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Investigation of the antimicrobial activity of a short cationic peptide against promastigote and amastigote forms of *Leishmania major* (MHRO/IR/75/ER): An *in vitro* study

Sara khalili[a], Elahe Ebrahimzade[b,*], Mehdi Mohebali[c], Parviz Shayan[a], Samira Mohammadi-Yeganeh[d], Mehrdad Moosazadeh Moghaddam[e], Samira Elikaee[c], Behnaz Akhoundi[c], Mohammad Kazem Sharifi-Yazdi[f]

[a] Department of Parasitology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
[b] Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran
[c] Department of Medical Science, School of Public Health, Tehran University of Medical Science, Tehran, Iran
[d] Biotechnology Department, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[e] Applied Biotechnology Research Center, Baqiyatallah University of Medical Science, Tehran, Iran
[f] Zoonosis Research Center, Tehran University of Medical Sciences, Tehran, Iran

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ABSTRACT

Cutaneous leishmaniasis is one of the most endemic global health problems in many countries all around the world. Pentavalent antimonial drugs constitute the first line of leishmaniasis treatment; however, resistance to these drugs is a serious problem. Therefore, new therapies with new modes of action are urgently needed. In the current study, we examined antimicrobial activity of CM11 hybrid peptide (WKLFKKILKVL-NH2) against promastigote and amastigote forms of *L. major* (MHRO/IR/75/ER). In vitro anti-leishmanial activity was identified against *L. major* by parasite viability and metabolic activity after exposure to different peptide concentration. In the presentt study, we demostrated that different concentrations of CM11 result in dose dependent growth inhibition of *Leishmania* promastigotes. Furthermore, we demostrated that CM11 peptide has significant anti-leishmanial activities on amastigotes. Our results demonstrated that CM11 antimicrobial peptide may provide an alternative therapeutic approach for *L. major* treatment.

1. Introduction

Cutaneous leishmaniasis (CL) is a typical form of leishmaniasis considered as an important public health problems to people globally. According to the World Health Organization (WHO), leishmaniasis is endemic in 98 countries, and 90% of CL occur in Iran, Afghanistan, Brazil, Saudi Arabia, Peru and Syria (WHO, 2017). There has been no efficient *Leishmania* vaccine available in humans so far; however, the original drugs used against leishmaniasis, such as meglumine antimonate and sodium stibogluconate that has its oen inevitably considerable adverse effects (Kedzierski, 2010). In recent years, many reports have shown drug resistance in treatment of *Leishmaniasis* (Chakravarty and Sundar, 2010; Hadighi et al., 2006; Rojas et al., 2006; Zarean et al., 2015). Although accessibility to alternative drugs such as amphotericin B or miltefosine exists, there is an urgent need to research in order to discover new strategies to cope with limitations of traditional anti-leishmanial drugs and also replace old and less effective drugs with new ones (Croft and Coombs, 2003).

Antimicrobial peptides (AMPs) are important members of innate immune systems as well as a novel therapeutic agent with special mechanism of action which has been studied and evaluated considerably in recent years (Moghaddam et al., 2015; Wang et al., 2011). Previous studies have shown that in vitro activities of some antimicrobial peptides are powerful anti-parasitic agents. Therefore, exploiting them in the development of new generation of drugs for topical or systemic treatment of important parasitic diseases has been a promising approach hope in the recent century.

AMPs are promising and excellent candidates for novel anti-protozoal therapies. These compounds have a broad ability to kill microorganisms with different mechanisms of action (Cobb and Denny, 2010; Rivas et al., 2009). Researches have indicated that anti-leishmanial activity of AMPs are based on permeability of biological membranes.

* Corresponding author.
E-mail address: eebrahimzade@um.ac.ir (E. Ebrahimzade).

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Therefore, AMPs-based anti-leishmanial agents may establish a novel drug with the ability to overcome the resistance observed with current drugs (Cobb and Denny, 2010; Marr et al., 2006).

With regard to leishmaniasis, good results have been obtained with in vitro use of antimicrobial peptides such as temporin (Abbassi et al., 2008), dermaseptins (Feder et al., 2000; Gaidukov et al., 2003), cecropins (Akuffo et al., 1998; Diaz-Achidica et al., 1998), melittin (Akuffo et al., 1998), indolicidin (Bera et al., 2003), histatin (Luque-Ortega et al., 2008) and HNP-1(Human Neutrophil Peptide-1) (Dabirian et al., 2013); However, there is still a lack of enough information about AMPs activity against parasites (Dabirian et al., 2013; Vizioli and Salzet, 2002).

CM11 peptide is a hybride peptide with 11-residue sequence (WKLFKKILKVL-NH2) and consists of N-terminal domain of cecropin A (2–8 residues) and hydrophobic C-terminal domain of melittin (6–9 residues). Cecropin peptide type A has 37 amino acids isolated from hemolymph giant silk moth and has melittin and 26 amino acids component of honey bee venom (Amani et al., 2015; Moghaddam et al., 2014). In the present study, we examined the efficacy of CM11 cationic peptide against promastigote and amastigote forms of *Leishmania major* parasite in an in vitro assay using parasite cultures.

