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Effectiveness of harmaline along with meglumine antimoniate on *Leishmania major*

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Abstract

Leishmaniasis is a disease caused by *Leishmania* species and transmitted *via* sandflies. Current control strategies against reservoir hosts and vectors are not eco-friendly. Using harmaline (HA) from *Peganum harmala*, and meglumine antimoniate (MA) could be a promising therapy. The study aimed to explore the potential treatment outcomes and action mechanisms of HA and MA against *Leishmania major* stages by investigating their effectiveness through molecular docking, anti-leishmanial effects, safety assessment, and apoptotic profile evaluations. According to the molecular docking results, the protein-ligand interaction profiler identified that Bcl-2 interacts with HA mainly through hydrogen bonds, while Bax uses both hydrogen and hydrophobic interactions, indicating a stronger binding of HA to Bax compared to Bcl-2. The HA combined with MA (HA/MA) showed potent anti-leishmanial activity without toxicity. *In vitro* studies significantly demonstrated that HA inhibited the growth of promastigotes and amastigotes. The HA/MA was more effective in inhibiting parasite growth. Based on the study findings, HA and HA/MA mixture can be considered a viable treatment option for cutaneous Leishmaniasis.

Introduction

Leishmaniasis is a protozoan disease affecting humans and animals in approximately 98 countries worldwide.[1] Currently, around 12 million individuals are infected with various forms of leishmaniasis, including cutaneous, mucocutaneous, and visceral, while an estimated 1.00 billion people are at the risk of exposure. Notably, 90.00% of cases occur in just seven countries, including Afghanistan, Iran, Algeria, Peru, Brazil, Saudi Arabia, and Syria.[1,2] Since 1911, antimony compounds, like meglumine antimoniate (MA; Glucantime®), have been the primary leishmaniasis treatment.[3] However, these treatments come with significant drawbacks, including lengthy treatment durations, high costs, ineffective responses in about 10.00 to 15.00% of cases, and potential severe toxicity, affecting the heart, liver, and kidneys.[4,5]

The second-choice treatments for leishmaniasis, such as amphotericin B, paromomycin, pentamidine, and terbinafine, face similar challenges, including high costs, substantial dosages, prolonged administration periods, limited efficacy, and the development of resistance in parasites.[6] Various methods have been historically employed to treat cutaneous leishmaniasis (CL), including topical radiotherapy, lesion burning, cryotherapy, and local infiltration; however, these approaches have been largely ineffective.[7] Currently, there are no vaccines or safe and effective medications available for all forms of visceral leishmaniasis, CL, and mucocutaneous leishmaniasis, and it remains a major medical and veterinary disease with serious social implications.[8] Therefore, it seems necessary and vital to study about potential of new drugs that have no previous problems and side effects.

Harmaline (HA) is a beta-carboline alkaloid predominantly found in the seeds of *P. harmala*, also known as Syrian rue. This compound has garnered attention as a result of its diverse biological properties,[9,10] including various therapeutic applications, such as alleviating psoriasis,[11] providing immunosuppression,[12] offering anti-pruritic effects,[12] analgesia,[13] reducing inflammation,[14] protecting against radiation,[15] and exhibiting anti-parasitic,[16] anti-fungal,[17] anti-viral,[18] anti-bacterial,[19] and

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anti-leishmanial activities.[20] Additionally, it has shown anti-cancer,[21] and anti-oxidant effects.[22] The anti-tumor properties of HA are believed to stem from its ability to influence cellular processes and signaling pathways related to oxidative balance, DNA integrity, cell cycle regulation (by inducing apoptosis through increased expression of the Bax protein and decreased expression of Bcl-2),[23] and apoptosis.[24] Research has demonstrated that HA exhibits anti-proliferative effects on human monocytes and displays anti-leishmanial activity against both the promastigote and amastigote stages of the parasite.[20,25,26]

The objective of this study was to evaluate the leishmanicidal properties and mechanisms of action of HA, MA, and their combinations. This included ligand-protein molecular docking, assessing anti-leishmanial effects, determining safety indices, and analyzing apoptotic profiles against different stages of *Leishmania major*.

