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**Journal of Chemical and Pharmaceutical Research, 2017, 9(1):112-117**

**Research Article**

**ISSN : 0975-7384**
**CODEN(USA) : JCPRC5**

# Antileishmanial activity of Yazd *Spirogyra* spp extracts Against *Leishmania (L) Major* [MRHO/IR/75/ER] Promastigotes: An *In-Vitro* Study

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**ABSTRACT**

*Leishmaniasis is still health treating problem in low health literacy regions of the world. On the other hand, toxicity, side-effects and expenses of the drugs, increased demand for natural sources material. Aim: Antileishmanial activity of Yazd spirogyra spp extracts (SSE) on the viability of Leishmania (L) major promastigote (PMs) an in vitro study. Sufficient root of spirogyra spp were minced, sterilized and prepared as tropica with concentration of 5, 25,125 and 725 µg. Leishmania major [MRHO/IR/75/ER] cultured then Amastigote was transferred to screw capped vials containing 5 ml of $RPMI_{1640}$ media to which different concentrations of 5, 25,125 and 725 µg SSE were added and each concentration was done in triplicates. Each run also included control. The mortality of parasitoids was measured by the enzyme-linked assay (ELISA) methods. The percentage viability of SSE and showed that viability of parasites in PMs significantly decreased in SSE 5, 25,125 and 725 µg treatment compare to control group (P=0.000, F=30.65). As shown, after 48hour, the percentage inhibition of spirogyra spp and stationary and logarithmic phases of Leishmania major [MRHO/IR/75/ER] that the percent inhibition is density in time (P=0.000, F=16.03).Then 24 h after, the Antileishmanial bioassay were determined by XTT detecting kit using chemiluminescent assay. According to the results dose dependent decrease detected on variety on Cutaneous Leishmaniasis using different levels of Glocantime and hydroalcholic extracts of green prevalent algae (P=0.000, F=24.985). Also, no significant difference detected on anti-Leishmania activity of the SSE compared to Glocantime (P>0.05). These results suggest green prevalent algae had medical potential similar to the Glocantime.*

**Keywords**: *Spirogyra* spp; Cutaneous leishmaniasis; Leishmania major; Yazd

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## INTRODUCTION

*Leishmania*, a flagellated genus protozoan parasite, is the causative agent of human cutaneous Leishmaniasis. In this infectious disease, a high proportion of cases evolve to severe anergic diffuse cutaneous Leishmaniasis, which is severely debilitating and disfiguring [1]. To date several medications used against Leishmaniasis, such as sodium stibogluconate (Pentostam), Meglumine antimoniate (Glocantime), amphotericin B and Pentamidine [2]. Based on the toxicity, side-effects and expenses of the synthetic medications, point towards the need to develop novel biomedical agents [3]. Algae appear to be an interesting source for ethno medicinal and photochemical studies. Algae are rich in dietary fiber, minerals, lipids, proteins, omega-3 fatty acids, essential amino acids, polysaccharides and vitamins [4]. Some algae were reported to produce chemicals that have potent biological effects [5]. *Spirogyra Spp.* is a common filamentous macro alga found in freshwater habitats that is worldwide [6]. The genus *Spirogyra* has recently drawn attention to researchers due to its various biotechnological and industrial applications, mostly based on its sorption properties. *Spirogyra* filaments remove heavy metals [7] and other toxic compounds from effluents [8]. Free-radical-scavenging assays using green algae revealed antioxidant properties for the sesquiterpenoids from *Ulva fasciata Delile* [9]. Green algae are rich in flavonoids and have potent antioxidant properties and reduced hepatic oxidative stress. Data obtained from animal model studies has started to shed light on the fact that the free radical scavenging effects of a hot water extract of *Ulva reticulate* reduced hepatic oxidative stress [10]. Based on the literature, algae are potential producers of compounds with antibacterial, antifungal, antiviral, ant oxidative, anti-inflammatory, and antitumor activity [3]. Scarce information exists on anti-leishmanial activity of the algae. In a study, Fouladvand et al. [11] determined anti-leishmanial activity of some brown, green and red algae from the Persian Gulf. They reported isolated extracts of the different Persian Gulf algae had anti-leishmanial activity. In contrast, red algae have important advantages for the development of new anti-leishmanial chemotherapies [12]. Based on the literature, scarce information exists on role of *SSE* against *Leishmania*. So, the aim of the current study was to determine Antileishmanial activity of SSE against *Leishmania (L) major* [MRHO/IR/75/ER] PMs: An *in vitro* study.

