Archives of Razi Institute, Vol. 74, No. 4 (2019) 413-422

Original Article

Determination of the Effective Dose of Curcumin alone and in Combination with Antimicrobial Peptide CM11 on Promastigote Forms of Iranian Strain of *L. major* (MRHO / IR / 75 / ER)

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Received 18 June 2018; Accepted 30 August 2018
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ABSTRACT

Zoonotic cutaneous leishmaniasis t caused by *Leishmania major* is spread in focal areas of more than 90 countries in the tropics, subtropics, and southern Europe. In the absence of any effective vaccine, the only means to treat and control leishmaniasis is conventional medication. Glucantime is the first choice of anti-leishmanial drug, has serious side effects like high toxicity, exorbitant cost, problems with the administration and development of resistance. Curcumin is the active component from the rhizome of herb Curcuma longa, possessing many pharmacological and biological activities with antiprotozoal and anti-proliferative effects which make it a good alternative to existing therapy. Antimicrobial peptides like CM11, a small peptide consisting of 11 amino acids, are also novel potential drugs against at least wide spectrum of microbial organisms. The aim of this study was to evaluate the effect of curcumin alone and in combination with CM11 on promastigote form of *L. major* (MRHO / IR / 75 / ER) for 12h and 24h *in vitro*. The results of Giemsa staining showed that the morphology of the flagellum and cell shape increased changed with increasing concentration of curcumin (5 µM, 10 µM, 20 µM, 40 µM and 80 µM). MTT and Trypan blue results demonstrated that the promastigotes were susceptible against curcumin in dose and time dependent manner, while CM11 alone at concentration of 8 µM as well as in combination with 10 and 20 µM curcumin had no significant effect on promastigotes. Our results revealed that curcumin can provide a new curative candidate against cutaneous leishmaniasis.

Keywords: Curcumin, Antimicrobial Peptide CM11, Promastigote, *Leishmania. major,* Glucantime

Détermination de la Dose Efficace de Curcumine Seule et en Association avec le Peptide Antimicrobien CM11 sur la Forme Promastigote de la Souche Iranienne *Leishmania major* (MRHO/IR/75/ER)

Résumé: La leishmaniose cutanée zoonotique causée par *Leishmania major* est répandue dans certaines zones de plus de 90 pays situés dans des régions tropicales, subtropicales et du sud de l'Europe. En l'absence de vaccin efficace, le seul moyen de traiter et de contrôler la leishmaniose est le traitement conventionnel. Le glucantime constitue le premier choix parmi les agents anti-leishmaniens, mais exerce des effets secondaires graves, notamment une toxicité élevée, des coûts exorbitants, des problèmes d'administration et le développement de résistances. La curcumine est le composant actif du rhizome de la plante *Curcuma longa* et possède de nombreux impacts pharmacologiques et biologiques. Les effets antiprotozoaires et anti-prolifératifs et anti-

prolifératifs de cet agent en font une alternative appropriée au traitement existant. De plus, les peptides antimicrobiens, tels que le CM11, qui est un petit peptide composé de 11 acides aminés, sont de nouveaux médicaments potentiels efficaces contre un large spectre d'organismes microbiens. Le but de cette étude était donc d'évaluer l'influence de la curcumine seule et en association avec le peptide antimicrobien CM11 sur la forme promastigote de *L. major* (MRHO/IR/75/ER) après 12 et 24 heures d'exposition *in vitro*. La coloration de Giemsa a indiqué des modifications morphologiques au niveau des cellules du flagelle associées à l'augmentation de la concentration de curcumine administrée (5, 10, 20, 40 et 80 µM). Les résultats du test MTT et du bleu de trypan ont montré que les promastigotes étaient sensibles à la curcumine en fonction de la dose et du temps. De plus, le CM11 seul à la concentration de 8 µM ainsi qu'en association avec 10 et 20 µM de curcumine n'a pas eu d'effet significatif sur les promastigotes. Nos résultats ont révélé que la curcumine peut être considérée comme un nouveau candidat curatif contre la leishmaniose cutanée.

