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Original Article

Evaluation of Apoptotic Effect of Transgenic *Leishmania tropica* Expressing MLLO-Bax-Smac Fusion Gene in Infected Macrophages, an In Vitro Study

Maryam Aghaei [1], Hossein Khanahmad [2], Shahrzad Aghaei [2,3], Akram Jalali [4], Zabihollah Shahmoradi [5], Nooshin Hashemi [6], Akram Sarmadi [2], Shima Aboutalebian [7], Mohammad Ali Mohammadi [8], Sayed Mohsen Hosseini [9], *Seyed Hossein Hejazi [10]

1. *Skin Diseases and Leishmaniasis Research Centre, Isfahan University of Medical Sciences, Isfahan, Iran*
2. *Department of Genetics and Molecular Biology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran*
3. *Department of Molecular Medicine, School of Advanced Technologies, Shahrekord University of Medical Sciences, Shahrekord, Iran*
4. *Research Center for Molecular Medicine, Hamadan University of Medical Sciences, Hamadan, Iran*
5. *Skin Diseases and Leishmaniasis Research Centre, Department of Dermatology, Isfahan University of Medical Sciences, Isfahan, Iran*
6. *School of Medicine, Bam University of Medical Sciences, Bam, Iran*
7. *Department of Medical Parasitology and Mycology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran*
8. *Research Center for Hydatid Disease in Iran, Kerman University of Medical Sciences, Kerman, Iran*
9. *Department of Biostatistics & Epidemiology, School of Public Health, Isfahan University of Medical Sciences, Isfahan, Iran*
10. *Department of Parasitology and Mycology, Skin Diseases and Leishmaniasis Research Center, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran*

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***Correspondence**
Email:
hejazi@med.mui.ac.ir

Abstract

Background: In the leishmaniasis parasitic infection, parasite escapes from the immune system of host or prevents macrophage apoptosis. It seems generating transgenic parasites to express proapoptotic proteins can accelerate the apoptosis of infected macrophage and prevent *Leishmania* differentiation. Hence, we investigated the efficacy of transgenic *L. tropica* expressing mLLO-BAX-SMAC in expediting macrophage apoptosis.

Methods: This study was performed at the Department of Parasitology and Mycology, School of Medicine, Isfahan University of Medical Sciences (2022 to 2023), Isfahan, Iran. m*LLO-Bax-Smac* coding sequence cloned in the pLexyNeo2 was entered into the *L. tropica* genome using homologous recombination. PCR, Western blot, and hemolysis tests were used to confirm integration accuracy. In addition, the apoptosis percentage of transgenic *L. tropica*- infected macrophages was assayed by flow-cytometry.

Results: The proper integration of the m*LLO-Bax-Smac* fragment into the 18srRNA locus of *L. tropica* and mLLO -BAX-SMAC fusion protein expression was approved. Furthermore, results of flow cytometry showed the mean percentage of apoptosis among the groups is different and transgenic *L. tropica* leads to a decrease in the apoptosis time of infected macrophages compared to the wild type of *Leishmania* and to an increase in the immune system response.

Conclusion: The data suggest the transgenic *L. tropica* can be used as an experimental model to expedite apoptosis of *Leishmania*-infected macrophage.

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Introduction

Leishmaniasis is a parasitic infection merged by 21 different species of *Leishmania* (a kinetoplastid and obligate intracellular parasite) that transferred by sting of infected female sand- fly to humans and resides in macrophages (1). Leishmaniasis is divided into three major types of cutaneous (CL), visceral (VL) (both are most common), and mucocutaneous (ML) (2). Cutaneous leishmaniasis is divided to zoonotic (human and animal disease) and Anthroponotic types that caused by *L. major* and *L. tropica,* respectively (3).

L. tropica is endemic in northern Africa, Europe, bordering the Mediterranean Sea, and Asian countries like India, China, Israel, and Iran (4). This parasite infects humans and hyraxes and causes non-healing, non-ulcerating and chronic disease (5), although the pathogenicity of *L. tropica* in mammalian hosts is partly known because the establishment of infection *in vivo* is difficult (6). Unlike *L. major, L. tropica* conforms a human-to-human transmission cycle or anthroponotic and more incline to cause satellite lesions and has generally less responsive to treatment (7).

