in the Logarithmic phase in terms of groups.

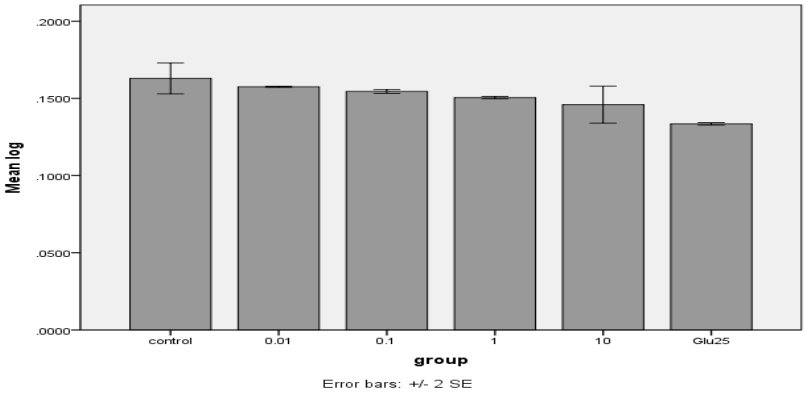

Figure 4: The effect of SBE on survival of Leishmania major in the Stationary phase according to group.

4. **Discussion**

Immigration, natural disasters, development of civilization and civil life, malnutrition, progressive increase of AIDS and lack of sterilized safety upon affliction with it, failure in preparation of vaccines, drugs, and effective pesticides against any species of Leishmania, specifically when there is HIV co-infection, all lead to a mortality rate increased by 100 times so that Leishmania is rendered as one of the three stubborn diseases the treatment of which imposes many problems on the healthcare system according to WHO (13, 14). Cases of both rural and urban cutaneous leishmaniasis are reported from all over the country while visceral leishmaniasis or Kala Azar are reported from Ardebil, Azerbaijan-e Sharqi, Fars, and Bushehr provinces locally and as idiopathic (single) cases from other parts of the country. The struggle for treating and controlling leishmaniasis dates back to more than one century ago; yet, there is no efficient medicine, vaccine, or pesticide available to fight this disorder. The four- and five-valency antimony compounds were used as the first option against leishmaniasis since 1940s onward. The emergence of resistance in some cases necessitates the rapid preparation of new agents. There are various approaches to preparing antileishmanial drugs, the phytomedicine preparations being one of the most important of these strategies (15). A species of snapdragon with the scientific name Scrophularia, called locally "Teshnehdari" (Ilam & Khuzestan provinces) (16), and Scrophularia bavanatia, locally named "Bender", is a wild self-growing multi-year herb which grows in Fars, Bavanat (17). Th chemical constituents of this herb are not identified yet. However, it is used by the local people practically in various forms such as ptisan or decoction, aerosol, and poultice to treat inflammation and infections of the eyes, ears, burns, putrid wounds, colds, etc. The numerous therapeutic characteristics of extract of this plant are due to ingredients such as tanons, phenol compounds, and the like. So far, some ingredients of snapdragon sect like alkaloid, glycoside resin, eridoid, and cryptophilic acid have been isolated and identified. These ingredients are usually found in various parts of the herb such as roots, leaves, sprouts, sapling, and bark (18-20). In this study conducted for the first time, SBE was used with 0.01, 0.1, 1, and 10 µg/mL concentrations and 25 µg Glucantime concentrations in culture medium containing urban Leishmaniasis major promastigotes in the stationary growth phase and logarithmic phase. The parasite promastigotes showed a significant difference with the control group at 24 and 48 h in the logarithmic phase in all concentrations used indicating that SBE with the least concentration of 0.01 µg/mL to the largest concentration of 10 µg/mL significantly hindered the growth of promastigotes compared to the control group that received no drug to

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fight promastigotes. Naserifar et al. (2012) investigated the effect of a special species of this herb simultaneously in macrophage and in culture medium and found that, similar to SBE, the extract of this plant prevented the growth of Leishmaniasis major promastigotes (21). In line with our findings, Sherafati et al. (2008) investigated the aqueous and hydro alcoholic extract of Scrophularia striata on Escherichia coli in vitro and found that the extract of this herb stops the growth of E. coli bacteria (22). Fernandez et al. (1996) demonstrated the antibacterial activity of the phenol distilled from Scrophularia striata (23). Moreover, Steven et al. (2002) demonstrated the wound healing effect induced by glycosides distilled from Scrophularia striata (24). Also, Gins et al. (2000) investigated and demonstrated the anti-inflammatory effects of glycotripnoids isolated from SBE (25). All these studies indicate the anti-parasitic, antibacterial, anti-inflammatory, and would-healing effects of this extract. Indeed, the local people of Bavanat, Fars province, Iran, use traditionally the poultice prepared from crushed leaves and stems of this herb. Our study indicated that the amount of SBE needed for exerting any effect on the stationary growth phase promastigotes is much greater than that needed for exerting the same effect on the logarithmic phase promastigotes, since the stationary form promastigotes are more powerful and resistant against the extract compared to the logarithmic form, a point which must be taken into account in future studies.

5. Conclusion

Leishmaniasis is a major health problem involving many tropical and subtropical regions. A great challenge in treating this annoying disorder is the selection of low-complication drugs with less toxicity and greater sensitivity of the parasite to it. Most chemical drugs suffer from unexpected complications and high toxicity. Hence, the need for a proper substitute for chemical medicines is felt more than ever. Our results suggested green prevalent SBE had a medical potential similar to the Glucantime and induced a better and more tangible effect on promastigotes survival without parasite resistance against it, without any complications compared to Glucantime, and with greater availability. However, further studies are necessary and should be evaluated in cell culture and *in vivo* conditions to confirm it.

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Ethical Considerations: This paper was distilled from a research project approved at the Committee of Ethics in Human Research at School of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran with code of ethics no.: R.SSU.MEDICINE.REC.1395.176**.**

Conflicts of interests: The authors declare no conflict of interests.

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