Mots-clés: Peptide antimicrobien CM11, Curcumine, Glucantime, *Leishmania major*, Promastigote

INTRODUCTION

Leishmaniasis is a group of endemic diseases and are mostly prevalent in poor developing countries (Berman, 1997). The *Leishmania* protozoan was first described in 1903 separately by Leishmana and Donova. This organism has been found to be a complex grouping of species, causing visceral leishmaniasis, cutaneous disease or both (Markle and Makhoul, 2004). The promastigote form of the parasite is a motile form with an anterior flagellum that develops in the sand fly. The promastigote form develops into metacyclic infectious form over approximately 10 days. The infectious form can then infect the macrophages of the host as amastigote form which is obligate intracellular, non-motile and about 2.5 to 7 microns in diameter (Chappuis et al., 2007). Cutaneous leishmaniasis can become disseminated (diffuse cutaneous leishmaniasis) especially in immunosuppressed persons and cause chronic leishmaniasis. Other unusual types of cutaneous disease include recidiva leishmaniasis, in which small nodules develop around a healed scar. The Kala-azar leishmaniasis is a visceral infection which sometimes is accompanied with the widespread cutaneous lesions (Hunter and Strickland, 2000). Zoonotic cutaneous leishmaniasis caused by leishmaniasis *L. major* is an important health problem in many countries of North Africa, Eastern Mediterranean region and South West Asia (Desjeux, 1996; Ahmad, 2002). The number of new cases per year of cutaneous leishmaniasis estimates of the number of cases range from approximately 0.7 million to 1.2 million (WHO, 2018), WHO has reported about Afghanistan, Algeria, Brazil, Colombia, the Islamic Republic of Iran, Pakistan, Peru, Saudi Arabia and the Syrian Arab Republic as countries with the high risk countries for leishmaniasis with mostly multiple lesions on the exposed part of the body and ninety present of cutaneous leishmaniasis cases occur in this countries (Desjeux, 2004). Meglumine antimoniate is considered first-line drugs in the treatment cutaneous leishmaniasis caused by L. major (Organization, 1995), their efficacy and toxicity are thought to be related to their content of pentavalent antimony (Reynolds, 1989). Pentavalent antimony compounds with leishmanicidal activity, but which are not effective against free flagellated forms *in vitro* (Organization, 1995). In the absence of any effective vaccine, the only means to treat and control leishmaniasis is conventional medication. Most of the drugs, like Glucantime, currently being used for leishmaniasis are regarding their high toxicity, development of resistance, exorbitant cost, and problems with the administration, difficult to use (Singh and Sivakumar, 2004). Therefore, there is an urgent need for safe, more effective and economically feasible drugs for the treatment of leishmaniasis.

Herbal medicine can most probably help in this way. Research with medicinal plants constitutes a very viable strategy for drug discovery. Curcumin is the active component from the rhizome of herb Curcuma longa, possessing many pharmacological and biological activities. This compound has anti-inflammatory, antioxidant, antiviral, anti-infectious, and anti-carcinogenic effects Toda et al. (1985); Araujo et al. (1999); Maheshwari et al. (2006); Buhrmann et al. (2014); Mashebe et al. (2014) have reported about curcumin as parasitcidal agent. Antimicrobial peptides (AMPs) represent an alternative to classical antibiotics. AMPs are present in all types of life and are ancient components of the innate immunity and represent the first line of defense in an infection (Zasloff, 2002; Maróti et al., 2011). Antimicrobial peptides and proteins are promising new drugs. Peptides and proteins are produced by a wide range of organisms, from bacteria to plants and humans and protect the host against infections (Aerts et al., 2011). The hybrid CM11peptide is a small peptide consisting of 11 amino acids (WKLFKKILKVL-NH2) with alpha helical structure (the first 7 amino acids are derived from Cecropin occurring in hem lymph of silk butterfly [from amino acid 2 to 8] and 4 amino acids derived from melittin occurring in Apis mellifera [from amino acid 6 to 9] with antimicrobial activity (Moore et al., 1996; Tamang and Saier Jr, 2006; Moghaddam et al., 2012). In the present report, the effect of curcumin alone and in combination with CM11 peptide was studies on promastigote form of *L. major*.

MATERIAL AND METHODS

Sample. Iran's Standard Strain *L. major* (MRHO / IR / 75 / ER) was prepared in the Department of Parasitology of Tehran University of Medical Sciences. Leishmania major promastigotes were grown at 25 °C in RPMI-1640 medium (CAISSON company) supplemented with penicillin-streptomycin 100 µg /ml (gibco company) and 10% fetal calf serum (PAA company).