Materials and Methods

Forecasting functional residues of Bax and Bcl-2 proteins. To identify the functional residues in the structures of Bax and Bcl-2 proteins, the CASTp Software (http://sts.bioe.uic.edu/castp/index.html?1ycs) was utilized. This tool is specifically designed to predict functional residues by integrating evolutionary, functional, and structural data from various bioinformatics databases. Before docking, it is essential to delineate and measure the concave surface areas on three-dimensional (3D) protein structures. The Molegro Virtual Docker Software (version 7.0; Molexus, Odder, Denmark) was employed as a cavity search tool to locate pockets on protein surfaces and cavities within proteins.

Protein-ligand docking. The 3D structure of HA was obtained from PubChem (CID 3562; [https://pubchem.ncbi.nlm.nih.gov/compound/Harmaline]). The 3D structures of Bax and Bcl-2 were sourced from the Protein Data Bank (PDB: Bax[4BD2, 1F16] and Bcl-2[6GL8]); https://doi.org/10.2210/pdb1F16/pdb and https://doi.org/10.2210/pdb6GL8/pdb). Before conducting experiments, molecular docking was performed using Molegro Virtual Docker Software. Initially, docking was carried out within a defined search space surrounding the potential binding site. The graphical representations were created using Molegro Molecular Viewer (version 2.5.0; Molegro ApS, Aarhus, Denmark), focusing on docking configurations selected for their binding affinity.

Drug preparation. This research was conducted as a case-control study. Harmaline, sourced from Sigma-Aldrich (St. Louis, USA) was acquired from commercial suppliers in Iran. Meglumine antimoniate (Sanofi-Aventis, Paris, France) served as a positive control drug and was used in combination with HA. Serial dilutions were prepared to achieve concentrations of 12.50, 25.00, 50.00, 100, 200, and 400 µg mL^{-1}.

Parasite culture. *Leishmania major* promastigotes from the standard strain (MRHO/IR/75/ER) were maintained at the Kerman Leishmaniasis Research Center, Kerman, Iran, and cultured in Roswell Park Memorial Institute Medium-1640 medium (Biosera, Nuaille, France) supplemented with 10.00% fetal bovine serum (FBS; Merck) and penicillin-streptomycin antibiotics (Merck), being incubated at 25.00 ± 1.00 ˚C.

Macrophage culture. Human macrophages (THP1 human monocyte cell line) were obtained from the Pasteur Institute, Tehran, Iran. These cells were cultivated in Dulbecco's Modified Eagle Medium medium (Sigma-Aldrich) enriched with 10.00% fetal bovine serumand penicillin along with 0.50% streptomycin (Biosera). The cells were maintained at 37.00 ˚C in a 5.00% CO_2.

Anti-promastigote activity. The effectiveness of HA, MA, and their combination against *L. major* promastigotes was evaluated using the MTT assay. In this procedure, 1.00 × 10^5 promastigotes *per* mL in the logarithmic growth phase were counted and cultured in 96-well plates. The promastigotes were treated with various concentrations (12.50, 25.00, 50.00, 100, 200, and 400 µg mL^{-1}) of the drugs and incubated at 25.00 ± 1.00 ˚C for 72 hr. Following this incubation, 10.00 µL of MTT solution (Merck) was added to each well and allowed to react for 3 hr. Subsequently, 100 µL of dimethyl sulfoxide (DMSO) was added to each well, and the optical density (OD) absorbance was measured at 490 nm using a Microplate Spectrophotometer (BioTek Epoch; Agilent Technologies, Santa Clara, USA). The 50.00% inhibitory concentration (IC_{50}) was then calculated using SPSS Software (version 22.0, IBM Corp., Armonk, USA).

