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Original Article

Effect of *Staphylococcus Aureus* and *Streptococcus beta-haemolytic* Supernatants' on *Leishmania Major* Promastigotes Viability: An *In Vitro* Study

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Background and Aims: Leishmaniasis is an intracellular protozoan- parasitic disease, the common vector of transmission. Both zoonotic and anthroponotic cutaneous leishmaniasis (CL) are endemic in different foci. With regard to the cutaneous form, 1.0-1.5 million cases were reported annually with 90% of the cases. Although antimony-containing compounds that are the main drugs used to treat Leishmaniasis has been recommended for CL treatment by the World Health Organization, but there are some restrictions in this case, including high expense, side effects, frequent injections need, and incomplete efficacy. The current research was conducted the effect of *Staphylococcus aureus* and *Streptococcus beta-haemolytic* Supernatants' on *Leishmania major* promastigotes (PMs) viability: an *in vitro* study.

Materials and Methods: *Staphylococcus aureus* and *Streptococcus beta-haemolytic* cultured for preparing supernatant, then *Leishmania (L) major* strain [MRHO/IR/75/ER] PMs cultured in Novy-Nicolle-Mac Neal (NNN) and roswell park memorial institute 1640 media. The cell proliferation of enzyme-linked immunosorbent assay, BrdU (Chemiluminescent) was performed as described by Roche Diagnostics. The mean of the viability PMs of *Leishmania (L) major* strain [MRHO/IR/75/ER] in culture according to *Staphylococcus aureus* and *Streptococcus beta-haemolytic* supernatant, Glocantime concentrations and in the control group (Glocantime) was obtained.

Results: It was shown that there was a statistical significant difference among *Staphylococcus aureus* and *Streptococcus beta-haemolytic* supernatant inhibits growth of *Leishmania (L) major* strain [MRHO/IR/75/ER] PMs with the control group ($p<0.05$) .

Conclusions: These exciting results suggest that *Staphylococcus aureus* and *Streptococcus beta-haemolytic* Supernatants have significant therapeutic potential as novel anti-leishmanial.

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Introduction

Leishmaniasis is a complex-parasitic disease, in which the sand fly is the most common vector of transmission. With regard to the Cutaneous Leishmaniasis (CL) form, 1.0–1.5 million cases are reported annually with 90% of the cases occurring in 8 countries, including Afghanistan, Algeria, Iran, Iraq, Saudi Arabia, Syria, Brazil and Peru [1-3]. CL is regarded as a major problem of the World Health Organization (WHO), this disease is still considered as one of the most severe affections by this. The treatment of Leishmaniasis is difficult because of the intramacrophagic location of the infectious form [4]. In the absence of a vaccine, there is an urgent need for effective drugs to replace or supplement those in the current use. The clinically used drugs, many of which are based on pentavalent antimony compounds, were developed before 1959. Other drugs, such as amphotericin B and pentamidine, also are used commonly [5-6]. When, the crust that covers the nodules of CL lesions falls off, an ulcer forms that frequently becomes affected with other microbial infections. Such secondary infections can reduce the number of amastigote to submicroscopic levels, and may overshadow the original leishmanial infection. In contrast, in the areas where CL is endemic, these kinds of bacterial or fungal cutaneous infections may be diagnosed on the basis of their clinical features, and be treated with drugs that usually produce side effects. Since many of the clinically suspected CL lesions in individuals who had been referred to the libratory for parasitological testing, it was carried out bacteriological examinations as well in order to determine the role of bacterial infections in suspected CL lesions [7-8]. Bacterial colonization occurs mainly due to staphylococci and streptococci in more than 50% of the patients with open CL ulcers and bacterial colonization did not impair the healing process [9-10]. There is no good guidance on when and how to use antibiotics as adjunct therapy for CL. It would add antibiotics on gram-positive bacteria to the specific antileishmanial therapy only when pain and erythematic are present. In chronic leg ulcers, these signs shown that the superficial bacterial colonization leads to dermal infection, with the latter requiring systemic antibiotics for cure. However, the situation of case we report extends beyond the control of dermal bacterial infection [11]. Secondary bacterial infection is one of the complications of CL and bacterial infections are often seen in the patients with Leishmaniasis, especially on sore of CL. The treatment of secondary bacterial infection is essential for healing. On the other hand, the secondary bacterial infection of the CL will increase the tissue destruction and the resulting scar [12-15]. Secondary bacterial infections are one of the major complications of CL. Some authors emphasize on the rarity of this finding, secondary bacterial infections can prolong the disease duration, increase tissue destruction and the resulting scar [16]. Several studies in Iran and elsewhere have been performed on the rate of secondary bacterial contamination in CL lesions where, in some studies, the improvement rate has been evaluated by the