Treatment with Curcumin, CM11 and Glucantime. Curcumin with a purity of more than 95% was purchased from Indsaff (Punjab, India). This commercial source of curcumin contains three major components: diferuloyl-methane (the most abundant and active component of turmeric) (82%) and its derivatives demethoxycurcumin (15%) and bisdemethoxycurcumin (3%), together referred to as curcuminoids (Bharti et al., 2003), Curcumin dissolved by DMSO (dimethylsulfoxide) as a stock concentration of 5000 µM and kept at -80°C and CM11 peptide, a small cationic peptide with formula CM11 (WKLFKKILKVL-NH2) was prepared by Biomatik company, Glucantime as a commercial product (Rorer Rhone-Poulenc Specia Paris, France) was prepared by the Department of Parasitology of Tehran University of Medical Sciences. The number of 2.5×10^6 cell/ml promastigotes on the logarithmic phase of the Iran's Standard Strain *L. major* (MRHO / IR / 75 / ER) were cultured in RPMI1640 medium and treated with different concentrations of curcumin (5 µM, 10 µM, 20 µM, 40 µM and 80 µM) for 12 h and 24 h. In the next experiment, 2.5×10^6 cell/ml were treated simultaneously with 8 µM CM11, 8 µM CM11/10 µM and 8 µM CM11/ 20 µM curcumin. It is to mention that the peptide at 8 µM concentration was chosen because CM11 at this concentration had no effect eukaryotic cells like fibroblast cells (data not shown). In other experiment 2.5×10^6 cell/ml were treated with 123.5 µl (10.5 mg pentavalent antimony)/ml Glucantime for 12 h and 24 h. Subsequently, the promastigotes were fixed in 2% formaldehyde /phosphate-buffered saline and counted using Neubauer chambers and stained.

Trypan blue staining and Giemsa staining. The effects of different concentrations of curcumin alone and in combination with the minimum concentration of curcumin with minimal effective concentration of CM11 peptide, CM11 peptide and Glucantime without curcumin on the parasite (promastigote form) evaluated after 12hour and 24 hour and estimated the parasite growth microscopically by Trypan blue dye exclusion

test that showed viability and mobility of the parasite (JG, 2011). The evaluation of parasite survival was determined by counting the cells by hemocytometer chamber (Neubauer chamber) before and after treatments as described above. For the analysis of the viability of the promastigotes, the living and dead cells after 12 and 24 hours treatment were separately compared to the number of the cells in control group at the mentioned culture times. The morphological alterations of promastigotes were analyzed by Giemsa staining (Brown et al., 1993; Guerrant et al., 2006) in treated and untreated cultures in the giving times.

MTT Assay. The promastigotes viability was determined by colorimetric MTT assay test (Díaz-Achirica et al., 1998; Shakibaei et al., 2013; Shakibaei et al., 2014) by treated and untreated promastigotes. Briefly, 2.5× 10^6 cell/ml promastigotes on the logarithmic phase of the Iran's Standard Strain *L. major* (MRHO / IR / 75 / ER) were cultured in RPMI1640 medium and treated with different concentrations of curcumin (5 µM, 10 µM, 20 µM, 40 µM and 80 µM) for 12 h and 24 h. To determine the destructive effect of CM11 on promastigotes, the promastigotes were treated with 2, 4, 8, 16, 32, 64 and 128 µM CM11 for 24 hours. Since the destructive effect of CM11 begun at concentration of 8 µM on promastigotes, in the next experiment, 2.5× 10^6 cell/ml were treated simultaneously with 8 µM CM11, 8 µM CM11/10 µM and 8 µM CM11/ 20 µM curcumin. In other experiment 2.5× 10^6 cell/ml were treated with 123.5 µl (10.5 mg pentavalent antimony) / ml Glucantime for 12 h and 24 h after incubation, 200 µL of various cultured sample was added to a 96-well tissue culture plate and centrifuged at 2500 x g for 10 minutes at 4°C. After centrifugation, the supernatant was removed. 100 µl from diluted MTT (10µl MTT+90 µl RPMI without FCS) was added to each pellet. 100 µl culture medium was used as blank sample. The micro titer plate was incubated for 3-4 hour in dark space at 25 °C. Subsequently, the plate was centrifuged 2500 x g for 10 minutes. After centrifugation, the supernatant was removed and 100µl (HCl + Isopropanol) was added to the each wells and incubated additionally for 30 minutes at 25°C under shaking condition. At the end, the colorimetric reaction was measured for each well at 560 nm using an ELISA reader (BioTek-ELX800, USA). The percentage of the cell viability was calculated by specific formula (mitochondrial activity= [AT-AB] / [AC-AB] ×100. In this formula, AT is the optical density measured for each well containing treated cells, AB is the optical density of blank wells, and AC is the optical density of control wells (Delavari et al., 2014). MTT test repeated three times.