Two distinct life cycles of *Leishmania* include *leptomonad* promastigotes and leishmanial amastigotes in the sand- fly and the mammalian host, respectively. When an infected female phlebotamine sand-fly sucks a blood meal, the macrophages of host tissue rapidly take up promastigotes in the parasitophorous vacuoles and *Leishmania* differentiates to the non-moving amastigote within 12 to 24 hours. Following the macrophage destruction, released amastigotes infect new macrophages and elicit leishmaniasis lesions (8). In contrast, infected macrophages knock parasites by oxidative burst and introduce the *Leishmania* antigens to the TCD4+ cells. TCD4+ cells produce IFN-γ that induces nitric oxide synthesis in infected macrophages to destroy parasites (9). In contrary, the *Leishmania* inhibits macrophage function through the proton pump, increasing the regulatory cytokines like IL-10, TGF-β, and down regulating MHC class II (10).

Apoptosis is another pathway which infected macrophages use for rapid elimination of *Leishmania* (11). As, *Leishmania* causes cellular stress response in macrophages and actives C-JUN/AP-1 and JNK signaling pathways, then the macrophages-expressed Fas (in response to TNF-α and IFN-γ) deletes infected macrophage and reduces parasitic burden (12). Also, in the apoptosis process, infected macrophages secrete apoptotic bodies containing *Leishmania* antigens that are engaged by non-infected dendritic cells, processed and presented via MHCI and subsequently activate $CD8^+$ T cells through cross-priming mechanism (13, 14).

In contrast, parasite lingers macrophage apoptosis by many mechanisms as pathways activation of ERK1/2 (15) and PIK3/ACK, prohibition of caspases 3 and 7 processes, induction of anti-apoptotic signals and inhibition of BAX gene expression and cytochrome C secretion (16).

The BAXα gene with 6 exons in the 19q13.3-q13.4, δ) (17) expresses in mitochondrial outer membrane (MOM) (18) and upon apoptosis, reacts with activator BID and induces BAX oligomerization. The activated BAX enters into the MOM to open the mitochondrial voltage-dependent anion channel (VDAC), moreover make an oligomeric pore mitochondrial apoptosis-caused channel (MAC) in MOM that results in secret apoptogenic factors to the cytosol (19). Furthermore, another mitochondrial intermembrane space protein, SMAC (Second Mitochondrial-derived Activator of caspases) elevates cytochrome C and TNF receptor-dependent activation of apoptosis through prohibiting IAPs (Inhibitor of Apoptosis Proteins) until cytochrome C commits the cell to apoptosis through caspase cascade activation (20).

Versus, *Leishmania* inhibits BAX homo-oligomerization and results in deficient translocation to mitochondria (21). Since the balanced expression between *Bcl-2* and *Bax* genes results in apoptosis inhibition, *Leishmania* deactivates the

macrophage apoptosis through up- regulating the *Bcl-2* and down- regulating the *Bax* genes (22).

Vaccination is one of the most affecting ways for controlling leishmaniasis. One of the recent vaccination policies is the creation of transgenic *Leishmania*. As, the strategy of deleting or entering genes into this organism could be possible by homologous recombination (23). It seems that transgenic *L. tropica* expressing proapoptotic proteins like BAX would be accelerate the macrophage apoptosis, decrease the wide spread of the parasite and the lesion generation without side effects on else cells. In present study, transgenic *L. tropica* expressing mLLO-BAX-SMAC proteins of host was generated and pathology of transgenic and wild-type parasites were contrasted *in vitro*.

Materials and Methods

This study was approved and supported (No.394791) by the Institutional Review Boards (IRB) of the Isfahan University of Medical Sciences with ethical approval numbers of IR.MUI.REC.1394.3.791.

The plasmid including mLLO-Bax-Smac gene

Plexy-Neo2 (Jena Bioscience, Germany), containing signal sequence of Secreted acid phosphatase 1 (SAP1) of *Leishmania* for secretion of the fusion gene, was used as the vector to integrate coding sequences (CDS) of murine *Bax* (alpha) gene [Accession Number (AF339055)- University California Santa Cruz (UCSC)] in *L. tropica* genome. The gene construct contains mutated listeriolysin O (m*LLO*) sequence (which works in pH ≥7), and also *Smac* gene (8aa) sequence was embedded at the 3'end of *Bax* CDS (Fig. 1). LLO (a pore-forming protein) acts within the acidic phagosomes and can lysis the phagosome membrane to escape *L. tropica* into the cytosol and grow into the cell. Also, furin cleavage site was embedded between *mLLO* and *Bax* sequences to cut mLLO protein from recombinant BAX-SMAC using host phagosome. The coding sequence of the construct was codon optimized through web server's optimizer (http://www.genoms.uvr.es) according to codon usage of *L. tropica*. Furthermore, the codon adaptation Index (CAI) was utilized to estimate the designed sequences to the Gene script web site (http://gene script.com). The optimized sequence of m*LLO-Bax-Smac* with 2292bp was synthesized by Genecust Company (Luxembourg) and cloned into the pUC57 vector. The vector was digested by *Sal*I and *Kpn*I, and *Sal*I-*mLLO-Bax-Smac-Kpn*I fragment was sub-cloned into the same enzymatic sites of pLEXSY-Neo2 vector. Next, the fragments were ligated by T4 DNA ligase enzyme (Thermo fisher-USA) and vector was transformed in chemically competent *E.coli* heat shock protocol (24) and separated on Luria-Bertani (LB) medium agar containing 100µg/ml ampicillin.

