The Potential Role of Lipopeptide Biosurfactant Generated by *Acinetobacter junii* B6 on *Leishmania Tropica*: The Synergy of Lipopeptide Biosurfactant and Glucantime

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Abstract

An in vitro investigation was carried out to assess how the lipopeptide biosurfactant (LPB) produced by Acinetobacter junii B6 affects Leishmania tropica infection and the associated cytokine gene expression in macrophages infected with L. tropica. Glucantime® (meglumine antimoniate, MA) and LPB were investigated for their leishmanicidal effect, alone and in combination (LPB + MA), using a colorimetric test and a macrophage model. Immunomodulatory impact was also evaluated through analysis of Th1 and Th2 cytokine gene expression in infected macrophages after treatment with MA and LPB, individually and in combination. The MA/LPB combination showed higher inhibitory impacts on L. tropica amastigotes and promastigotes than each alone. Cytokine gene expression confirmed LPB's affinity to IFN-γ, affirming the elevated IL-12p40 and IFN- γ concentrations in addition to a reduction in the secretion of IL-10 in a dose-dependent manner, particularly in combined treatment. The results indicated higher effectiveness of LPB along with MA in the reduction of the parasite growth and promoting the immune reaction level, which may be considered as a possible therapeutic strategy to treat those with anthroponotic cutaneous leishmaniasis.

Keywords Lipopeptide biosurfactant · Acinetobacter junii B6 · Leishmania tropica · Glucantime

Introduction

Leishmaniasis is an important global disease and health concern with no effective treatment. Eco-effective compounds against cutaneous leishmaniasis are highly sought after. The usage of existing treatments of leishmaniasis such as Glucantime® (meglumine antimoniate, MA) has consequently expanded, however is has also resulted in increasing environmental concerns because of the toxicity and structure of the drug (Bailey et al. 2019). Searching for sustainable and renewable treatment or control drug or methods, such as employing environment-friendly treatments, has pointed to the potential application of biosurfactants as possible solutions (Mohanty et al. 2021). Biosurfactants have been reported to have some antibacterial, antifungal and anticancer properties (Ohadi et al. 2020). Biosurfactants are surface-active compounds characterized by hydrophobic and hydrophilic parts; they can be produced by various microorganisms, like yeasts, bacteria, and fungi, and are divided into glycolipids and lipopeptide groups (Adetunji and Olaniran 2021). Because the compounds exhibit higher biodegradability and lower toxicity, they can be used in many biomedical fields such as antileishmanial activities (Abd Burghal et al. 2021). Recent studies have shown that LPB (lipopeptide

biosurfactant) or combination therapies of existing medications with synergistic impacts may highlight a novel approach to treat pathogens (Rivardo et al. 2011). It has been shown that these amphiphilic compounds may be able to inhibit *Plasmodium falciparum* development through interfering with Sirtuins 2 (SIR2) activity (Chakrabarty et al. 2008). Sirtuins are NAD-dependent nonhistone and histone deacetylases that regulate important cellular processes in parasites and promote the amastigotes survival through the prevention of programmed cell death (Vergnes et al. 2002). Recently, biosurfactants have been considered as possible biomedical agents with expressed immunological properties (Subramaniam et al. 2020). Biosurfactants have been shown to have immunomodulatory properties, supporting the development of the Th1 immune response. The importance of CD_{4+} Th1 cells in generating IL-12 - and IFN, as well as infection healing, has been demonstrated in animal model studies, as opposed to Th2 cells' function in disease development through IL-10 (Sajid et al. 2020). Therefore, the disease outcome in mice affected by cutaneous leishmaniasis can be associated with the balance of Th1/Th2 cytokines (Oliaee et al. 2020). Since traditional therapy has presented significant obstacles, substituting new drugs or combining therapies may be a viable alternative for an improved treatment (Oliaee et al. 2020). LPB produced by *Acinetobacter junii* B6 is diverse and complex structures including a hydrophobic fatty acid part, linking to a hydrophilic peptide chain. In addition to their high surface activity, these amphiphilic molecules have shown a wide biological activity spectrum such as antimicrobial, antibiofilm, and cytotoxicity activities (Ohadi et al. 2017, 2020). Using colorimetric methods, this study primarily aimed to evaluate the inhibitory effects of LPB in vitro, alone and combined with MA, on amastigote and promastigote types of *L. tropica*. The intra-macrophage amastigote was applied for assessing their immunomodulatory impact in combination and individually via measurements of cytokine gene expression profiles.