## MATERIAL AND METHODS

### Preparation of SSE

*Spirogyra spp* variety was collected along the Yazd city, Yazd province, Iran during 2015-16. Approximately, 500gr of green prevalent algae was washed with tap water to remove sand particles [11]. Two types of the extracts (methanol and hydro) obtained in this study. The plants (100 gr) were chopped into small pieces and soaked in 1 liter of methanol alcohol (70%) for 3 days at room temperature [15]. About 10 g of each fresh alga, corresponding to 1 g of dry alga material was homogenized in 100 ml cold double distilled water and the same amount in hot double distilled water. The mixture was clarified by filtration using Whatman No. 1 filter paper. Then the crude extract was sterilized by filtering (0.22 µm). The sterilized *Spirogyra spp* variety was stored in –80°C until the date of use [11].

### Parasite culture

*Leishmania* major PMs [MRHO/IR/75/ER] were cultivated in RPMI $_{1640}$ (Sigma, St. Louis, MO, USA) medium supplemented with 10% fetal calf serum (Sigma, St. Louis, USA) and 50 mg/ml Ampicillin (Sigma, Germany) at $25^{\circ C}$. PM forms in stationary phase were suspended in fresh $RPMI_{1640}$ medium to a final concentration of $4\times10^6$ cells/ml. The test was performed in 96-well microtitre plates and each well was filled with 100 µl of culture medium and the plates were incubated at $27^{\circ C}$ for 1 h before algae extracts addition [11].

### Study groups

To determine anti-Leishmania activity of the green prevalent algae, 11 experimental groups were designed as follows:

Group 1 (control): 200 µl of $RPMI_{1640}$ + $4\times10^6$ cells/ml PMs

Group 2: 200 µl of $RPMI_{1640}$ + $4\times10^6$ cells/ml PMs + 1 µg Hydroalcholic *SSE*

Group 3: 200 µl of $RPMI_{1640}$ + $4\times10^6$ cells/ml PMs+ 5 µg Hydroalcholic *SSE*

Group 4: 200 µl of $RPMI_{1640}$ + $4\times10^6$ cells/ml PMs+ 25 µg Hydroalcholic *SSE*

Group 5: 200 µl of $RPMI_{1640}$ + $4\times10^6$ cells/ml PMs + 125 µg Hydroalcholic *SSE*

Group 6: 200 µl of $RPMI_{1640}$ + $4\times10^6$ cells/ml PMs+ 725 µg Hydroalcholic *SSE*

Group 7: 200 µl of $RPMI_{1640}$ + $4\times10^6$ cells/ml PMs+ 1 µg Glocantime

Group 8: 200 µl of $RPMI^{1640}$ + $4\times10^6$ cells/ml PMs+ 5 µg Glocantime

Group 9: 200 µl of $RPMI_{1640}$ + $4\times10^6$ cells/ml PMs+ 25 µg Glocantime

Group 10: 200 µl of $RPMI_{1640}$ + $4 \times 10^6$ cells/ml PMs+ 125 µg Glocantime
Group 11: 200 µl of $RPMI_{1640}$ + $4 \times 10^6$ cells/ml PMs+ 725 µg Glocantime

**Cell proliferation ELISA, Brdu (Chemiluminescent) method**

After 24 h, the anti-leishmanial bioassay was done by XTT (Sigma, St. Louis, MO, USA) detecting kit using chemiluminescent assay. The Nucleobases are nitrogen-containing biological compounds included 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 and 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 solving the formazan crystals, 150 µl of acidic isopropanol was added to each well. The plate was read on an ELISA reader using 540 nm as test wavelength and 630 nm as the reference wavelength [11].