Anti-amastigote activity. For the anti-amastigote activity assessment, 1.00 × 10^5 macrophages *per* mL were counted and cultured on a glass microscope slide, incubating for 24 hr at 37.00 ˚C with 5.00% CO_2. Following this, 1.00 × 10^6 stationary-phase promastigotes were added to the macrophages at a ratio of 10 : 1 and incubated for another 24 hr to allow the parasites to transform into amastigote form. Afterward, various concentrations (12.50, 25.00, 50.00, 100, 200, and 400 µg mL^{-1}) of HA, MA, and their combination were introduced and incubated for 72 hr. The samples were dried, fixed with methanol, and stained with Wright-Giemsa. The number of intra-macrophage amastigotes was counted in 100 macrophages for each concentration, and the average was used to calculate the IC_{50} values for the amastigotes.

Cytotoxic effects of HA and MA. To assess cytotoxicity in macrophages, various concentrations (12.50, 25.00, 50.00, 100, 200, and 400 µg mL^{-1}) of HA, MA, and their combination were added to the cells and incubated for 72 hr at 37.00 ˚C with 5.00% CO_2 in 96-well plates. After incubation, 10.00 µL of MTT solution was introduced to each well and allowed to react for 3 hr. Following this, 100 µL of dimethyl sulfoxide was added to each well, and the

optical density values were measured at 490 nm using a Bio-Tek enzyme-linked immunosorbent assay reader. Each concentration was tested in triplicate and the results were compared with an untreated control group.

Combination index (CI) of drugs. The CI was calculated to evaluate the potential synergy of HA and MA. The formula used for CI calculation was as follows:

$$CI = (D)/(Dx)i + (D)/(Dx)ii,$$

where, *(Dx)i* and *(Dx)ii* show the concentrations of HA and MA, respectively, and *(D)* represents a combination of HA and MA. The CI value was used to quantitatively define the degree of synergism (CI < 1.00), additive outcome (CI = 1.00), or antagonism (CI > 1.00) between two medications. The theoretic IC_{50} was calculated using the following formula:[27]

$$Theoretic\ IC_{50} = (IC_{50}\ MA/2) + (IC_{50}\ HA/2)$$

Statistical analyses. Statistical analyses were performed using SPSS Software and GraphPad Prism (version 8.0; GraphPad Software Inc., San Diego, USA) to determine differences between groups, employing a paired *t*-test and one-way ANOVA. A significance threshold was set at a *p*-value of less than 0.05.

Results

Docking analyses presented the ability of HA to bind to Bax and Bcl-2. According to the Molegro results, HA binds with the active site residues Met99, Asp102, Asn104, Phe 105, and Asn106, for Bax, and Lys22, Ala 72, Glu111, Gly 114, and Val 115 for Bcl-2. According to the software's algorithm, these amino acids were highly prone to mutation within access tunnels and catalytic pockets (Figs. 1A and 1C).

Prediction of structural pockets on protein surfaces. Surface pockets, specific topological and geometric features, indicated the structural foundation for functional studies. Representing these features can be beneficial in the treatment advancement. The amino acids predicted to be involved in the pocket formation of Bax and Bcl-2 are exhibited in Table 1.

Molecular docking evaluated the two-dimensional interaction diagrams, revealing the most likely hydrophobic cavities along with various nearby hydrophobic residues and ligand-cavity hydrogen bonds. It should be mentioned that hydrophobic interactions and hydrogen bonds play a key role in displaying the binding energy between the ligand and target protein. In this study, the protein-ligand interaction profiler online server was employed to identify the amino acids involved in the interactions between the central pocket of Bax, Bcl-2, and HA metabolites (Figs. 1B and 1D). Notably, the interaction between Bcl-2 and HA is only mediated by hydrogen bonds in contrast with Bax which has both hydrogen and hydrophobic interactions (Table 1). These results can be an indicator of HA's stronger binds to Bax than Bcl-2.