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application of antibiotic therapy. The results have been controversial [17-19]. Evaluation of secondary bacterial infections in CL lesions and the effect of its elimination or prevention on the lesions' improvement rate is important. In the the present study carried out the effect of *Staphylococcus aureus* and *Streptococcus beta-haemolytic* Supernatants' on *Leishmania major* promastigotes (PMs) viability: an *in vitro* study.

Materials and Methods

Staphylococcus aureus and *Streptococcus beta-haemolytic* cultured for preparing supernatant

The design of the present study was experimental (laboratory-trial) based on Iranian endemic species of *Leishmania (L) major* [MRHO/IR/75/ER]. The strains were cultured for 3, 6, 17, or 24 h in tryptic soy broth (TSB) medium with shaking (200 rpm) at 37°C. Supernatants were collected by centrifugation of cultures (10,000×*g*, 10 min., 4°C) and sterile filtration (0.2-µm pore size) to remove all cells. Vivaspin 6 ml columns with 5- and 10-kDa molecular-mass cutoff filters (Sartorius Stedim Biotech GmbH) were used to fractionate the *Staphylococcus aureus* and *Streptococcus beta-haemolytic* JE2 WT supernatant. The supernatants were kept at -20°C until use [20-21]. A fixed initial density of the parasites was transferred to screw-capped vials containing 5 ml of liquid medium to which 10 µg of *Staphylococcus aureus***,** 10 µg, *Streptococcus beta-haemolytic* and 10 µg Glocantime (Control) were added. Each concentration was done and each run included control cells grown in 96-well tissue-culture micro plates are labeled by the addition of BrdU for 2-24 hours triplicates. During this labeling period, BrdU is incorporated in place of thymidine into the DNA of cycling cells. After removing the labeling medium, the cells are fixed, and the DNA is denatured in one step by adding FixDenat. After removing FixDenat, the anti-BrdU-POD antibody is added, and then bound to the BrdU incorporated into the newly synthesized cellular DNA. The immune complexes are detected by the subsequent substrate reaction. The reaction product is quantified by measuring the light emission using a scanning multi-well luminometer (enzyme-linked immunosorbent assay (ELISA) reader, synergyxHTX, USA).

Source of parasites

Leishmania (L) major strain [MRHO/IR/75/ER] PMs were obtained from the medical parasitology department, school of medicine, Shahid Sadoughi university of medical sciences. It cultured in Novy-Nicolle-Mac Neal (NNN). The Third passage PMs from NNN medium were progressively adapted to roswell park memorial institute (RPMI) 1640 media (Gibco) with antibiotics, glutamine and fetal calf serum (FCS) supplemented with penicillin (100 U/ml), streptomycin (100 µg /ml) and 20% heat-inactivated FCS at 25°C [22-24].

Cell proliferation ELISA, BrdU method

The cell proliferation of ELISA, BrdU (Chemiluminescent) was performed as described by Roche Diagnostics GmbH Roche Applied Science 68298 Mannheim Germany (Version march 2016, Cat. No. 1027640). The cell proliferation ELISA, BrdU (Chemiluminescent) method is a quantitative

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determination of DNA synthesis in cell cultures is now a routine procedure in many laboratories. Protocols are available for various applications, especially in cell culture systems [25].

Statistical analysis

The results were expressed as mean±SEM. Comparisons among the experimental groups were done by one-way ANOVA test using graph pad prism5 software program. The upper level of significance was chosen as $p<0.05$.