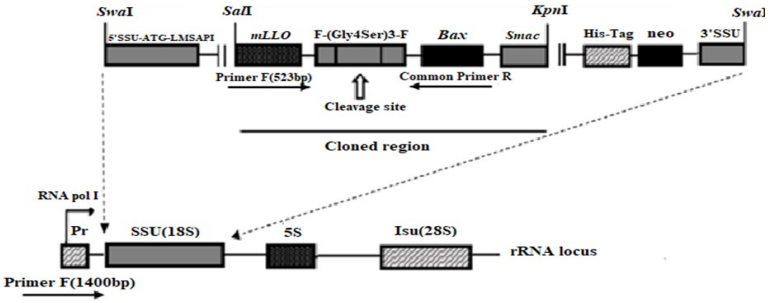


Fig. 1: Schematic illustration of a codon optimized DNA fragment encoding m*LLO-Bax-Smac* was subcloned in *Sal*I/*Kpn*I sites of pLEXSY-Neo2 vector. Furin-(Gly4ser) 3-Furin cleavage site was designed to cut mLLO protein from recombinant BAX-SMAC using host phagosome. The linearized plasmid by *Swa*I restriction enzyme will integrate into the 18srRNA locus of the electroporated *L. tropica* genome through homologous recombination. F: forward and R: reverse primers were marked

Plasmid extraction

The colonies of transformed bacteria including pLEXY-m*LLO-Bax-Smac* were cultured in new LB medium contain 100µg/ml ampicillin, at 37°C and 200rpm overnight (25). Then, the recombinant plasmids DNA were extracted by plasmid extraction kit (SolGent, Korea) and were digested with *Swa*I (Thermo Scientific,USA). The digested plasmids were dissociated by 1% agarose gel electrophoresis. The upper band (about 7911 bp) including m*LLO-Bax-Smac* + flank of up and down of plasmid backbone fragments was purified by a gel purification Kit (Bioneer, Korea).

Parasite culture

L. tropica promastigotes (MHOM/IR/99/YAZ1) were received from Department of Parasitology and Mycology, Isfahan University of Medical Sciences, Iran, and grown at 23-24℃ in culture medium involving 2 ml NNN and 2 ml RPMI 1640 medium (Gibco, USA) enriched with 10% FBS (Fetal Bovin Serum), 100 µg/ml streptomycin and 100 U/ml penicillin (Gibco, Pen-Strep15140). The medium sub-cultured in fresh media every 2 days (26).

Transfection and the selection of transfected promastigotes

L. tropica parasites were transfected with linearized plasmid (50 µg) using the electroporation protocol of Jena Bioscience (Jena Bioscience, Germany). Briefly, 1×108 parasites from logarithmic growth phase were washed with 500ml cold electroporation buffer for 2 times (containing 2.5 gr HEPES [pH 7.5], 4gr NaCl, 0.05 gr Na_2Hpo_4, 0.594 gr Glucose, and 0.185 gr KCl). The parasites were centrifuged and resuspended in 450µl of cold electroporation buffer and mixed with 50µg of the plasmid fragment extracted of gel in a 4mm cuvette. Then, cuvettes were incubated on ice for 10 min and were pulsed three times using an Electeroporator (BioRad, USA) and through the following conditions: 1600 v, 25 µF, 1.2 ms. Moreover, as a negative control, wild-type parasites were electroporated without any plasmid (8). Parasites were transferred to fresh media containing RPMI, FBS and cultured for 24 h at 25°C without any antibiotic prior to selection. The transfected promastigotes were centrifuged (4 °C, 10 min, 3000 rpm) and cultured in liquid RPMI 1640, LB broth and BHI media enriched with 10% FBS, 100µg/ml streptomycin, 100U/ml penicillin and exposed to different concentrations of Geneticin (G418) (Roche, Germany). The stringent selection was continued through rising the concentration of Geneticin from 25 µg/ml up to 100µg/ml for 2 weeks (8).

MTT assay

MTT assay was done to detecting the effect of the 18SrRNA gene insertion on the viability and growth rate of the transgenic parasites (8).