Materials and Methods

Preparation of the LPBs

The LPB was prepared according to Ohadi et al. (2017, 2018). Briefly, LPB was prepared by *A. junii* B6 (GenBank accession KT946907) already separated from oil-contaminated soil. The prepared LPB was subjected to purification in two steps acid precipitation and solvent extraction. Based on the analytical characterization indicated via spectroscopy assessment, the proposed structure was approved.

The Cytotoxicity Assays

The J774A.1 murine macrophage cell line was prepared from Pasteur Institute, Iran. Cells were kept in RPMI 1640 medium treated with 100 U/ mL penicillin G, 15% fetal bovine serum (FBS), and 100 µg/mL streptomycin at 37 °C in a humidified room with 5% CO_2. For evaluating the cytotoxicity of LPBs and MA, cell lines were treated with various levels of drugs (5-500 µg/mL) for 72 h in 5% CO_2 at 37 °C in 96-well tissue culture plates. The control group wells contained cells and the medium without any drugs. After 72 h incubation, MTT [3- (4, 5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide] (5 µg/mL, Sigma Aldrich, USA) powder was used according to manufacturer instructions to perform the MTT test on the J774A.1 cell line. Reading of optical density (OD) was carried out at 490 nm by the BioTek-ELX800 ELISA reader. The 50% inhibition level (IC_{50}) cytotoxicity value was calculated by SPSS 21 after the viability inhibition rate was plotted against concentration. The 50% cytotoxicity concentration (CC_{50}) was assessed by the Probit test. The experiments were carried out in triplicates (Paris et al. 2004).

Parasite Culture and Anti-Promastigote Test

Promastigotes of *L. tropica* (MHOM/IR/ 2002/Mash2) were prepared by the Research and Training in Skin Diseases and Leprosy Center, Iran. For mass production, the promastigotes were grown in 24- well microtiter plates containing RPMI1640 media (Biosera, France) supplemented with 10% heat-inactivated FBS (Gibco, Germany) and antibiotics (penicillin (100 IU/ml) and streptomycin (100 µg/ml). Anti-promastigote assessments were carried out using direct counting assay according to growth inhibition. The promastigotes were grown at an initial level similar to the level of early log phase (10_6 promastigotes/ml) and followed by multiplying for 72 h at 25 °C. Afterward, the promastigotes were treated in different groups: (1) LPBs (50–300 µg/mL), (2) LPBs plus MA as test groups, and (3) MA as a reference agent (50–300 µg/mL). Promastigotes with no drug and complete

medium with no organism were respectively applied as positive and negative controls. The MTT solution (10 µl) was mixed with each well after incubation for 4 h at 25 °C. Isopropyl alcohol was used to terminate the reaction, which was then assessed using the BioTek-ELX800 ELISA reader at 490 nm. The value of IC_{50} was determined through the Probit test and SPSS 21. All experimental procedures were carried out three times (Oliaee et al. 2020).

Anti-Amastigote Assay

Following the placement of sterile 1 cm^2 coverslips in the wells, 6-chamber slides (Lab-Tek, Nalge Nunc International NY, USA) recieved 100 µl murine macrophages (10^6 cells/ mL), followed by incubation for 4 h at 37 °C with 5% CO_2. Then, non-adherent cells were removed and stationary-phase Leishmania + macrophage was added to the wells (10:1 ratio) for 24 h under similar conditions (Mohseni et al. 2022). Following exposure and removing free parasites, various levels of 50–300 µg/mL LPBs, MA, and the combination of LPBs and MA were applied for treating the affected cells in 5% CO_2 at 37 °C for 72 h. Then, six dried slides were stained using methanol and Giemsa, and a light microscope was used to evaluate them. Macrophages alone were considered negative controls and infected macrophages without drugs were considered positive controls. The drug leishmanicidal impact was assessed using the average number of amastigotes per 100 macrophages (Oliaee et al. 2020).