## STATISTICAL ANALYSIS

All data are reported as mean ± standard deviation. Statistical analysis was done using SPSS 21.0 for windows package. One-way analysis of variance (ANOVA) was performed followed by post-hoc LSD. The $p < 0.05$ level to assess significant protection in treatment groups.

## RESULTS

The results of Antileishmanial activity of Hydroalcoholic *SSE* on cutaneous *Leishmaniasis* in Yazd city is provided in tables 1-12 and figures 1-3. According to the table 1, a dose dependent decrease observed in on Cutaneous *Leishmaniasis* Viability using different levels of SSE *(P=0.000, F=30.65).*

**Table 1: Effect of different levels of SSE compared to negative control against viability Cutaneous *Leishmaniasis* PMs in stationary phase**

| | Mean ± SD | Min | Max |
|---|---|---|---|
| **1 (µg/ml)** | 90.6667±.33 | 89.23 | 92.1 |
| **5 (µg/ml)** | 88.0000 ±.00 | 88 | 88 |
| **25 (µg/ml)** | 85.0000 ±.00 | 85 | 85 |
| **125 (µg/ml)** | 76.3333 ±.33 | 74.89 | 77.76 |
| **725 (µg/ml)** | 90.0 ± 4.00 | 80.06 | 99.93 |
| **Total** | 86.0000±1.45 | 82.88 | 89.11 |

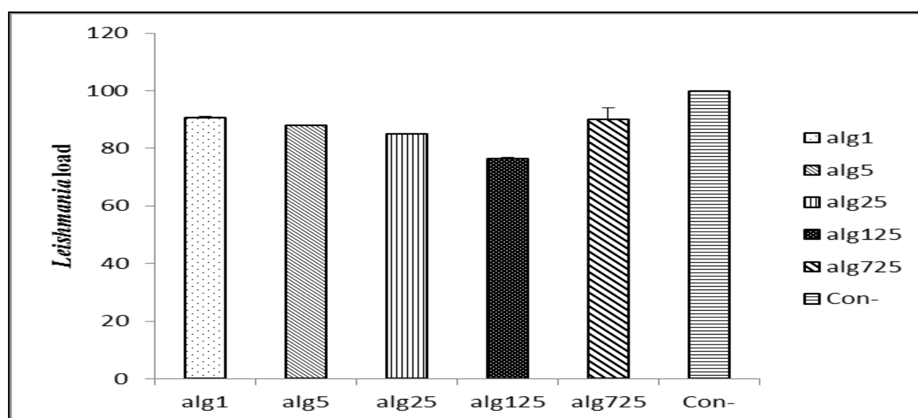


**Figure 1: Effect of different levels of SSE compared to negative control against viability Cutaneous Leishmaniasis PMs in stationary phase**

As seen in figure 1, a dose dependent decrease observed on Cutaneous *Leishmaniasis* Viability using different levels of SSE in comparison to control group *(P=0.000, F=30.65).* The effect of different levels of Glocantime on Cutaneous *Leishmaniasis* Viability is presented in table and figures 2. As seen, a dose dependent decrease identified on Cutaneous *Leishmaniasis* Viability using 1, 5, 25, 125 and 725 µg/ml of the Glocantime (*P=0.000, F=16.03).*