Evaluation of the transfected L. tropica using PCRs

Molecular confirmation of construct was performed by PCR. As, 10ml from medium containing log-phase promastigotes (OD=2-3) was subjected to extract genomic DNA using Genetbio kit (South Korea). Long range PCR was performed using primers hybridizing within the expression cassette and upstream of the 18srRNA locus on genomic DNA of *L. tropica* that not present on the plasmid. The detailed primers are shown in the supplementary Table S1. PCR for some part amplification of m*LLO-Bax-Smac* was also performed.

Protein preparation, SDS-PAGE and Western blotting

The cultured transgenic and wild-type parasites were centrifuged and transferred to conditioned media (serum and bicarbonate sodium free-RPMI 1640 (Gibco, USA) enriched with BHI and LB broth, 100µg/ml streptomycin, 100U/ml penicillin, and 100µg/mL gentamycin) and incubated 48h at 25°C. The supernatants of 1×10^8 parasites from late-log/stationary-phase were harvested and pro-

cessed according to Cuervo procedures with some modification (27). The equal amounts of concentrated proteins of the transgenic and wild-type parasites were loaded on 12% Sodium Dodecyl Sulfate polyacrylamide gel electrophoresis (SDS-PAGE and transferred onto a nitrocellulose membrane for 90min at 4°C and 80V. The membrane was blocked in 5% skim milk and incubated with a dilution of mouse anti-(6×His) HRP cojugated antibody (1:1000, Sigma,USA) overnight. Finally, the his-taged antigen conjugated to HRP-IgG was visualized using the Tetramethylbenzidine (TMB) substrate (8).

Hemolysis test

Hemolysis test of the transfected *L. tropica* as well as wild-type parasite (control) was performed through linear culture on the blood agar plate including defibrinated sheep blood, nutrient agar, rifampicin, ampicillin and incubation at 24-26 °C for 7 days (28).

Apoptosis assessment with Flow cytometry

To analyze macrophage apoptosis, J774A cell lines were purchased from Pasteur Institute of Iran and 10^5 macrophages were cultured in cell culture flasks containing RPMI 1640 medium enriched with FCS 10%, 100µg/ml streptomycin, 100U/ml penicillin and incubated in 5% CO2, at 37 °C for 5–6 days. At a parasite to cell ratio of 10:1, macrophages were infected with late-stationary-phase promastigotes of transgenic *L. tropica* expressing m*LLO- Bax- Smac* gene. The supernatants and macrophage lysates were harvested at 12, 24 and 48h post infection and the apoptosis of infected macrophages was analyzed using flow cytometry (BD Biosciences kit, Roch) (29, 30). Macrophage cultures were examined through Wright stain and 5% Giemsa stain and using light microscopy (x1, 000) (31).

Statistical analysis

Results were presented as means ± SEM (standard error of mean). The data analysis was performed using the ANOVA and Tukey tests. Furthermore, 16 SPSS software was used for the statistical analyses and differences considered significant when P-value<0.05.

Results

Codon optimization and cloning:

The CAI of the codon optimization m*LLO-Bax-Smac* sequence increased from 0.56 to 0.92 that was an appropriate index of expression. After subcloning of the fragment m*LLO-Bax-Smac* CDS, the authenticity of pLEXSY-neo2-m*LLO-Bax-Smac* vector was confirmed using *Sal*I /*Kpn*I restriction enzymes. The 7911bp and 2292bp bands belong to the backbone of the plasmid and m*LLO-Bax-Smac* CDS were separated on agarose gel (Fig. 2).

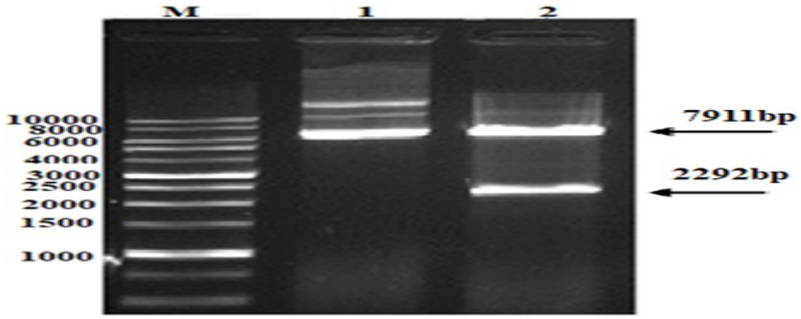


Fig. 2: Electrophoresis of the plasmid pLEXY-*mLLO-Bax-Smac* using *Sal*I/*Kpn*I restriction enzymes. M: DNA Ladder 1kb (thermo fisher, USA), 1: undigested pLEXY-*mLLO-Bax-Smac* vector, 2: pLEXY-m*LLO-Bax-Smac* vector digested with *Sal*I /*Kpn*I restriction enzymes. The 7911 bp and 2292 bp bands belong to the backbone of plasmid and m*LLO-Bax-Smac* CDS, respectively