Effect of HA, MA, and HA/MA combination on *L. major* promastigotes. The MTT assay was used to assess the anti-leishmanial effects of HA, MA, and HA plus MA combination against the promastigotes of *L. major*. As shown in Figure 1, the IC_{50} values of HA, MA, and HA plus MA were 313.70 ± 7.60, 209.70 ± 9.90, and 154.40 ± 5.30 µg mL^{-1}, respectively. These results revealed that IC_{50} of various concentrations of HA (IC_{50} = 4.89), MA (IC_{50} = 5.13), and HA plus MA (IC_{50} = 9.59) in comparison with the control group showed significant difference values (p < 0.001). Additionally, the inhibitory activity exhibited a dose-dependent pattern. The HA and MA combination had a significantly better leishmanicidal effect than various concentrations of HA and MA alone (p < 0.001). The graph showed the value of three independent experiments along with the standard deviation (Fig. 2).

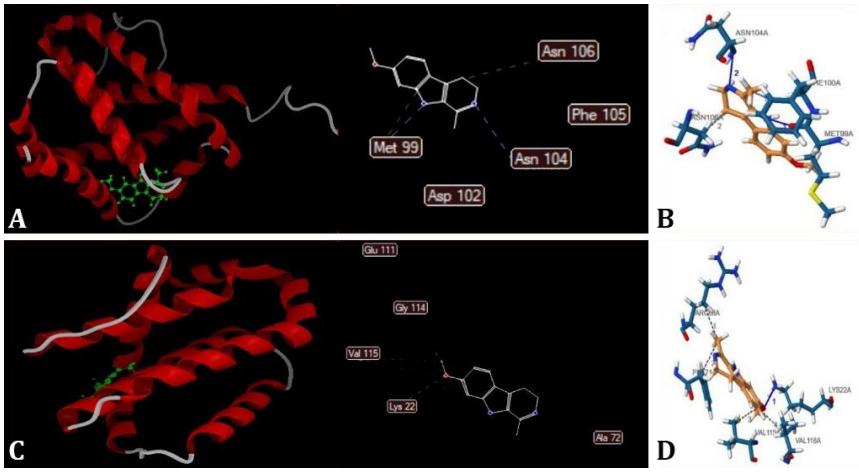

Fig. 1. Molecular docking. **A)** The Bax + harmaline (HA) molecular docking by Molegro Virtual Docker Software (version 7.0, Molexus, Odder, Denmark); **B)** The Bax + HA predicted amino acids in pocket formation by protein-ligand interaction profiler (PLIP) web tool; **C)** The Bcl-2 molecular docking by Molegro Virtual Docker Software; **D)** The Bcl-2 + HA predicted amino acids in pocket formation by protein-ligand interaction profiler web tool (projects.biotec.tu-dresden.de).

Table 1. Contribution of the Bax and Bcl-2 residues/molecule.

	Hydrophobic interactions						
	Index	**Residue**	**AA**	**Distance**	**Ligand atom**	**Protein atom**	
Bax	**1**	100A	PHE	3.95	2,992	1,478	
	2	106A	ASN	3.40	2,984	1,559	
	Hydrogen bonds						
	Index	**Residue**	**AA**	**Distance H-A**	**Distance D-A**	**Donor angle**	**Donor atom**	**Acceptor atom**
	1	99A	MET	1.55	2.44	144.21	2,980 [Npl]	1,457 [O2]
	2	104A	ASN	1.88	2.64	132.38	1,521 [Nam]	2,981 [N2]
	Hydrophobic interactions						
	Index	**Residue**	**AA**	**Distance**	**Ligand atom**	**Protein atom**	
Bcl-2	1	68A	ARG	3.54	2,260	704	
	2	71A	PHE	3.66	2,260	763	
	3	115A	VAL	3.32	2,261	1,445	
	4	118A	VAL	3.78	2,261	1,487	
	Hydrogen bonds						
	Index	**Residue**	**AA**	**Distance H-A**	**Distance D-A**	**Donor angle**	**Donor atom**	**Acceptor atom**
	1	22A	LYS	1.68	2.51	134.71	227 [N3+]	2,247 [O2]

AA: Amino acid, ASN: Asparagine, ARG: Arginine, Lys: Lysine, Met: Methionine, PHE: Phenylalanine, Val: Valine.