Evaluation of the Expression Level of Cytokines

Control samples and treated macrophages were assessed by quantitative real-time PCR (qPCR) analysis for variations in IL-12p40, IFN-γ, and IL-10 expression levels. The Th-1 pathway was presented by genes corresponding to IFN-γ and IL-12 p40 whereas the Th-2 pathway was presented by the IL-10 gene (Nooshadokht et al. 2022). In brief, the extraction of total RNA was carried out using treated and untreated groups by the RNeasy Mini Kit (Cat. No. 74,106, Qiagen, Germany) as per instructions. The quality and quantity of extracted RNA were calculated using 2% agarose gel electrophoresis and Nanodrop (ND-2000, Thermo Scientific Fisher, US), respectively. Extracted RNA (500 ng) in a FlexCycler (Analytik Jena, Germany) was used with the RT reagent Kit (Takara, Japan) at 37 °C for 15 min to synthesize cDNA. A qPCR system (Rotor- Gene Q, Qiagen) was used according to manufacturer instructions to perform the qPCR reaction in 15 µl using SYBR® Premix Ex TaqTM II (Japan). The reference gene sequences, and specific primers are shown in Table 1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene for normalization purposes with a no-cDNA specimen as the negative control. The experiments were carried out in triplicate.

Statistical Analysis

Values were reported as mean ± SD. One-way ANOVA was applied for comparison between the experiment and control groups. Variations of IC_{50} in Nam and MA groups were compared with a t-test at $p < 0.05$. Prism 7.01 software was used for data analyses.

Results

Cytotoxic Assay

The drug was applied to macrophages at different concentrations (50–300 µg/mL), and the CC_{50} was determined for each drug (Table 2). LPB had less cytotoxicity than MA on macrophages, whereas the CC_{50} of LPB was 315.5 ± 0.19 µg/ ml and MA was 214.2 ± 0.61 µg/ml (Table 2).

Anti-Promastigote Activity

The anti-promastigote activities of LPB, MA and LPB + MA on the *L. tropica* promastigote form were determined by MTT assay (Fig. 1a). The inhibition percentages were dose-dependent, with increased inhibition at increasing concentrations. The LPB, MA, and LPB + MA IC_{50}s were 88.3 ± 0.01, 208.9 ± 0.03, and 31.9 ± 0.01 µg/mL, respectively (Table 2). LPB + MA showed a lower IC_{50} value than the positive control (MA) ($p < 0.001$) (Table 2). LPB + MA displayed the highest inhibitory effects compared with LPB or MA alone (Fig. 1a).

Anti-Amastigote Activity

The anti-amastigote activity of drugs was evaluated in *L. tropica* -infected macrophage cultures (Fig. 1b). Calculation of the IC_{50} values for LPB, MA and LPB + MA against amastigotes revealed IC_{50} of 38.5 ± 0.01 μg/ml, 65.9 ± 0.03 μg/ mL, and 19.04 ± 0.01 μg/mL respectively. The combination of LPB + MA showed lower IC_{50} value in comparison to the LPB or MA treatment alone (Table 2). The combination of LPB/MA has a selectivity index (SI) value of 13.9, indicating that it does not even have a substantial cytotoxicity effect on mammalian macrophages.

Gene Expression

T cell-mediated immune response gene expression was examined in MA, LPB, the combination of LPB and MA, and untreated control (UC) cells. IL-10 (Th2 cell-related parameter) and IL-12P40 and IFN-γ (Th1 cell-related parameters) were all expressed (Fig. 2a-c). Compared to untreated cells, Th1-related parameter transcription factors and expression were markedly higher in the LPB, MA, and LPB + MA groups. When LPB and MA were combined, Th1 transcription factors and cytokines significantly increased while there was substantial reduction in Th2 cytokine expression when compared to LPB and MA alone ($P < 0.001$). The effects of Th2-related cytokine downregulation and Th1-related transcription factor and cytokine overexpression on survival of parasites are shown in Fig. 2.

Discussion

Leishmaniasis typically affects infected human's health for many years, which is a major public health problem globally. The use of existing treatments of leishmaniasis are extremely challenging because of their toxicity, high cost, low efficacy, and side effects (Imane et al. 2022). Treatment using combinations of available drugs and natural products has attracted interest in recent years as an excellent alternative to address the various issues associated with the management of this parasitic infection (Souza et al. 2022). Since safety and biocompatibility are two ideal features of antiparasitic therapeutic agent, biosurfactants were considered an potential promising alternative therapies because of their lower toxicity and higher biodegradability properties (Al-Jubury et al. 2018). The main effect of biosurfactants have been evaluated in various protozoa, such as *Plasmodium falciparum* (Chakrabarty et al. 2008), the *Nosema ceranae* (Porrini et al. 2010), and the *Ichthyophthirius multifiliis* (Al-Jubury et al. 2018). In our study, the antileishmanial effect and cytokine expression levels of LPB, alone and combined with MA, against *L. tropica* were assessed. Our results indicated that the use of LPB + MA (compared to MA or LPB alone) on promastigotes resulted in the highest inhibition and the lowest IC_{50}. Also, the average intra-macrophage amastigote count was markedly lower when LPB was combined with MA compared when they were used alone. In general, combination therapies are more effective compared to a single drug treatment (Rivardo et al. 2011).