The digestion of pLEXY-mLLO-Bax-Smac using SwaI

After digestion of recombinant plasmid using *Swa*I, two desired fragments 3000bp and 7880 bp were obtained (Fig. 3). Then, promastigotes successfully were transfected with 7880 bp fragment (include linear pLEXSY-m*LLO-Bax-Smac* vector). To investigate the metabolic rate of the parasites, an MTT test was carried out. The same growth curve in both transgenic and wild-type parasites were obtained, as insertion did not affect metabolic rate and transgenic parasites showed the same health as the wild-type parasites (p=0.929) (Fig. 4).

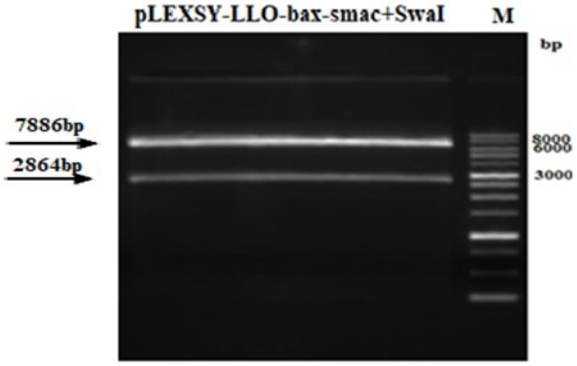


Fig. 3: Electrophoresis of pLEXY-m*LLO-Bax-Smac* digestion using *Swa*I. M: 1kb DNA ladder. The 7886 bp and 2864bp bands respective belong to the specific fragment of m*LLO-Bax-Smac* /homologous stems and backbone of plasmid

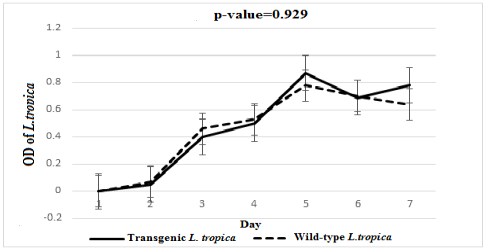


Fig. 4: Growth curves of transgenic *L. tropica* and wild-type *L. tropica*. Transgenic parasites display the same characteristics as the wild-type parasite

The genomic analysis of the extracted DNA of electroporated parasites resistant to geniticin, showed the proper integration of the m*LLO-Bax-Smac* CDS into the 18srRNA ribosomal locus of *L. tropica* genome. As, long range PCR showed 1400kb band belong to some of the segments of m*LLO-Bax-Smac* gene, plasmid backbone and upstream of 18srRNA coding genomic DNA (Fig. 5A). Furthermore, the 523 bp band using m*LLO-Bax-Smac* specific primers was showed only in the transgenic reactions (Fig. 5B).

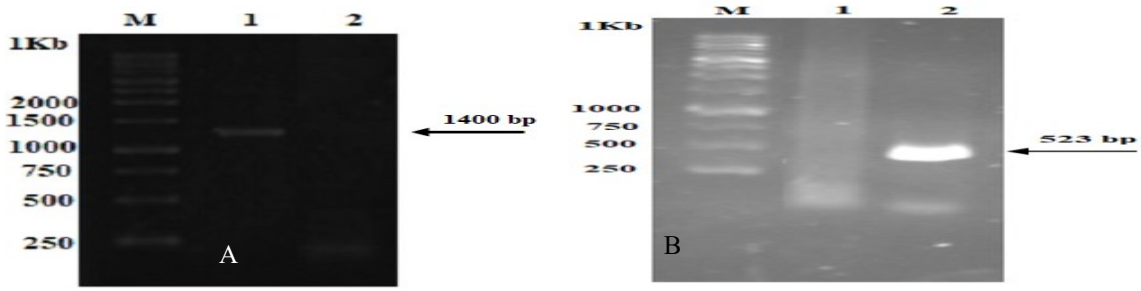


Fig. 5: (A) The PCR on genomic DNA of transgenic *L. tropica*. M: 1kb DNA ladder; 1: 1.4 kbp band confirmed homologous recombination;2: Wild-type *L. tropica*. **(B):** The PCR on genomic DNA of transgenic *L. tropica* with specific primers for m*LLO-Bax-Smac* construct. M: 1kb DNA ladder; 1: Wild-type *L. tropica*. (Negative control); 2: The 523 bp band approved random and also homologous integration

The BAX- SMAC expression confirmation using SDS–PAGE & Western blotting

The SDS–PAGE gel of the transgenic *L. tropica* supernatant proteins showed a ~23 kDa band respective to BAX-SMAC fusion protein (Fig. 6). Furthermore, a ~23 kDa band in Western blotting confirmed the BAX-SMAC-6_His band (Fig. 7).