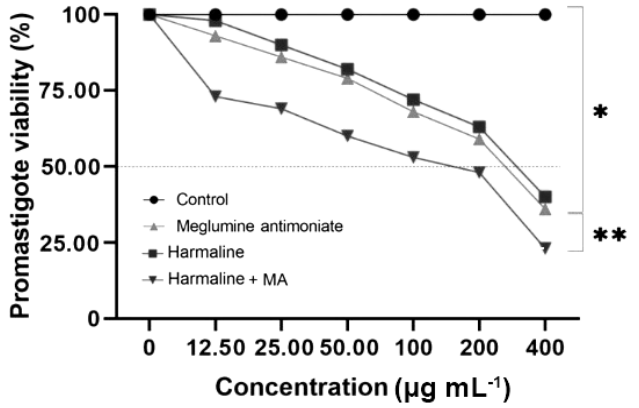

Fig. 2. The survival rate of *Leishmania major* promastigotes treated with different concentrations of harmaline and meglumine antimoniate (MA) alone and in combined treatment compared to the control group. *Significant difference from the control group ($p < 0.0001$); ** Significant difference from the combined treatment ($p < 0.001$).

Effect of HA, MA, and HA/MA combination on *L. major* intra-macrophage amastigotes load. The effects of HA, MA, and their combination on the load of L. major amastigotes within macrophages were assessed. Table 2 presents the average number of amastigotes found in macrophages after treatment with various concentrations (12.50, 25.00, 50.00, 100, 200, and 400 µg mL^{-1}) of HA, MA, and the HA/MA combination compared to an untreated control group. Both HA and MA demonstrated a significant reduction in the number of amastigotes in a dose-dependent manner compared to the untreated control group ($p < 0.001$). The inhibitory rates of intra-macro-phage parasites also showed a dose-dependent pattern. The combination of HA and MA resulted in a marked decrease in the number of amastigotes per macrophage compared to both the control group and individual drugs ($p < 0.01$), particularly at a concentration of 200 µg mL^{-1} where no amastigotes were detected (N = 0). Furthermore, at a concentration of 400 µg mL^{-1}, the HA/MA combination completely eradicated all amastigotes ($p < 0.001$), as shown in Table 3.

Table 3. The effect of different concentrations of harmaline + meglumine antimonate on the mean number of intra-macrophage amastigotes. Data are presented as mean ± standard deviation.

Concentrations (µg mL^{-1})	**Number**	***p*-value**
0.00 (Control)	63.30 ± 3.80	**Not related**
12.50 + 12.50	57.30 ± 2.10	***p* < 0.001**
25.00 + 25.00	51.70 ± 1.50	***p* < 0.001**
50.00 + 50.00	44.30 ± 0.40	***p* < 0.001**
100 + 100	32.36 ± 2.50	***p* < 0.001**
200 + 200	16.30 ± 1.50	***p* < 0.001**
400 + 400	**0.00 ± 0.00**	***p* < 0.001**

Table 2. The impact of different concentrations of harmaline and meglumine antimonate on the average number of intra-macrophage amastigotes. Data are presented as mean ± standard deviation.

Concentrations (µg mL^{-1})	**Harmaline**		**Meglumine antimonate**	
	Number	***p*-value**	**Number**	***p*-value**
0.00 (Control)	63.30 ± 3.80	Not related	63.30 ± 3.80	**Not related**
12.50	44.70 ± 3.10	$p > 0.05$	51.30 ± 1.50	***p* < 0.001**
25.00	38.30 ± 2.50	$p < 0.01$	45.70 ± 2.10	***p* < 0.001**
50.00	25.30 ±3.10	$p < 0.001$	37.7 0 ± 2.10	***p* < 0.001**
100	16.70 ± 4.20	$p < 0.001$	30.30 ± 1.50	***p* < 0.001**
200	0.00 ± 0.00	$p < 0.001$	7.70 ± 1.50	***p* < 0.001**
400	**0.00 ± 0.00**	***p* < 0.001**	**0.00 ± 0.00**	***p* < 0.001**