2. Materials and methods

2.1. Peptide synthesis

CM11 hybrid peptide was synthesized as a C-terminal carboxamide on a Rink-p-methylbenzhy-drylamine resin by solid-phase synthesis method using standard protocols (Badosa et al., 2007). The peptide was purified with reversed-phase semi preparative HPLC on C18 Tracer column, using linear gradient from 10 to 60% acetonitrile in water and 0.1% trifluoroacetic acid over 50 min. HPLC purity electrospray ionization mass spectrometry was used to confirm peptide identity. CM11 was obtained with more than 95% purity.

2.2. Parasites and cultures

The standard strain of *L. major* promastigote form (MRHO/IR/75/ER) was provided from the School of Public Health (Tehran University of Medical Sciences, Tehran, Iran). Promastigotes were maintained at 25 ± 1 °C with weekly transfer from RPMI 1640 Glutamax (Gibco, USA) medium with 20 mM HEPES sodium salt (pH = 7.4), 10% inactivated fetal bovine serum (Gibco,USA) and antibiotics. Promastigotes were cultured until they reached the logarithmic phase. Late log phase promastigotes *L. major* at 2.5×10^5 cells/ml were cultured in complete RPMI 1640 medium with different concentration of CM11 (2,4,8, 16, 32, 64, 100 and 128 μM). Untreated parasites were used as negative control and incubated at 25 ± 1 °C for 24, 48 and 72 h.

2.3. Evaluation of inhibitory effects of CM11 on L. major promastigotes

2.3.1. Trypan blue assay

Viability test was carried out by adding 100 μL of trypan blue solution (0.2% in saline containing 0.01% sodium azide) to 100 μL of cell suspension. The number of promastigotes and their morphology were checked using Neubauer Hemocytometer under light microscope, and viability was calculated using the following formula: %Viability = live counted cells/all counted cells) × 100.

2.3.2. MTT assay

The cytotoxicity of CM11 peptide on *L. major* promastigotes was tested by using colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) metabolic activity assay (Vaucher et al., 2010). Briefly, after the treatments, 100 μL of MTT (0.5 mg/ml) was added to each well and incubated at 37 °C for 4 h. The wells were centrifuged at 2000 rpm for 10 min and the supernatant was removed, the pellet was dissolved in acidified isopropanol and absorbance was measured at 540 nm using an ELISA reader (Awareness Technology, USA). The viability percentage was calculated by using the following formula (AT-AB/AC-AB × 100), where AT, AC and AB are absorbance of treated cells, negative control and blank respectively. The IC50 value of peptides were calculated by Graphpad Prism Software.

2.3.3. Morphological evaluation

Logarithmic-phase of *L. major* promastigotes were exposed to CM11 peptide at different concentrations as well as different time points (24,48 and 72 h). Smear was prepared and fixed with methanol for 1 min, stained with solution of Giemsa for 20 min at room temperature, and finally, washed with water and air dried. The stained smear was observed under oil immersion with 1000_X objective.

2.4. Anti-amastigote assay

2.4.1. Evaluation of inhibitory effects of the CM11 peptide on L. major amastigotes

Macrophage cell line RAW264.7 (ATCC number TIB-71) was obtained from Iranian Biological Resource Center, Tehran, Iran. Macrophages were cultured in complete RPMI medium containing 10% fetal bovine serum (FBS); 2×10^4 cells/well of macrophages were cultured in 8-chamber slide (SPL, Korea) and allowed to adhere at 37 °C with 5% CO2 for 6 h. Subsequently, the macrophages were infected with *L. major* stationary phase promastigotes in a ratio of 1:10 (macrophage/promastigote) and incubated for 18 h. Free promastigotes were removed by washing macrophages with RPMI medium for three times. CM11 peptide solutions were used at concentrations of 8, 16, and 32 μM for 24, 48 and 72 h. Non-infected macrophages and treatment-free infected macrophages were used as the negative controls, and glucantime® (Rorer Rhone-Poulenc Specia, Paris, France) in concentrations of 123.5 μM/ml, which was previously calculated in another study (data hasnot shown), was used as positive control. Plates were stained with 10% Giemsa solution. Each test was carried out in triplicates. Anti-amastigotes effect of CM11 peptide was assessed by counting the number of amastigotes in each infected macrophage through examining 100 macrophages in comparison with controls. Inhibitory concentration at 50% (IC50) was assessed by using Graphpad Prism program.