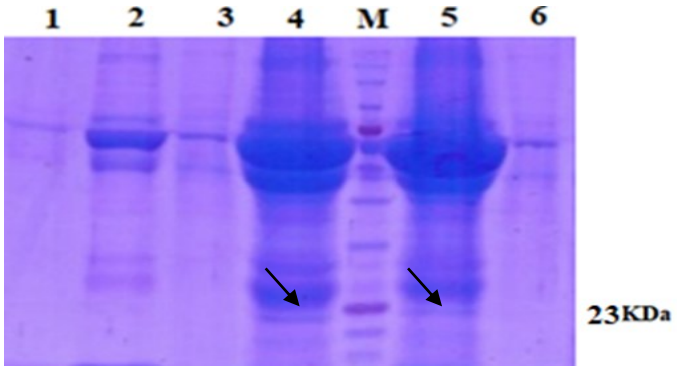


Fig. 6: SDS–PAGE analysis of secretory proteins of *L. tropica* transfected with pLEXY-m*LLO-Bax-Smac*. M: Prestaining protein Marker (SMO431);1: Secretory protein of wild-type *L. tropica* unconcentrated.2: Secretory protein of wild-type *L. tropica* concentrated with TCA 20%.3: Secretory protein of wild-type *L. tropica* concentrated with TCA 10%.4 &5: secretory proteins of transgenic *L. tropica* concentrated with TCA 20%.6: secretory proteins of transgenic *L. tropica* unconcentrated. The desired band was determined with arrow

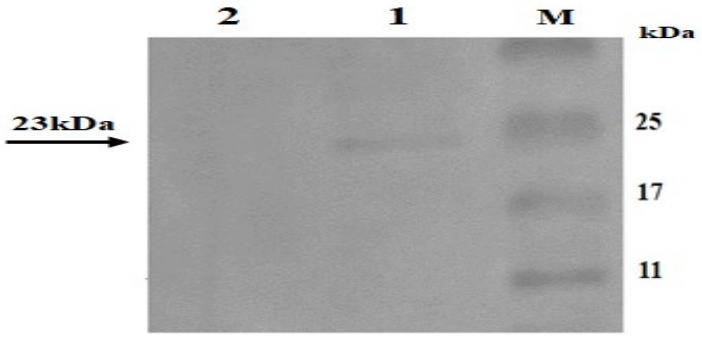


Fig. 7: The Western-blotting analysis of BAX-SMAC-6His protein. M: Marker protein (SMO431).1: The concentrated secretory protein with TCA 20%. of transgenic *L. tropica* 2: The concentrated secretory protein with TCA 20%. of wild-type *L. tropica*. The desired band was determined with arrow

Hemolysis test results

The hemolysis on the blood agar medium of the transgenic *L. tropica* come to be visible after 7 days compared to wild-type *L. tropica* (Fig. 8).

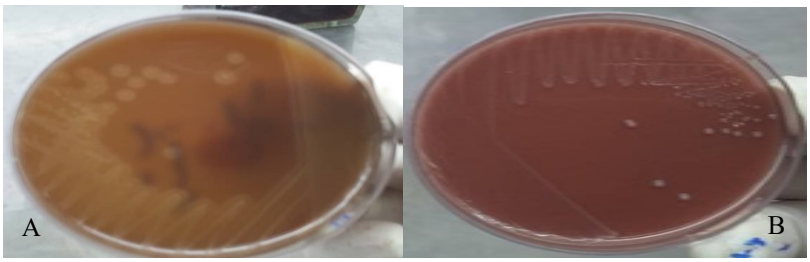


Fig. 8: The linear culture of *L. tropica* on the Blood Agar medium. **A:** LLO expression on the Blood Agar medium of transgenic *L. tropica* caused hemolysis. B: The Lack of LLO in wild-type *L. tropica* did not cause hemolysis

The results of flow cytometry

The establishment of parasitic infection in the J744 cells revealed using Giemsa staining of macrophages infected with transgenic and wild-type *L. tropica* (Fig. 9). Moreover, the flow cytometry displayed statistical difference of the apoptosis percentage mean among the 3 groups at 12, 24 and 48h (p=0.000) (supplementary Table S2), as the mean showed increased and accelerated apoptosis rate of macrophages infected with transgenic *L. tropica* compared to the wild-type *L. tropica*- infected macrophages and the non-infected macrophages. Furthermore, there was no difference between non-infected macrophages and wild-type *L. tropica* -infected macrophages at 12, 24 and 48h (p=0.293, 0.851 and 0.894), respectively (supplementary Table S3 and Figs. 10, 11).