Results

Mean of viability PMs of *Leishmania (L) major* strain [MRHO/IR/75/ER] in culture according to *Staphylococcus aureus* and *Streptococcus beta-haemolytic* supernatant by the cell proliferation ELISA, BrdU (Chemiluminescent) method: Affect of *Staphylococcus aureus*, *Streptococcus beta-haemolytic* and both of them together supernatant against *Leishmania (L) major* strain [MRHO/IR/75/ER] of stationary phase PMs that ANOVA test was run shows statistically there was a significant difference (Table1).

The effect of *Staphylococcus aureus*, *Streptococcus beta-haemolytic* and both of them together supernatant in comparison with the control group against *Leishmania (L) major* strain [MRHO/IR/75/ER] of stationary phase PMs that ANOVA test was run showed there was a statistical significant difference ($p<0.5$) (Fig. 1).

Mean of viability PMs of *Leishmania (L) major* strain [MRHO/IR/75/ER] in culture according to Glocantime concentrations of 125, 250, 500 and 1000 µg /ml by the cell proliferation ELISA, BrdU (Chemiluminescent) method: the effect of glocantime concentrations against *Leishmania (L) major* strain [MRHO/IR/75/ER] of stationary phase PMs that ANOVA test was run showed there was a statistical significant difference ($p<0.5$) (Table2).

Table1. Affect of *Staphylococcus aureus*, *Streptococcus beta-haemolytic* and Both of them together supernatant against *Leishmania (L) major*

Bacteria name	Mean±SD	P value
***Streptococcus beta-haemolytic* A group**	43.53±5	0.003
Staphylococcus aureus	41.74±9.78	0.009
Both of them together	26.37±1.12	0.000

Table 2. Effect of Glocantime concentrations 125, 250, 500 and 1000 µg /ml against *Leishmania (L) major*

Glocantime concentrations	Mean±SD
125	88.14±5.23
250	88.18±4.11
500	73.77±5.06
1000	60.93±3.07

($p<0.5$)

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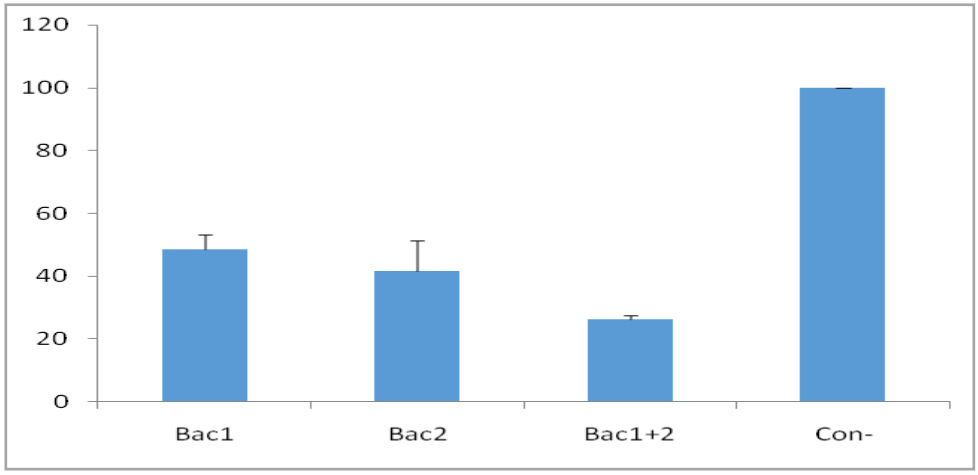


Fig. 1. Effect of *Staphylococcus aureus, Streptococcus beta-haemolytic* and both of them together supernatant in comparison with the control group against *Leishmania (L) major* ($p<0.5$). Bac1= *Staphylococcus aureus;* Bac2= *Streptococcus beta-haemolytic;* Bac1+Bac2= *Staphylococcus aureus* + *Streptococcus beta-haemolytic*; Con-= control

Mean of viability of PMs of Leishmania (L) major strain [MRHO/IR/75/ER] in culture according to Glocantime concentrations of 125, 250, 500 and 1000 µg /ml has been compared with the control group by the cell proliferation ELISA, BrdU (Chemiluminescent) method: the effect of Glocantime concentrations of 125, 250, 500 and 1000 µg /ml against *Leishmania (L) major* strain [MRHO/IR/75/ER] of stationary phase PMs in comparison with the control group through ANOVA test showed a statistical significant difference (Fig. 2).