2.4.2. DNA extraction and real time PCR

After infecting RAW264.7 cells with stationary phase of *L. major* promastigotes, treatment with CM11 peptide solution was done at different concentrations (8, 16 and 32 μM) for 24, 48 and 72 h. The culture conditions were the same as described previously. In 24-well cultured plates, Infected macrophages were carefully recovered with a cell scraper (SPL, Korea) from cultured plates, and were washed with PBS and kept at −80 °C until DNA extraction. Total DNA was extracted using DNA Extraction Kit according to the manufacturer's instructions (MBST, Iran, Tehran). DNA concentration and quality was determined by NanoDrop®ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

2.4.3. Standards preparation

Two separate plasmid constructs were prepared. A fragment of *DNA pol* gene from *L. major* (for quantitation of parasite load in macrophages) and a fragment of *β-acin (ACTB)* gene from normal mice genome (for the preparation of a quantitation standard) were separately amplified with their specific primers and then ligated into PTZ 57R/T according to the instructions of T/A Cloning Kit (Thermo Fisher Scientific, USA(. Briefly, DNA samples from *leishmania* promastigote were submitted to conventional PCR for *DNA pol* gene sequencing. The sequences of primers were as follows: forward:5′CGCCTTGTTGTGGAC TCCTACT-3′and reverse: 5′ TGTTGCTGCCCTTTGTAATCC-3´.

Primers originated from *β-actin* genes of mice genome were used as internal control. The sequences of primers were as forward:5′-AGAGG GAAATCGTGCGTGAC-3′ and revered: 5′-CAATAGTGATGACCTGGC CGT-3′. The PCRs were performed in 25 μL total volume of 1 ng DNA template and 0.5 μL of each primers (20 μM); 12.5 μL of PCR master mix (SinaClon, Iran).

Reactions were carried out in automated thermal cyclers (Rotor-Gene Q) with the following program: 5 min incubation at 95 ˚C to denature double-stranded DNA, followed by 35 cycles of 45 s at 94 ˚C (denaturation), 45 s at 52 ˚C (annealing) and 45& s at 72 ˚C (extension).

Finally, PCR was terminated by an additional extension step at 72 ˚C for 10 min. The PCR products were electrophoresed in 1.5% Agarose gel and visualized under UV-light. PCR product was then cloned into TA cloning kit according to manufacturer's instructions. The ligated vectors were used to transform into *Escherichia coli* TOP10 (Invitrogen, USA), and plated into LB broth agar plates with ampicillin as the selected antibiotic. The concentrations of *DNA pol* and *β-actin* plasmids were determined according to their Optical Density (OD260 nm) by Nano Drop 1000 Spectrophotometer. Tenfold serial dilutions of plasmids were prepared from 10 to 10^5 copy (s)/rxn(reaction). *β-actin* plasmid followed the same approach as *DNA pol* serial dilutions, ranging from 10^2 to 10^9 copies/rxn. Specific Real-time PCR reactions for both *DNA pol, β-actin* and all prepared dilutions were performed in triplicated to determine the standard curve, linearity and efficiency.

2.4.4. Determining the load of parasites after treatments

SYBR Green I-based Real-time PCR was performed to determine the absolute load of parasites after different treatments. Real-time PCR reaction mixture of 12.5 μL Master Mix (RealQ Plus 2x Master Mix Green, Amplicon, Denmark), 1 μL each of primers (20 μM), 1 ng/μl of template DNA, and distilled water was added to a final volume of 20 μL. Thermal cycling was performed on Rotor-Gene (Corbett 2000, Australia) using the following cycling Conditions: 95 ˚C for 5 min, followed by 40 cycles of 94 ˚C for 15 s, 51.5 ˚C for 15 s, and 72 ˚C for 15 s. These reactions were also performed for three times.

2.5. Evaluation of cytotoxicity effect of CM11 on macrophages

MTT assay was used to determine cytotoxic effects of CM11 peptide on macrophage. RAW264.7 macrophages were transferred into 96-well plates (5×10^4 cells/well) in RPMI 1640 medium with 10% fetal bovine serum (FBS) and incubated for cellular adhesion for 6 h at 37 ˚C with 5% CO_2. Afterwards, non-adhered cells were removed by washing the cells for three times with RPMI medium. Several concentrations of CM11 peptide (8, 16, 32 and 64 μM) were added into each well in triplicate for 24, 48 and 72 h. Macrophages alone in RPMI medium were used as the negative control. Then, 100 μL of MTT (0.5 mg/ml) were added to each well and the microplate was incubated for 4 h at 37 ˚C. Subsequently, 100 μL of acidified isopropanol was added as solvent and plates were incubated for 30 min. Absorbance was measured at 540 nm on ELISA reader. The IC50 value of peptide were calculated by Graphpad Prism software.

2.6. Statistical analysis

Repeating all of the experiments for three times, differences among control and peptide-treated samples were analysed using ANOVA and Dunnett's post–hoc test. GraphPad Prism software (version 6, USA, 2015) was used to perform statistical analysis of calculated IC50 and presented graphically. p value < 0.05 was considered statistically significant.