Evaluation of cytotoxicity. The mortality profile of *L. major* promastigotes treated with various substances is summarized in Table 4. The 50.00% cytotoxic concentration (CC_{50}) values indicated that the combination of HA and MA had a significantly greater effect on promastigotes compared to either HA or MA alone ($p < 0.0001$ *versus* $p < 0.001$), relative to the untreated group. Different concentrations of the drugs (ranging from 0.00 to 400 µg mL^{-1}) were tested on human cell lines in Dulbecco's Modified Eagle Medium culture media, and the CC_{50} rates were determined by counting the intra-cellular amastigotes. The assessment of cytotoxicity at these concentrations revealed that the drugs did not exhibit lethal effects, as indicated by the selectivity index (SI), calculated as CC_{50}/IC_{50}, which remained within acceptable limits, with an SI value of at least 1.00. Specifically, the SI values for HA, MA, and the HA/MA combination were 4.89, 5.13, and 9.59, respectively, all indicating safety (SI ≥ 1.00) and no toxic effects (SI = CC_{50} (macrophages) / IC_{50} (promastigotes) ≥ 1.00). These findings suggest that HA selectively targets parasites without adversely affecting human macrophages (Table 4).

Combination index. The CI was used to predict the degree of drug interactions. Based on the equation, the index was calculated to be additive (equal to 1.00) when the sum of the effects of the chemicals acted self-sufficiently. Significant differences were observed in the indices of *L. major* intra-cellular amastigotes compared to the negative group ($p < 0.001$). Table 4 provides the values for IC_{50}, CC_{50}, and SI of HA, MA, and the combination of both. The CI index was determined as 0.948, defined as synergism (CI < 1.00). The analysis verified that the theoretical IC_{50} of HA alone (108.20 µg mL^{-1}) had a significant difference from the experimental IC_{50} of HA/MA mixture (48.20 µg mL^{-1}; $p < 0.001$), representing a synergistic effect in the combination activity (Table 4).

Discussion

Leishmaniasis represents a significant public health challenge, with chemical treatments often associated with numerous side effects and the emergence of parasite resistance.[28] Despite extensive efforts, developing an effective and affordable vaccine has not succeeded.[29] Controlling the various vectors and hosts involved in the transmission of the disease is also impractical.[30] Therefore, the primary management strategy relies heavily on chemotherapeutic agents.[31] Current challenges necessitate the exploration of more effective treatment options and enhancement of novel drugs, whether used individually or in combination, for leishmaniasis treatment.[32]

In silico study showed that the anti-leishmanial activity of HA was demonstrated *via* different aspects. Our study showed that HA binds with the active site residues Met99, Asp102, Asn104, Phe 105, and Asn106 for Bax, and Lys22, Ala 72, Glu111, Gly 114, and Val 115 for Bcl-2, indicating the structural foundation for the amino acids predicted to be involved in the pocket formation of Bax and Bcl-2. According to the molecular docking results, the protein-ligand interaction profiler identified that Bcl-2 interacts with HA primarily through hydrogen bonds, while Bax utilizes both hydrogen and hydrophobic interactions.

This suggests that HA binds more strongly to Bax than to Bcl-2. The Bax promotes apoptosis by facilitating mitochondrial outer membrane permeabilization, releasing pro-apoptotic factors during chemotherapy-induced cellular stress. In contrast, Bcl-2 inhibits apoptosis by maintaining mitochondrial integrity. The ratio of Bax to Bcl-2 can predict chemotherapy response; a higher ratio indicates better treatment outcomes due to the increased pro-apoptotic activity. Increasing Bax and decreasing Bcl-2 expression can induce apoptosis in infected cells in leishmaniasis treatment.[33] Harmaline may enhance this effect by increasing the Bax/Bcl-2 ratio, promoting programmed cell death in *Leishmania*-infected cells.[34] Harmaline combined with other drugs that affect Bcl-2 and Bax could serve as a combined therapeutic approach for leishmaniasis.[26] This strategy may increase the effectiveness of treatment, reduce side effects, and provide a hypothesis for the development and discovery of an effective vaccine.