RESULTS

Four millions cells from cryopreserved of promastigote form of *L. major* was cultured in 10 ml RPMI / 10%FCS for 4 days to achieve the logarithmic phase resulting in the rosette forming structure. 2.5× 10^6 cells/ml of above mentioned culture were treated with 5 µM, 10 µM, 20 µM, 40 µM and 80 µM curcumin and as control with 123.5 µl (10.5 mg pentavalent antimony)/ml Glucantime for 12 and 24 hours respectively and subsequently the cells were analyzed by Giemsa staining, trypan blue staining and MTT test. The results showed that the Glucantime had no significant effect on the promastigote growth after 12 hours and 24 hours respectively (figures 1 & 2). In contrast to the Glucantime, curcumin had destructive effect on promastigote in dose dependent manner. The morphological changes have been observed by Giemsa staining including changes in the morphology of the flagellum and the degradation of the normal cell shape. The promastigotes lost their typical morphology and formed clumps of amorphous material. In concentration of 40µM and 80µM/ml of curcumin this morphological change was strongly pronounced (figures 1 & 2). Treatment of the promastigotes with 8µM CM11 showed mild effects in typical morphology structure of parasite whereas 8µM CM11in combination with 10µM or 20µM curcumin could not enhance the destructive effect of curcumin alone (figure 3). The MTT analysis of the treated compared to the untreated cells for 12 hours showed that promastigotes were

susceptible against curcumin with concentration of 20 µM and the effect of curcumin increased on promastigote in dose dependent manner. The mitochondrial activity of a lived promastigotes was only 23% after 12 hours of treatment with 80 µM curcumin. Approximately 10% mitochondrial activity had the promatigotes after 24 hours of treatment with 80 µM curcumin (figure 4). Counting of the promastigotes by trypan blue staining confirmed the results achieved by MTT analysis with more emphasis of disruption of the promastigotes (figure 5). The number of promastigotes decreased already after treatment with 5 µM curcumin demonstrated by trypan blue analysis (figure 5). The promastigotes were barely detected after treatment with 80 µM curcumin within 12 and 24 hours respectively (figure 5). Trypan blue analysis of the treated promastigotes for 24 hours showed that CM11 at concentrations of 2 and 4 µM had no effect on promastigotes, whereas the constructive effect started with concentration of 8 µM and continued in dose dependent manner. Within 24 hours culture, approximately 50% and 70% of the promastigotes, which were treated with 16 and 32 µM CM11, were dead respectively. Approximately all promastigotes

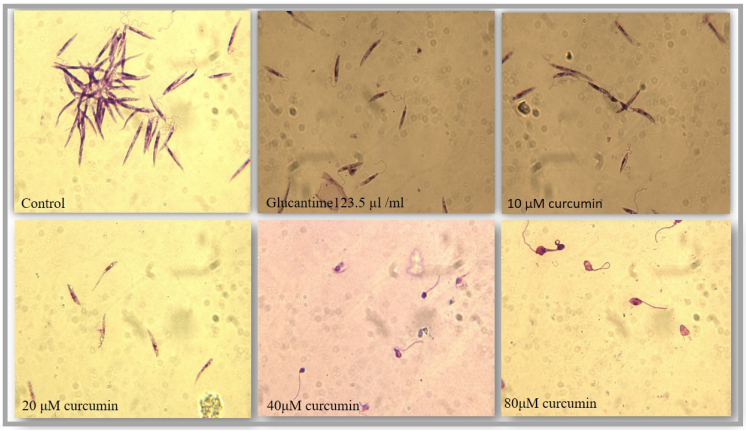

Figure 1. 2.5× 10^6 cell/ml promastigotes were cultured in RPMI164 medium and treated with different concentrations of curcumin (10 µM, 20 µM, 40 µM and 80 µM) and with 123.5 µl /ml Glucantime for 12 h and subsequently, stained with Giemsa and examined under light microscope 100X magnification.