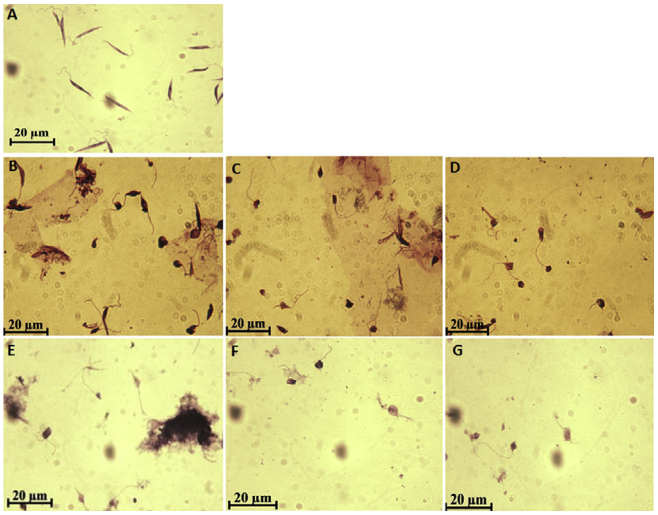

Fig. 1. Microscopic observation of *L. major* promastigotes in the presence and absence of the CM11 peptide after 24 h of incubation (Giemsa stain). The promastigotes were incubated with no CM11 peptide considered as a negative control (A). The effect of different concentrations of CM11 peptide including 8 μM (B), 16 μM (C), 32 μM (D), 64 μM (E), 100 μM (F) and 128 μM (G) on morphology of promastigote of *L. major* has been shown. Lysis and complete disruption of cellular bodies at high concentrations (64–128 μM) was observed (E,F,G). The magnification is 1000X. Scale bar indicates a distance of 20 μm.

3. Result

3.1. Effects of CM11 on promastigotes of L. major

3.1.1. Morphological evaluation

The effect of different concentrations of CM11 on morphology of promastigote *L. major* was evaluated through microscopic observation of Giemsa stained smears. Untreated parasites were used as negative control in triplicate samples. After 24 h incubation period, untreated control parasites revealed thin and slender form whilst parasites treated with 8 μM and higher concentrations of CM11 showed morphological changes. In fact, after exposure to CM11 peptide, most of the *L. major* promastigotes showed swelled and rounded-up cell bodies. In higher concentrations (64–128 μM) disruption of the cell membrane was observed (Fig. 1).

3.1.2. Trypan blue assay

When different concentrations of CM11 were added to 2.5×10^5 cells/ml of *L. major* promastigotes, and untreated parasites used as negative control, the results showed statistically significant reduction in parasite survival rate by trypan blue dye at 8 μM concentrations of CM11 peptide and higher, compared with the negative control. The peptide showed a dose-dependent effect with almost 100% death at a concentration of 32 μM after 48 h incubation (Fig. 2).

3.1.3. MTT assay

MTT assay was used to determine the effect of CM11 peptide on metabolism of *L. major*. This assay demonstrated the dose dependent cytotoxic effect of CM11 peptide on *L. major*. The IC50 of CM11 peptide was calculated which was 6.92 μM (~7 μM) after 48 h (Fig. 3).

3.2. Effects of CM11 on amastigotes

3.2.1. Anti-amastigote assay

Anti-amastigote effect of various concentrations of CM11 peptide was evaluated by counting the number of amastigotes in each infected macrophage through examining 100 macrophages in comparison with positive and negative controls. After 24 h exposure of macrophages to

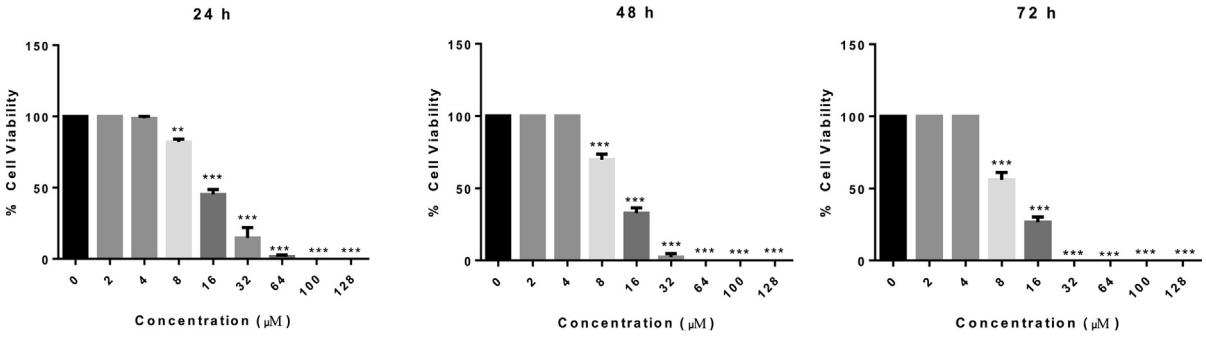

Fig. 2. Effects of different concentrations of antimicrobial peptides (CM11) on *L. major* promastigotes with trypan blue stained after 24, 48 and 72 h. Different superscripts differ significantly, (*) $p \leq 0.05$, (**) $P \leq 0.01$, (***) $p \leq 0.001$, error bar displays the standard error of mean value.