The present results demonstrated that HA significantly inhibited the proliferation rate of *L. major* intra-macrophage amastigotes and promastigotes alone and had more lethal effects in combination. Due to the higher IC_{50} value of the HA (313.70 ± 7.60 µg mL^{-1}) in the promastigote form of *L. major* compared to the IC_{50} of HA acting on the amastigote form (108.20 ± 0.50 µg mL^{-1}), it can be concluded that promastigotes were more sensitive to HA than the amastigote forms. Several factors may account for the

Table 4. Comparison of the effect of different concentrations of harmaline (HA) and meglumine antimonite (MA) alone and their combination (HA + MA) on the average number of intra-macrophage amastigotes compared to the untreated control group. Data are presented as mean ± standard deviation.

Drugs	Amastigote		Promastigote		Macrophage	SI
	IC_{50} (µg mL^{-1})	*p*-value	IC_{50} (µg mL^{-1})	*p*-value	CC_{50} (µg mL^{-1})	
MA	95.80 ± 8.20	Not related	209.70 ± 9.20	Not related	492.20 ± 18.40	**5.13**
HA	108.20 ± 0.50	$p < 0.001$	313.70 ± 7.60	$p < 0.001$	529.40 ± 12.30	**4.89**
MA + HA	**48.20 ± 3.60**	**$p < 0.001$**	**154.40 ± 5.30**	**$p < 0.001$**	**462.30 ± 17.40**	**9.59**

IC_{50}: 50.00% inhibitory concentration, drug concentration inhibited 50.00% of promastigotes and amastigotes growth; CC_{50}: 50.00% cytotoxic concentration, drug concentration inhibited 50.00% of macrophage growth; SI: Selectivity index (CC_{50}/IC_{50}).

difference in sensitivity between the two forms of *L. major*, including the phagolysosomal membrane of macrophages and inhibition of HA entry.[35] On the other hand, this study indicated that HA plus MA had a greater inhibitory effect on *L. major* than MA alone, showing a synergistic effect between HA and MA in inhibiting *L. major* stages. The results observed in the latter study suggested that inhibition of promastigote internalization within macrophages could result from inhibition of protein kinase C activity by HA.[36] Naturally, the inhibitory effect of a drug on an intra-cellular parasite is advantageous when it does not have a very pronounced effect on host cells. To explain this, the SI is calculated from the comparison of the effect of drug on the disease agent and host cell.[37] During the research to identify an effective and safe anti-leishmanial drug, it has been determined that there is a strong correlation between the results of evaluating toxicity to mammalian cells *in vitro* and toxicity results *in vivo*; therefore, in this study, the toxicity of HA, MA, and HA plus MA on macrophages was evaluated (4.89, 5.13, and 9.50 at 72 hr, respectively), and SI higher than 1.00 indicated the specificity of drug on the parasite and macrophages.[38]

Regarding the effect of HA in combination with a known anti-leishmanial drug, the present study showed that the CI value in amastigotes treated with all HA + MA formulations was less than 1.00. This could indicate a synergistic interaction between these two substances. The combination of herbal immunomodulators with standard drugs provides effective treatment of a variety of molecular targets, improving therapeutic efficacy and reducing toxicity.[39] Combination therapy, as emphasized in many studies, not only increases the effectiveness of CL treatment but also reduces the course and cost of treatment and diminishes adverse side effects, duration, and risk of parasite resistance.[40,41] Nevertheless, the topical application of HA, a suitable formulation for CL, can significantly reduce the concern of systemic side effects.

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Conflicts of interest

The authors declare no conflicts of interest.

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