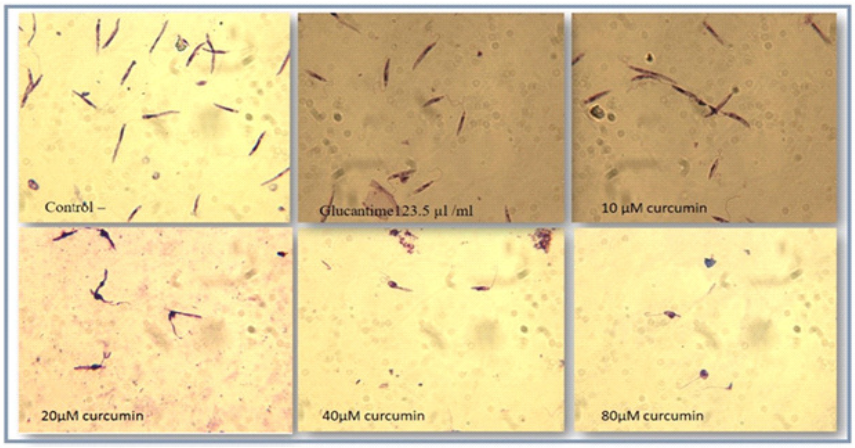

Figure 2. 2.5× 10^6 cell/ml promastigotes were cultured in RPMI164 medium and treated with different concentrations of curcumin (10 µM, 20 µM, 40 µM and 80 µM) and with 123.5 µl /ml Glucantime for 24 h and subsequently, stained with Giemsa and examined under light microscope 100X magnification.

were dead, if they were treated with 64 and 128 µM CM11 for 24 hours. Trypan blue analysis showed that 8 µM CM11 It has no destructive effect on promastigotes, as well in combination with 10 and 20 µM curcumin had not synergistic effect.

DISCUSSION

In most zoonotic cutaneous leishmaniasis healing occurs within 6-9 months without treatment. Unfortunately this infection leave permanent scars causing social stigma. The first line drugs, pentavalent antimony compounds, which are toxic and has considerable side effects with the possibility to develop drug resistance, indicate the urgent need for new and creative treatments without mentioned side effects. Curcumin represents as anti-inflammatory and anti-

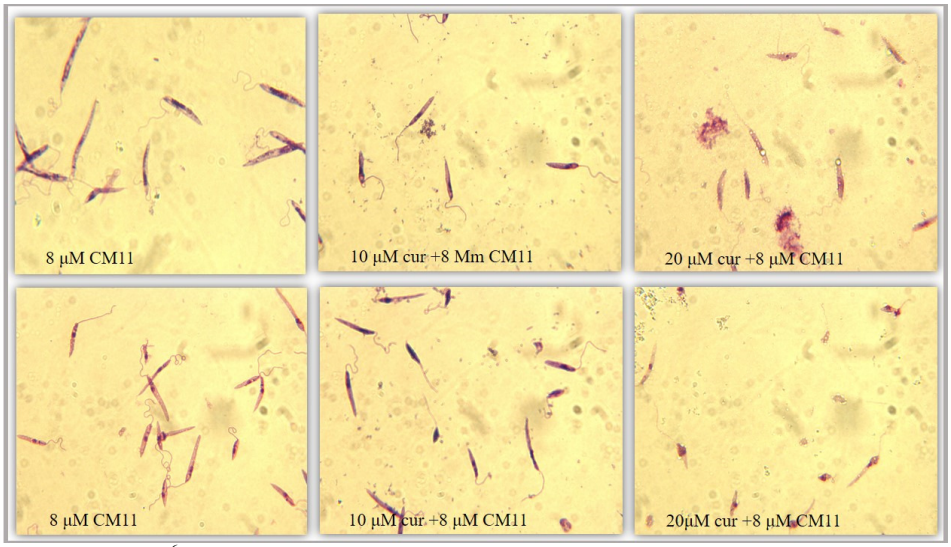

Figure 3. 2.5× 10^6 cell/ml promastigotes were cultured in RPMI1640 medium and treated with different concentrations of curcumin (8 µM CM11, 8 µM CM11+10 µM curcumin , 8 µM CM11+20 µM curcumin) for 12 and 24h and subsequently, stained with Giemsa and examined under light microscope 100X magnification.