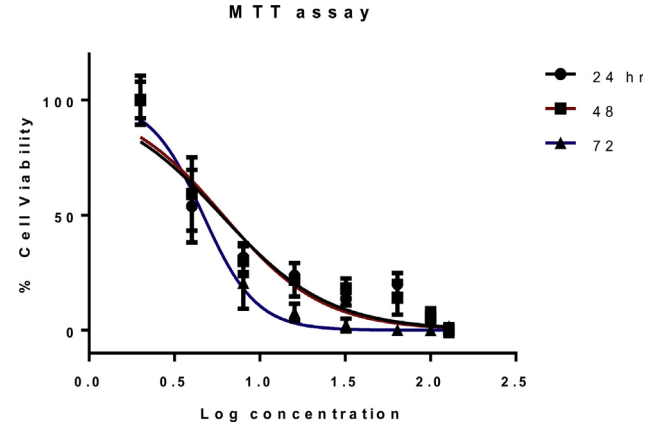

Fig. 3. Determination of the IC50 (Inhibitory concentration at 50%) of CM11 peptide on promastigotes *L. major*. Promastigotes were incubated with different concentrations of the peptide, and metabolic activity was evaluated by MTT assay after 24, 48 and 72 h.

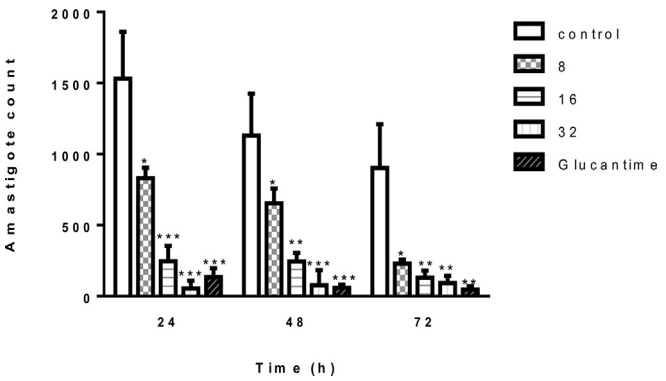

Fig. 4. Anti-amastigote effect of CM11 peptide against *L. major*. Amastigote number were counted three times in different days with different concentrations of the peptide. Glucantime was used as the positive control. The results were analysed by ANOVA, and indicated the statistically significant difference between negative control and all concentrations of CM11 peptide. Significance level of (*) $p \leq 0.05$, (**) $p \leq 0.01$, (***) $p \leq$& 0.001, error bar displays the standard error of mean value.

8 μM of CM11 peptide, anti-leishmanial effect against intra-macrophage amastigotes was observed. This effect sustained until 48 and 72 h post-exposure. Statistical analysis revealed dose-dependent anti-leishmanial activity of CM11 peptide (Fig. 4). Moreover, IC50 value of CM11 peptide against amastigote forms of *L. major* after 48 h was 9.015 μM (~9 μM).

3.2.2. Real time analysis

To evaluate the effect of CM11 peptide on viability and parasitic burden of macrophages, Real-time PCR was performed on macrophages which were infected by *Leishmania* and exposed to different concentrations (8, 16 and 32 μM) of the peptide. Standards were also applied to absolutely quantify the number of parasites in cells (2×10^5 cells/well). Serial dilutions of plasmids from both *DNA pol* and *β-actin* were prepared to set up and evaluate the efficiency and reproducibility of reaction. The results showed that standard curve prepared from serial dilutions of both *DNA pol* and *β-actin* have proper linearity and efficiency (more than 90%). Therefore, they were suitable controls for evaluation of parasite burden in samples. Regarding to the copy numbers of *DNA pol* and housekeeping (*β-actin*) in extracted tissue samples, results revealed that the *DNA pol* quantity in CM11-treated macrophage cells was significantly less than untreated group ($p < 0.05$). The best effect of CM11 peptide on parasite growth inhibition was observed at 48 h post-treatment. The number of copies of *DNA pol* gene was calculated (521285 ± 9917) at the concentration of 8 μM after 48 h incubation. In addition, the evaluation of the *DNA pol* quantity at 16 μM concentration (2246 ± 83/44) after 48 h showed a significant decrease compared to the negative control group (626239 ± 4919) (Fig. 5).