The LPB /MA mode of action is possibly because of interaction with the parasite membrane increasing the activity of antiparasitic drugs through the formation of pores in the outer membrane leading to facilitation of the MA entrance through the cytoplasmic membrane (Porrini et al. 2010; Rivardo et al. 2011) reported that *Bacillus licheniformis* V9T14 produces an amphiphilic LPB that can treat *Escherichia coli* CFT073 biofilm by delivering antibiotics into an extracellular polymeric substance to enhance the impact of the antibiotics against the biofilm. There are various mechanisms associated with growth inhibition by LPB in various parasites. The suggested hypothesis is that the amphiphilic features of LPBs destroy the cell structure through their action on the lipid bilayer and cell proteins, leading to the death of the parasite (Al-Jubury et al. 2018; Porrini et al. 2010). Previous studies showed that the LPB such as surfactin has a lethal effect on the *Plasmodium falciparum* by inhibiting Sir2. SIR2 protein is essential for the survival and proliferation of parasites. Surfactin is a new Sir2 inhibitor representing competitive inhibition considering NAD^+ and uncompetitive inhibition with acetylated peptide (Chakrabarty et al. 2008). Such inhibition pattern with surfactin can provide more support for the observed antiparasitic activity of LPB. It has been indicated that microbial surfactants possess an ability to regulate inflammation-associated genes, which suggests that biosurfactants may have an immunomodulatory effect (Sajid

et al. 2020). Inflammatory reactions were also associated with the parasite infection, which is in line with our results, indicating immune response progression through higher secretion of Th1 pathway cytokines (IL-12P40 and IFN-γ) and also lower secretion of the Th2 cytokine IL-10 in treatment by the LPB + MA combination compared to LPB or MA alone. Microbial biosurfactant molecules are usually identified by Toll-like receptors available on different kinds of cells. The bacterial cell wall components activate monocytes and neutrophils leading to high secretion of pro-inflammatory cytokines (IL-12, IFN-γ) (Gillrie et al. 2010). Konig et al. (König et al. 1992) reported similar findings where they proposed enhanced inflammatory mediator release by human platelets by *Pseudomonas aeruginosa* glycolipid.

Conclusion

The use of LPB + MA combination demonstrated higher inhibitory effectiveness against the *L. tropica* growth in a macrophage model through the promotion of immune response levels. Thus, LPB combined with MA is a valuable source as well as promising option for anti-leishmanial drug development in anthroponotic cutaneous leishmaniasis cases in clinical settings. In addition, our findings open a new area of exploration to develop novel therapeutic strategies to deal with different leishmaniasis variations, and, possibly, similar protozoan parasites.