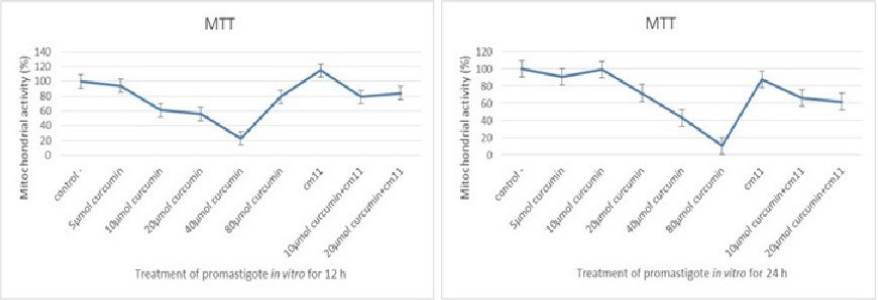

Figure4. Quantification of mitochondrial changes after treatment with different concentrations of curcumin (10 µM, 20 µM, 40 µM , 80 µM, 8 µM CM11, 8 µM CM11+10 µM curcumin and 8 µM CM11+20 µM curcumin) for 12 and 24h and subsequently.

oxidative effect reported to be a potent inhibitor of reactive oxygen species (ROS formation) generated by NADPH in mitochondria (Venkatesan et al., 2000; Biswas et al., 2005; Attia et al., 2014). Furthermore, anti-cancerous effects of curcumin has been reported previously as anti-proliferative, inhibitor of the transcription factor NF-kB and modulation of variety of growth factor receptors and cell adhesion molecules (Bharti et al., 2003; Posner, 2010; Wilken et al., 2011; Shakibaei et al., 2013) and even can potentiate the antitumor activity of anti-tumor agent like 5-fluorouracil (Toden et al., 2015). These actions lead to assumption that curcumin can be used as a preventive or therapeutic agent for several human diseases. Curcumin is extracted from rhizomes of turmeric and had three phenolic analogues (difeuloylmethane, desmetoxycurcumin and bidesmetoxycurcumin) and is called as curcuminoids (Anand et al., 2008). In the present study could show that promastigotes were susceptible against curcumin in dose and time dependent manner and the anti-proliferative and killing effect of curcumin on promastigotes has been shown *in vitro* determined by MTT, Trypan blue analysis as well as by Giemsa staining. Pinto et al (2016) had used curcumin at higher concentration (500µg/ml and 7.8µg/ml) against promastigotes of *L. major* and *L. braziliensis*, whereas in our study the promastigotes of *L. major* (MRHO / IR / 75 / ER) were used and the treatment was performed with curcumin concentration between 28.96 µg/ml (80µM) and 1.81 µg/ml (5µM). According to the difference in the methodic of determination of cell viability in both studies, it seems that curcumin act in the intensity of the effect on promastigotes of *L. major* differently. For the viability test in our experiments, the intact cells after 12 and 24 hours treatment were compared to the amount of the cells in control group, whereas in the study of Pinto et al. (2016), they compared the living cells with the total living and dead cells at the end time of experiment in the same culture test. We believe that with the method for calculation in our study, we should be able to show the inhibitory effect of curcumin in the proliferation of the promastigotes and the killing effect leading to the strong lysis of the promastigotes producing uncountable cell debris, which is easy to see in the cultures treated with 40 and 80 µM curcumin. Taken together, our results confirmed the results of Pinto et al. (2016). Our results confirmed also the results achieved by Koide et.al (2002) according to the LD50 value of curcumin on *L. major*. In contrast to the results reported by Chan et.al. (2005), our results showed the gross inhibitory effect of curcumin on promastigote in concentrations above 5 µM. This is may be due to the different strain of L. major used in both studies. The anti-parasitic activity of antimicrobial peptides (AMPs) was evaluated by Löfgren et al. (2008) against the