3.3. Murine macrophage toxicity assay

MTT assay was used to determine the cytotoxic effects of different concentrations (8, 16, 32 and 64 μM) of CM11 peptide on murine macrophage RAW264 after 24, 48 and 72 h exposure. Glucantime®(123.5 μM) was used as the positive control. 24 h after

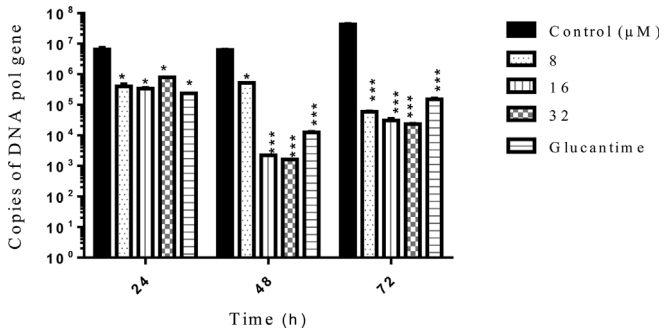

Fig. 5. Evaluating the effect of CM11 peptide on parasite burden of the infected macrophage cells in different time points (24h, 48h, 72h) by Real-time PCR. A fragment of DNApol gene, the target gene of the parasites, and a fragment of *β-acin*, one of the house keeping genes of macrophages were separately amplified with their specific primers. Glucantime was used as a positive control. Real-time PCR analysis indicated a statistically significant difference between negative control and the rest of concentrations) 8, 16, 32 μM) of CM11 peptide. Significance level of (*) $p \leq 0.05$, (***) $p \leq 0.001$, error bar displays the standard error of mean value.

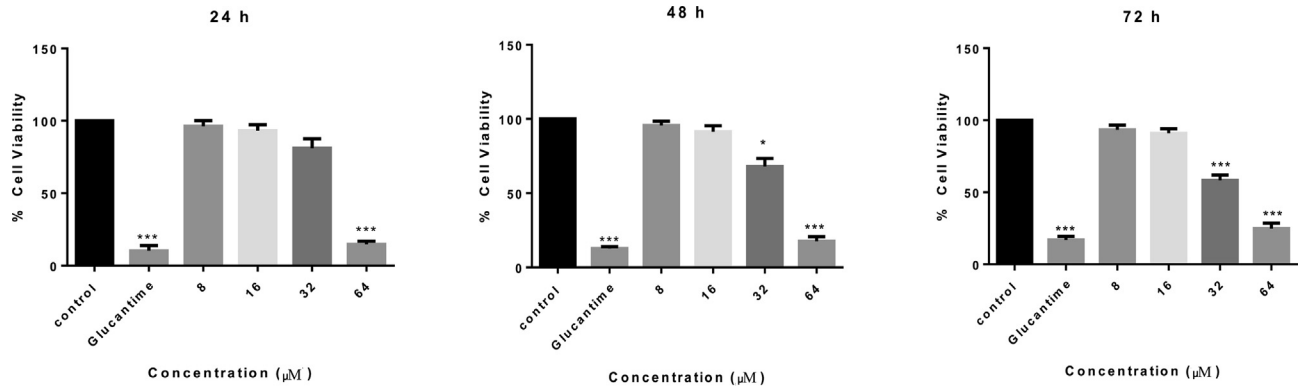

Fig. 6. Toxic effect different concentrations of CM11 peptide on murine macrophage RAW264 using MTT assay. Glucantime® was used as the positive control. Macrophages alone in RPMI medium were used as the negative control. The cytotoxic effect of pepetide was evaluated based on percentage of viable cells in culture after 24, 48 and 72 h. Significance level of (*) $p \leq 0.05$, (**) $p \leq 0.01$, (***) $p \leq 0.001$, error bar displays the standard error of mean value.

exposure, concentrations of 32 and 64 μM of CM11 peptide and glucantime® showed a statistically significant decrease in macrophage viability compared with the negative control (group without peptide or 0 μM). Glucantime® cytotoxicity lasted for 48 and 72 h; however, 8 and 16 μM of CM11 peptide did not show any cytotoxicity (Fig. 6). We have observed that CM11 also decreased macrophage viability with a IC50 value of 44.49 μM after 48 h.

4. Discussion

As stated previously, CM11 is an amphipathic hybrid peptide derived from cecropin A and melittin residues. Similar to this peptide, Diaz-Achidica et al., in 1998, reported anti-leishmanicidal activity against *Leishmania donovani* promastigotes in a synthetic cecropin and A-melittin hybrid peptide (CM26) comprising 1 to 8 cecropin A and 1 to 18 melittin residues (KWKLFKKIGIGAVLKVLTTGLPALIS-NH_2) (Diaz-Achidica et al., 1998). However, due to high production costs and toxicity of these long chain peptides, their usage were limited. To overcome these problems, peptide analogs with shorter chain sequences are being investigated as alternative to improve their biological properties and reduce their toxicity.