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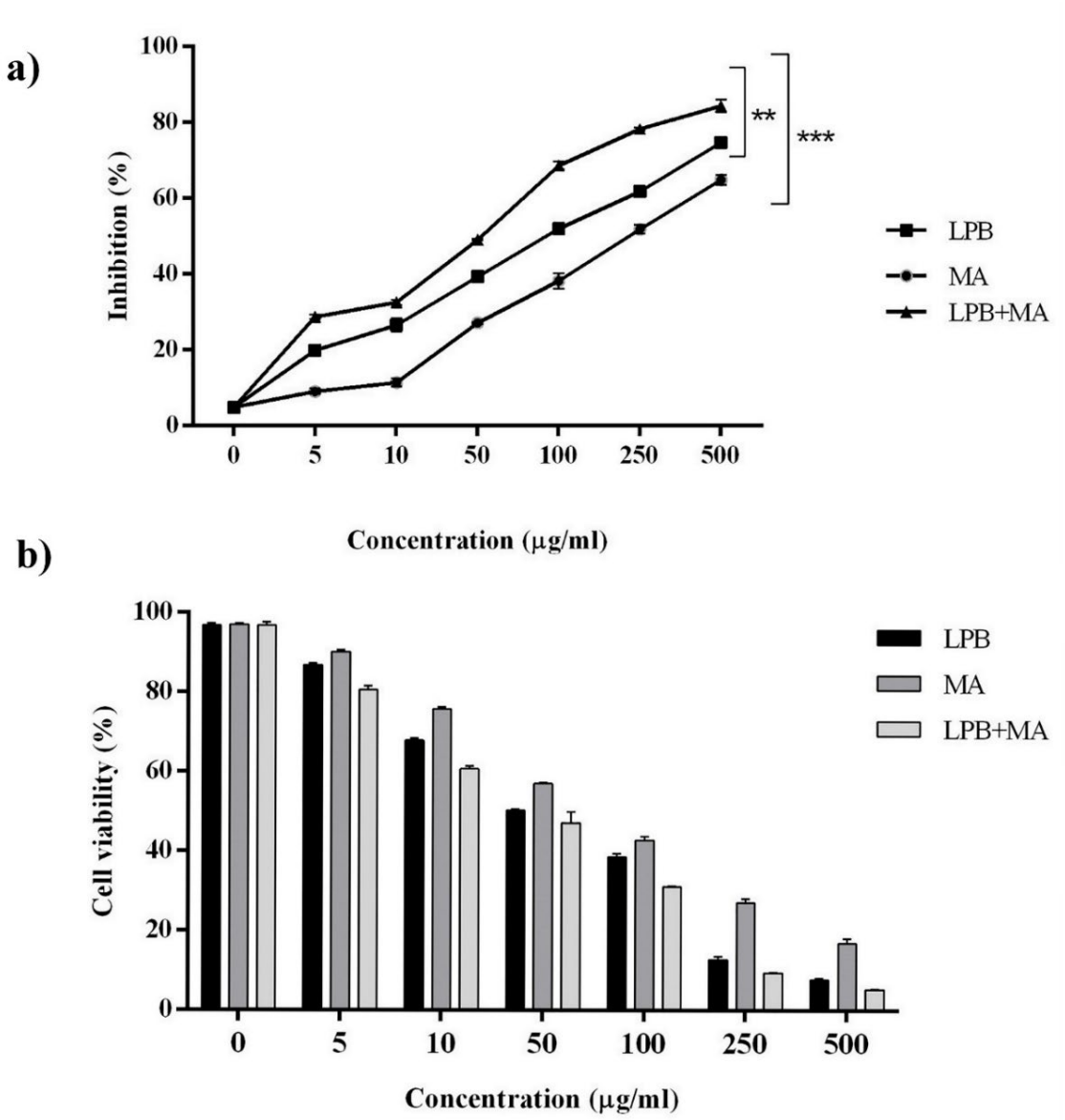
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Fig. 1.