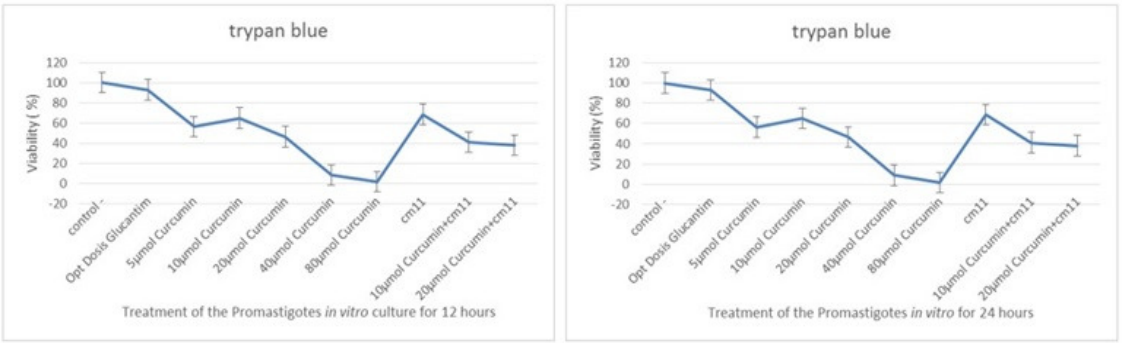

Figure 5. Promastigote viability analyzed by the trypan blue dye exclusion method after treatment with different concentrations of curcumin (10 µM, 20 µM, 40 µM , 80 µM, 8 µM CM11, 8 µM CM11+10 µM curcumin , 8 µM CM11+20 µM curcumin) and with 123.5 µl /ml Glucantime for 12 and 24h and subsequently.

promastigote form of *Leishmania braziliensis* and could show that one of these peptides named Tach was the most potent peptide killing completely *L. braziliensis* at concentration of 12.5 µM. Another AMP named CM11 had antifungal and antibacterial activities (Andreu et al., 1992; Moghaddam et al., 2012). Moghaddam et al. (2012) demonstrated small peptides (CM11, CM15) were highly active against most important hospital infection strains of bacteria. For this reason, beside curcumin, antimicrobial peptide CM11 was also used in our study. The destructive effect of CM11 on promastigotes was 100% at concentrations of 128 and 64 µM, whereas this effect was at concentration of 32 µM and 16 µM approximately 70% and 50% respectively. The CM11 at concentration of 8 µM had minimal effect on promastigotes. Buhrmann et al. (2014) showed that curcumin and 5-fluorouracil (5-FU) a drug used in chemotherapy treatment against colorectal cancer had synergistic effect on each other. This synergistic desirable effect could be achieved with also low concentrations of the mentioned components to reduce the side effects of 5-FU. Tiwari et al. (2017) also showed the synergistic effect of curcumin with miltefosine against visceral leishmaniasis caused by *Leishmania donovani.* In our study CM11 was used at concentration of 8 µM to investigate its possible synergistic effect with curcumin on promastigotes. For this aim curcumin at concentrations of 10 µM and 20 µM was used. Our results showed that curcumin and CM11 at the used concentrations after 12 and 24 hours of culture had no synergistic effect on promastigotes of *L. major*. Meglumine antimoniate drug (Glucantime) is still the first drugs of choice in the treatment of leishmaniasis in man. Unfortunately this drug has critical side effects and problems (painful, cost and some more). Our study showed that Glucantime had no remarkable effect on promastigote form of *L. major*. It is may be due to inability of promastigotes to reduce Sb(V) to Sb(III) of Glucantime but the amastigote form can do it (Shaked-Mishan et al., 2001; Haldar et al., 2011). Our preliminary not published data confirmed this assumption regarding the amastigote form of *Leishmania*. Because the used drugs against leishmaniasis are accompanied with serious problems, it is important to find a new drug with lesser side effects and curcumin can be considered as candidate for developing safe drugs for local administration at least for cutaneous leishmaniasis.

Curcumin can inhibit the growth and kill the promastigote form of *L. major in vitro*. Since curcumin in the used concentrations has nearly no side effect on the skin tissue, a drug formulated with curcumin as an effective component can be considered as candidate for at least treatment of cutaneous leishmaniasis.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

All authors listed have contributed sufficiently to the project to be included as authors, and all those who are qualified to be authors are listed in the author byline. To the best of our knowledge, no conflict of interest, financial or other, exists.

Grant Support

This study was funded by Tehran university research council and the Iran National Science Foundation (grant N: 7502016/6/15) and Investigating Group Molecular Biological System Transfer (MBST).

Acknowledgment

The authors thank the Ministry of Science Technology and Development for financial support. Also, the authors thank the Institute for Investigating Group Molecular Biological System Transfer (MBST) for the financial and scientific support.

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