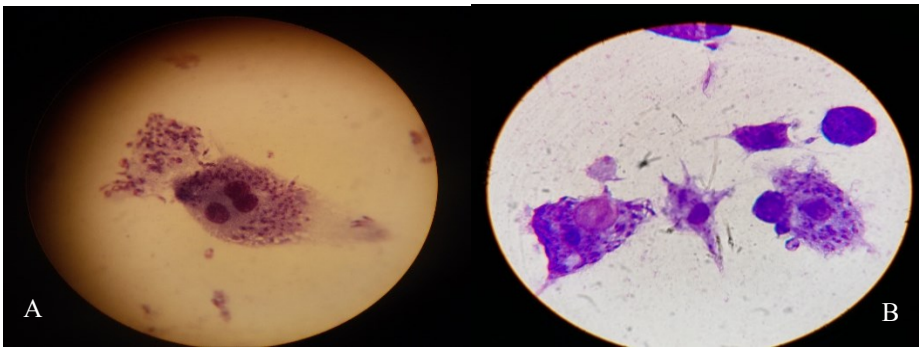


Fig. 9: Giemsa staining of macrophages infected with (A) transgenic *L. tropica* and (B) wild-type *L. tropica*, 8h post-infection

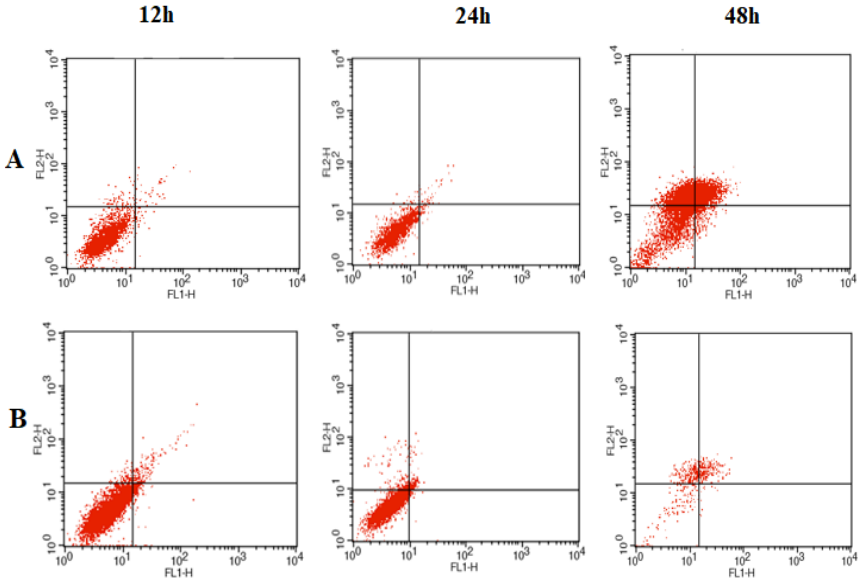


Fig. 10: Dot plots of macrophages infected with (A) wild-type and (B) transgenic *L. tropica* at 12, 24 and 48h post infection. Plots show data representative of 3 separate experiments for each group. Left bottom: viable cells; right bottom: early apoptosis; left top: necrosis; right top: late apoptosis. FACS plots are gated on macrophage cells. and X and Y Axes are related to Annexin V (apoptosis) and PI (necrosis)

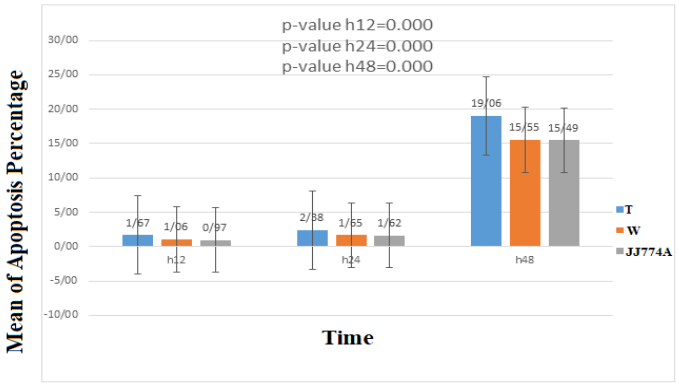


Fig. 11: Comparison the apoptosis percentage mean of the infected macrophages among 3 groups (T=Macrophages infected with transgenic *L. tropica*. W= Macrophages infected with wild-type *L. tropica*. J774A= non-infected macrophages) at 12, 24 and 48h post infection. The apoptosis percentage mean of macrophages infected with transgenic *L. tropica*, statistically raised at different hours compared to other groups, but there was no difference between non-infected macrophages and with wild- type *L. tropica*- infected macrophages

Discussion

With regard to parasite function to prevent or delay macrophage apoptosis, it is hoped that transgenic expression of the apoptosis regulators by *Leishmania* itself in infected macrophages may be one of the therapeutic strategies for leishmaniasis. In this study, we integrated the m*LLO-Bax-Smac* fusion gene in *L. tropica* genome and obtained accelerated apoptosis of infected macrophage compared to wild-type parasites *in vitro*.