In this study, we found potent anti-leishmanial activity of CM11 peptide against promastigote forms of *L. major*. Optical density (OD) and IC50 value for promastigote form, showed significant inhibitory effects of CM11 on promastigotes growth in a dose-dependent manner, with IC_{50} value of 7 μM or (~9.9 μg/ml) in our study. In our results, we found potent anti-leishmanial activity of peptide even after 48 and 72 h post-treatment. This findings were similar to findings reported by Moghaddam et al., 2012, 2014. Furthermore, morphological changes in the promastigote form of the parasite at low doses in our study indicate the peptide interactions with the promastigote membrane. Since amastigote form of *Leishmania* is pathogenic in vertebrate, and due to its membrane composition and structure is different from promastigote, anti-amastigote effect of CM11 was assessed with Giemsa staining and Real-time PCR in our study. Results of this investigation showed a significant anti-leishmanial activity on amastigotes with an IC50 value of 9 μM (12.7 μg/ml). According to these results, promastigote forms were more sensitive than amastigote forms. The difference in the impact of CM11 on promastigote and amastigote forms of *Leishmania*, could be due to their different composition and structure of the cell membrane. Studies have shown that in promastigote form, palasma membrane is strongly negative charged due to high levels of lipophosphoglycan (LPG), while amastigote forms do not have LPG but do contain several glycoconjugates compound related to this. Furthermore, promastigote form has higher percentage of anionic phospholipids than the standard mammalian membranes and amastigote form membrane, which results in more membrane sensitivity to cationic compounds such as CM11 peptide.

Diaz-Achidica et al., reported that electrostatic interaction between promastigote membrane and peptide was inhibited by anionic compounds such as polyanionic polysaccharides (Diaz-Achidica et al., 1998). Therefore, due to similarities between LPG of *Leishmania* and lipopolysaccharide of bacteria, the action of CA (1 ± 8) M (1 ± 18) on *Leishmania* promastigotes takes place by targeting plasma membrane. Similarly, many studies demonstrated that cationic peptides such as CM11 act as a cell-permeable agent. Since their amphipathic properties enable them to electrostatically interact with the poly-anionic surface of the cell and then penetrate to hydrophobic region of the membrane, which leads to pore formation causing leakages in cellular components or passage of hydrophobic molecules across the cell membrane.

According to studies by Shai and Ferre et al. it seems that CM11 peptide kills organism based on a "carpet like" mechanism (Ferre et al., 2006; Shai, 2002). According to this mechanism, after carpeting and thinning the membrane by peptide, at a critical threshold concentration peptide forms toroidal transient holes in the membrane and above this concentration, the membrane disintegrates and forms micelles after disruption of the bilayer curvature (Lee et al., 2008; Madani. et al., 2011; Papo and Shai, 2005).

As more accurate test, Real-time PCR analysis was also used in our study to determine the effect of CM11 peptide on viability of *Leishmania* burden on macrophages, by quantifying *DNApol* gene, previous studies have shown that DNA degredation rapidly occurs after parasite death (Prina et al., 2007). In this regard, quantitative DNA analysis is directly related with number of live amstigotes form of *Leishmania.* In our study, Real-time PCR analysis showed significant reduction of the parasite load with gene copies (2246 ± 83.44) versus (626239 ± 4919), ($p < 0.05$) at 16 μM after 48 h. In recent years, many studies have been conducted about anti-leishmanial activity of AMPs. Konno et al., evaluated anti-leishmanial activity of Decoralin (a linear cationic α-helical peptide from the venom of *Oreumenes decoratus*) and Decoralin-NH_2 on promastigotes form of *L. major*, and concluded that Decoralin (IC_{50} ~ 72 μM) was weakly active, while Decoralin-NH_2 (IC_{50} ~ 11 μM) showed potent anti-leishmanial activity similar with our finding, even though our finding showed much higher activity of CM11 peptide against promastigotes form (IC_{50} ~ 7 μM) compared to Decoralin-NH_2 (Konno et al., 2007).

In another study, Lynn et al., investigated anti-leishmanial activity of cathelicidin bovine myeloid antimicrobial peptide 28 (BMAP-28) and its isomers (L-, D- and RI-BMAP-28) against the promastigote and amastigote *L. major.* Of the three BMAP-28 variant forms, anti-leishmanial activity of D-BMAP-28 against *L. major* promastigote and amastigote forms were more severe than the other isoforms. These data demonstrated that amastigotes were killed upon the addition of the