**Table 2: Effect of different levels of Glocantime compared to negative control against viability Cutaneous *Leishmaniasis* PMs in stationary phase**

| | Mean ± SD | Min | Max |
|---|---|---|---|
| **1 (µg/ml)** | 96.66±1.52 | 92.87 | 100.46 |
| **5 (µg/ml)** | 93.33 ± 3.21 | 85.34 | 101.31 |
| **25 (µg/ml)** | 89.00 ±.00 | 89 | 89 |
| **125 (µg/ml)** | 88.00 ±.33 | 88 | 88 |
| **725 (µg/ml)** | 85.33 ± 2.51 | 79.08 | 91.58 |
| **Total** | 90.46 ± 4.48 | 87.98 | 92.95 |

Additionally, a dose dependent decrease on cutaneous *Leishmaniasis* viability using different levels of Glocantime (1, 5, 25, 125 and 725 µg/ml) compared to control group (*P=0.000, F=16.03).*

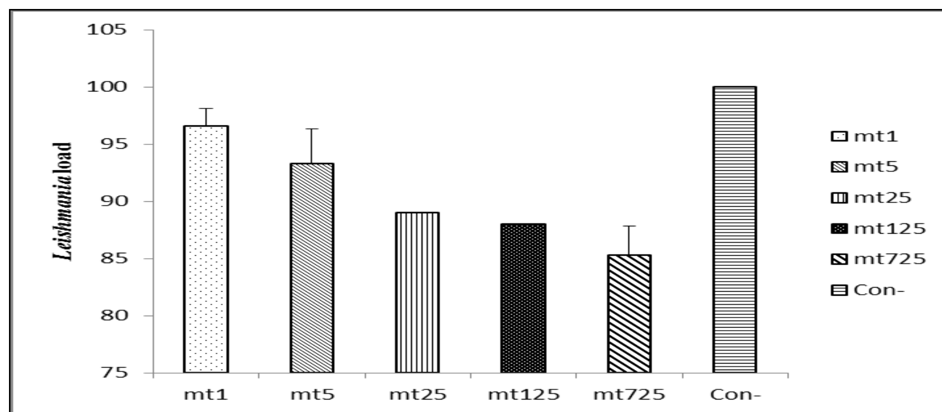


**Figure 2: Effect of different levels of Glocantime compared to negative control against viability Cutaneous *Leishmaniasis* PMs in stationary phase**

Effect of different levels of SSE compared to Glocantime against cutaneous *Leishmaniasis* viability in stationary phase is presented in figure 3. As seen, SSE has the same results compared to Glocantime against cutaneous *Leishmaniasis* viability compared to control group *(P=0.000, F=24.985).*

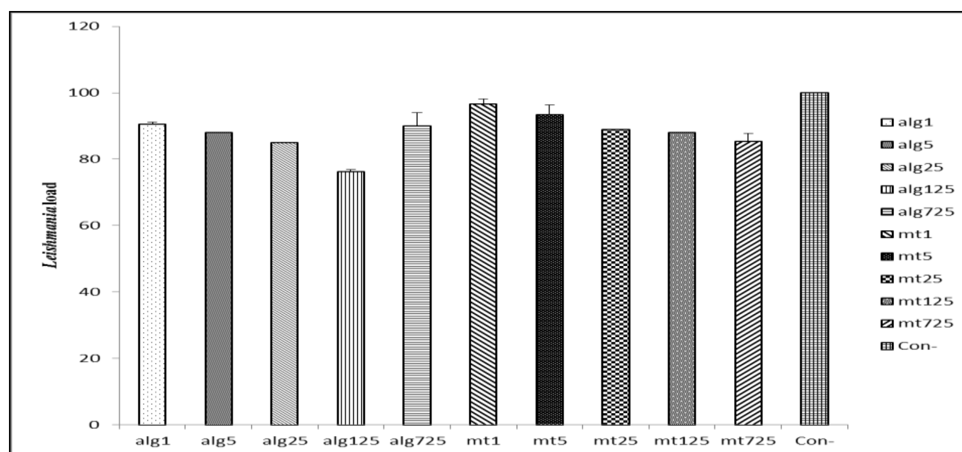


**Figure 3: Effect of different levels of SSE compared to Glocantime and negative control against viability Cutaneous Leishmaniasis PMs in stationary phase**

## DISCUSSION

To our knowledge, there is no previous study on Effect of different levels of SSE compared to Glocantime against Cutaneous *Leishmaniasis* Viability in stationary phase. These results suggest hydroalcholic *spirogyra spp* has medical potential similar to the Glocantime.