Our study strategies included using of the signal peptide of the secreted acid phosphatase 1 (SAP1) of the pLEXSY-neo2 vector for secreting the m*LLO-Bax-Smac* fusion protein, and also the *L. tropica* 18srRNA promoter for transferring the m*LLO-Bax-Smac* sequence in the 18srRNA region of *L. tropica* genome through homologous recombination (5'uss and 3'uss). Codon optimization increased CAI near to the optimal level of mLLo-BAX-SMAC expression by *L. tropica*. Furthermore, the molecular weight of the construct was determined ~84 kDa, and the secretory protein band (23kDa) in Western blotting showed that mLLO-BAX-SMAC fusion protein was cut in the cleavage site of furin by the furin-like proteases, as the protein size decreased from 84 to 23kDa that was related to BAX-SMAC fusion protein.

Moreover, the C-terminal fragment expression of a fusion protein (BAX-SMAC) approved the expression of N-terminal fragment (mLLO), as the emersion of hemolysis on the blood agar media of transgenic *L. tropica* versus wild-type *L. tropica* confirmed the expression of mLLO.

Furthermore, the correct integration of target gene (m*LLO-Bax-Smac*) into the 18srRNA gene of *L. tropica* genome through homologues recombination was confirmed using amplification of 1400bp and 523bp bands using PCR and growth curve of transgenic *L. tropica* was same to wild-type *L. tropica*. Similarly, Patel et al. (32) generated the transgenic *L. tropica* constitutively expressing Green fluorescent protein (GFP) and detected high level of GFP expression in the promastigote and amastigote stages. Also, they resulted that transgenic species have the identical growth pattern, sensitivity to reference drugs and also ability to infect mammalian host cells as their wild-type organisms.

In contrast to Jena Bioscience, the higher expression level is not guarantee using opti-

mized synthetic gene always, as despite the codon optimization, the expression level of secretory protein of transgenic parasites was determined ~1mg/ml, similar to our other studies in *L. major* (33) and *L. infantum* (34). These results are relatively small amounts compared to previous studies like kianmehr study (35) that may be due to low copy number of gene inserted into the host's genome. Therefore, the recombinant protein production could be enhanced using more optimization of expression conditions like culture method.

Flow cytometry results revealed the increased apoptosis rate of macrophages infected with transgenic *L. tropica* in comparison with wild-type *L. tropica*-infected macrophages. Furthermore, the positive result of the hemolysis test proposed that the mLLO protein results in the endocytic vesicles lysis before forming secondary lysosome with acidic pH. So, the difference apoptosis rate between two parasite strains can be of perforation the phagosomal membrane by the secreted mLLO from the recombinant strain that simplifies the transfer of phagosomal proteases and also parasite antigens into the cytosol. In addition, SMAC was considered as an adjuvant protein to BAX. It seems that as the same time other lysosomal proteases like cathepsin along with BAX-SMAC could release from the phagolysosomes into the cytosol and by activate caspases, evoke the internal pathway and raise apoptosis of transgenic *L. tropica*- infected macrophages. Thus, it can be suggested that observed apoptosis is not exclusively due to BAX-SMAC expression (36). As, in agreement to our study, Esseiva observed that recombinant expression of mammalian BAX at *T. brucei* induces trypanosomatoid apoptosis, and the apoptosis processes were transitional and reversible (37).

Conclusion

The constant insertion of the codon-optimized m*LL0-Bax-Smac* sequence into the *L. tropica* genome was performed successfully using the Plexy-Neo plasmid and the extracellular expression of recombinant protein observed. Moreover, compared to wild-type parasites, transgenic *L. tropica* could reduce the apoptosis time of infected macrophage and accelerate the reaction of the immune system against *Leishmania*. This achievement could be used in experimental studies for vaccine expansion against leishmaniasis.

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Conflicts of interest

The authors declare that they have no conflict of interest.

Supplementary materials

Supplementary file includes Tables S1, S2, S3. They might be requested from the corresponding author based on a reasonable application.

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