BMAP-28 isomers in a concentration dependent manner (Lynn et al., 2011). It seems that the potency of this peptides is greater than CM11 peptide but our peptide has shorter sequence in comparision with BMAP-28 (18 amino acid), which could be an advantage in production point of view. Wang et al., studied anti-leishmanial activity of recombinant human histone H2A and H2B on *L. major*, *L.braziliensis*, and *L.mexicana* (Wang et al., 2011). They reported that histones H2A and H2B had anti-leishmanial activity against promastigotes of three species in concentration dependent manner, but they were not active against amastigotes. Flow cytometry findings indicated that survival rates of histone H2A and H2B treated promastigotes decreased by 49% and 75% in *L. major* at the concentration of 100 μg/ml that is much higher than the effective concentration of CM11 against promastigotes in our study. In a similar study conducted by Dabirian et al., anti-parasitic activity of recombinant human neutrophil peptide-1 (rHNP-1) against *L. major* promastigotes and amastigotes indicated that about 60 μg/ml concentration is needed to kill 50% of promastigotes (IC_{50}) in comparison with about 10 μg/ml of CM11 in our study (Dabirian et al., 2013). In another study by Boumaizia et al., it was shown that camel hepcidin (a β-defensin-like peptide mainly produced by liver hepatocytes) analogs including DH1, DH3 and Met-HepcD as functional recombinant camel hepcidin-25, statistically inhibit *L. major* promastigote growth at 60%, 44% and 43% level when treated with 33 μM (100 μg/μL) of DH3, DH1 and Met-HepcD respectively (Boumaiza et al., 2015). According to our results, CM11 peptide has a stronger anti-leishmanial activity in comparison with these peptides. Recently, Marr et al., demonstrated anti-leishmanial activity of host defense peptide (LL-37) and three synthetic peptides E6, L-1018, and RI-1018, that act against promastigote and amastigote forms of *L.donovani* and *L. major* (Marr et al., 2016). Based on their report, promastigotes grown in the presence of E6, LL-37, L-1018, and RI-1018 at a final concentration of 20 μM had a significant reduction in viability. Similar to CM11 in our study, these peptides were effective on amastigotes in higher concentrations. Similarly, Erfe et al., also found anti-leishmanial activity of synthetic peptides RP-1 and AA-RP-1 (microbicidal α-helical domain of mammalian CXCL4 platelet kinocidins) on morphology of *L.infantumchagasi* promastigotes (Erfe et al., 2012). They reported considerable morphological changes after exposure to these peptides. Their study reported that most of the promastigotes showed swelled and rounded-up cell body, which are similar to our results. Also, like CM11 peptide, their results showed that both RP-1 and AA-RP-1 peptides caused a dose dependent decrease in number of promastigotes parasite after 72 h. Generaly, in recent years, various studies have been conducted on antimicrobial activity of peptides especially against *Leishmania* species. In all these studies, sensitivity of promastigotes to AMPs were higher than amastigotes as mentioned earlier, this could be due to the morphological and biochemical differences between the two life stages of the *Leishmania* parasite and their location within the phagolysosome of the macrophage (Marr et al., 2016; Diaz-Achidica et al., 1998). Although, these results are remarkable, clinical applications of AMPs have some limitations such as toxicity especially on macrophages. With regard to cytotoxicity of CM11, our results indicated that CM11 has no significant cytotoxicity on murine macrophage (RAW264) after 24, 48 and 72 h post treatment at 8 and 16 μM peptide concentrations, which are the effective doses for killing promastigotes and amastigotes. However, cell viability was strongly decreased at 64 μM peptide concentration after 24, 48 and 72 h post-treatment. This indicates that higher concentration of CM11 could elicit cytotoxicity. We also observed that CM11 decreased macrophage viability with IC_{50} value of (44.5 μM) after 48 h incubation compared to IC_{50} values of peptide on promastigote and amastigote forms, for 7 μM (~9.9 μg/ml) and 9 μM (~12.7 μg/ml) respectively, this is much weaker on macrophage cells. However, in previous study by Moghaddam et al., macrophage toxicity by MTT assay showed that CM11 peptide led to death of 50% of cells at 12 μM (16 μg/ml) concentrations after 48 h incubation in this concentration while macrophage viability was about 70% after 24 h (Moghaddam et al., 2014). This difference in results can be due to the methodology of experiments or as a result of the presence of stimuli or inhibitory agents (Rudenko and Wajdi, 2005). Furthermore, Moghaddam et al., used isolated primary macrophage cells from the peritoneal cavity of mouse.

It should be taken into considaration that various factors are involved in the cytotoxicity, efficacy and damaging properties of AMPs. The amphipathicity, hydrophobicity, charges, stereochemistry, and propensity of peptides to form barrels are possible factors. As a matter of fact, differences in the antimicrobial activity of peptides is associated with characteristics of their amino acid structures that causes interactions with the anionic membrane of microorganisms (Dolis et al., 1997; Matsuzaki et al., 1998). Furthermore, it has been suggested that sensitivity and viability of eukaryotic cells are associated with hydrophobicity. Membrane phospholipid composition with low levels of specific PLs are detrimental to AMPs actions, and the metabolic activity of cells (Pacor et al., 2002; Rivas et al., 2009; Vaucher et al., 2010). Previosuly, the association of helical content of CM11 peptide with their cytotoxicity has been indicated by some studies (Kaminski and Feix, 2011). It has been demonstrated that CM11 peptide show α-helical structure about 23%. However, bioinformstic and experimental studies are needed to clarify the association of helical content in CM11 structure with peptide cytotoxicity.

5. Conclusion

In conclusion, it seems that CM11 peptide may represent the basis for modeling new and safe anti-leishmanial drugs with additional utility against *L. major*. Further studies are needed to assess the activity of CM11 peptide against the intracellular amastigote stage of *leishmania* and its therapeutic activity in animal models of leishmaniasis.

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