Since past decades, pentavalent antimonials constitute the first-line treatment for all forms of *Leishmaniasis* and, in case of therapeutic resistance to these compounds, amphotericin B and liposomal amphotericin B may also be used. However, both of these treatments are not entirely satisfactory, due to being expensive and complicated by the fact that they are given intravenously or intramuscularly. Given the limitations of the current treatments, there is an urgent need for the development of new therapeutics [13]. Studies about anti-Leishmanial effects of Mediterranean red algae showed that halogenated compounds from these algae have strong *Leishmanicidal* activities [22]. Also, brown algae from British and Irish water have showed that all studied brown algae displayed anti-*Leishmanial* activities, but some species showed more potent *Leishmanicidal* activity [14]. Moreover, Sabina et al. [15] have reported the promising anti-*Leishmanial* effects of brown and green algae from the coast of Pakistan.

Limit information exist on effect of anti-*Leishmanial* effects of brown and green algae in Iran. In a sole study, Fouladvand et al. [11] reported crude extracts of brown, green and red species algae especially hot water crude extracts from "*Gracilaria corticata*, *Gracillaria salicornia* and *Sargassum oligocystum*" have *anti-Leishmanial* activity. Compounds isolated from plants with promising activity against the *Leishmania* genera and low toxicity as compared to the pentavalent antimonial drugs include chimanine B; 4-hydroxy-tetralone; 8-8'-biplumbagin; rolliniastatin-1; squamocin; dictyolamide A and B and 2-benzoxazolinone [16]. Also, anti-leishmanial activity reported using various seaweeds such as *Caulerpa faridii*, *Codium flabellatum* and *Melanothamnus afaqhusaini* [15]. Also, Vitale et al. [17] reported the red alga *Asparaguses taxiformis* (40 µg/mL) had 100% mortality of the parasite and the $LD_{50}$ value was detected using 25 µg/mL for PMs and 9 µg/mL for Amastigote.

The mechanism by which algae exerts anti-leishmanial effect is not clear. However, it has been reported that algae has an important role on the host nonspecific immunity by macrophage activation [18]. Thus, the healing of *Leishmania* infections involves the activation of host macrophages, leading to parasite killing as a result of the production of reactive oxygen and nitrogen metabolites by the activated cells [19]. Among immune cells, macrophages play major roles in the immune system and are capable of destroying microorganisms, mainly by the production of intermediate metabolites, such as $H_2O_2$ and NO [20].

These results suggest SSE have medical potential against *Leishmania* infections. Therefore, the development of new therapeutic protocols for the treatment of Leishmaniasis and new drugs with Leishmanicidal and immunomodulatory activity are needed, in order to achieve total elimination of the parasite [17]. Also, it is reported *sponge Plakortis sp.*, inhibited Leishmania mexicana proliferation (1 µg/mL) [21]. In conclusion these results suggest SSE have medical potential similar to the Glocantime. Further researches are needed to determine cellular and molecular mechanism of action.

## ACKNOWLEDGEMENT

The authors would like to thank the authorities of the school of Pharmacy for financial support also thanks to all people who have assisted the experimental procedure especially to Mrs Tayebe Karimi of medical Parasitology and Mycology department, the school of medicine too. This article is based on a thesis submitted No. 4125, in the school of Pharmacy, Yazd Shahid Sadoughi University of medical Sciences, Yazd, Iran.

Source of funding this research received specific grant based on. 4125, a thesis submitted in the school of pharmacy, Yazd Shahid Sadoughi University of medical sciences. Yazd, Iran. Conflicts of interest the authors have no conflict of interest.

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