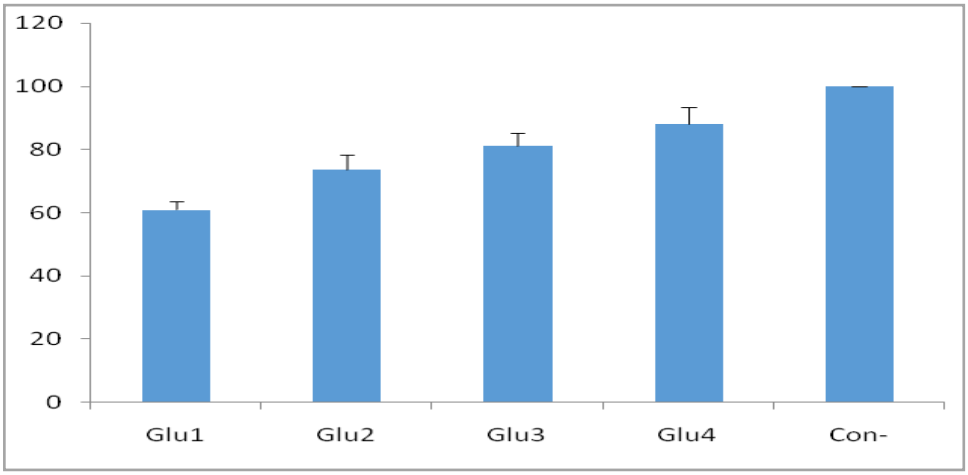


Fig. 2. Effect of Glocantime concentrations 125, 250, 500 and 1000 µg /ml against *Leishmania (L) major* ($p<0.5$).
Glu1= 125 µg /ml; Glu2= 250 µg /ml; Glu3= 500 µg /ml; Glu4= 1000 µg /ml

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Discussion

Although World Health Organization has been recommended antimony is the main drug used to treat Leishmaniasis, but there are some restrictions in this case, including high expense, side effects, frequent injections, and incomplete efficacy [26]. Despite the recent developments, the effective therapy for CL has been yet based on long parenteral courses of these drugs for six decades, even though these are fairly costly, toxic and inconvenient to use, along with inadequate knowledge on their pharmacokinetics or mechanism of action [27-28]. Many studies have been done for preparing drug to Leishmaniasis treatment, but none of these treatments, including herbal medicine, synthetics drugs, gene therapy has not been successed; and for this reason preparation of anti-Leishmaniasis is one of the most urgent needs.

Leishmaniasis still poses an enormous public health problem in throughout the world. Current measures are outdated and have some associated drug resistance, prompting the search into novel therapies. Several innovative approaches are under investigation, including the utilization of fungal and bacteria's, supernatant of culture media on the viability of Leishmania PMs. In a study performed by Miriam et al. (2011), the effect of BMAP-28 antimicrobial peptides on *Leishmania major* promastigote and amastigote growth: role of Leishmanolysin in parasite survival was investigated; their results demonstrate that BMAP-28 is a promising template for the development of anti-infective therapeutics targeting Leishmania [29]. In a research was done by Fabri et al. (2009), their results suggest that *M. frigidus* has interesting antimicrobial, antileishmanial and antioxidant activities [30]. So far, many studies have been carried out regarding the effect of *Staphylococcus aureus* and *Streptococcus beta-haemolytic* supernatants on parasitic infections. Among biological and pharmacological activities of Staphylococcus aureus and *Streptococcus beta-haemolytic* supernatants the most activities are antifungal [31], anti-inflammatory [32], antioxidant [33] and several other ones.

Conclusion

Interestingly, *Staphylococcus aureus* and *Streptococcus beta-haemolytic* supernatants appear to be the most potent anti-parasitic of the three isomers against type *L major* PMs and amastigotes. These exciting results suggest that *Staphylococcus aureus* and *Streptococcus beta-haemolytic* supernatants have significant therapeutic potential as a novel anti-leishmania.

Conflict of Interest

The authors declare no conflicts of interest.

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