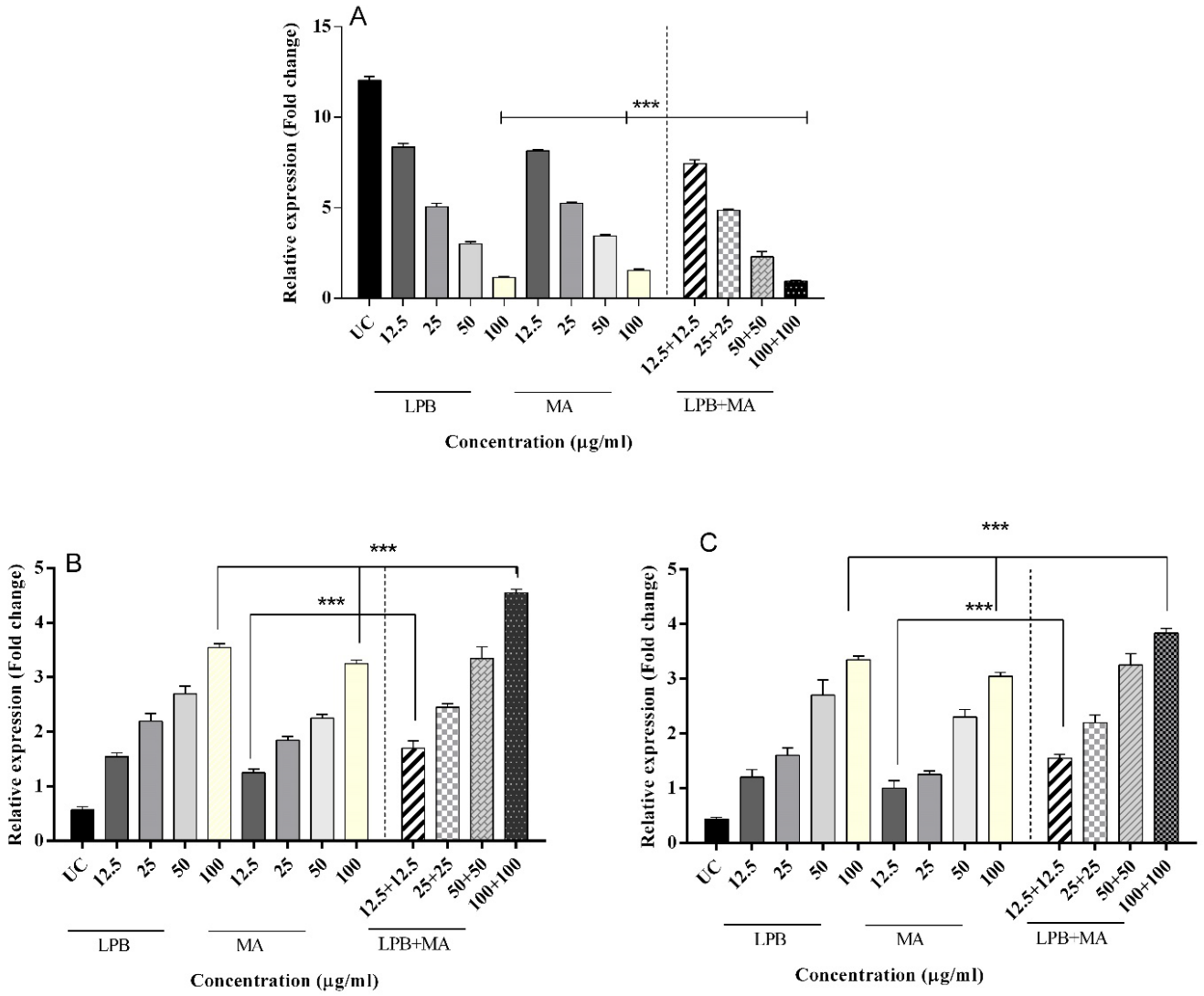
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Fig. 2.

Figure Captions:

Fig. 1. The inhibitory effect of LPB, MA alone and LPB + MA (B) on the promastigotes (a) and -amastigote (b) of *L. tropica* standard strain.

Fig. 2. Gene expression profiles of IL-10 (a), IL-12P40 (b), and IFN-γ (C) in macrophages treated with LPB, MA and LPB + MA, as compared to untreated group. Error bars are SD (**$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$). Each test was conducted in triplicate.

Table 1

The qPCR primers and reference gene sequences.

Template	Forward and reverse sequences (5′-3′)	Product size (bp)
IL-12P40	F-CTGGAGCACTCCCCATTCCTA	160
	R- GCAGACATTCCCGCCTTTG	
IL-10	F-CTTACTGACTGGCATGAGGATCA	101
	R- GCAGCTCTAGGAGCATGTGC	
IFN-γ	F-CAGCAAGGCGAAAAAGGATG	106
	R- TGGTGGACCACTCGGATGA	
GAPDH	F-AGCTTCGGCACATATTTCATCTG	89
	R- CGTTCACTCCCATGACAAACA	

Table 2 Evaluating the IC_{50} values of LPB in promastigote and amastigote forms of *L. tropica*

Drugs	**Amastigote**		**Promastigote**		**Macrophage**	**[c]SI**
	[a]IC_{50} ± SD (μg/mL)	P -value	[a]IC_{50} ± SD (μg/mL)	P -value	[b]CC_{50} (μg/mL)	(Selectivity Index)
LPB	38.5 ± 0.01	NR	88.3 ± 0.01	NR	315.5 ± 0.19	8.2
MA[d]	65.9 ± 0.03	P < 0.001	208.9 ± 0.03	P < 0.001	214.2 ± 0.61	3.5
LPB+ MA[d]	19.04±0.01	P < 0.001	31.9±0.01	P < 0.001	265.2± 0.36	13.9

compared to the positive control of MA (glucantime®) as well as the CC_{50} value of macrophage.

NR: Not related.
[a]IC_{50}: Concentration inhibiting 50% of promastigotes and amastigotes.
[b]CC_{50}: Concentration inhibiting 50% of macrophages.
[c]SI: Selectivity index (CC_{50}/IC_{50}).
[d]MA: Glucantime®.