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Research Article

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Biostimulated-sesame sprout extracts as potential agents against *Leishmania mexicana*

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Abstract

Leishmania mexicana is one of the causal agents of cutaneous leishmaniasis. Current antileishmanial chemotherapeutics have demonstrated adverse side effects; thus, alternative treatments are needed. In this study, we performed *in silico* and *in vitro* analyses of the leishmanicidal potential of the most abundant phenolic compounds identified in black sesame sprouts biostimulated with *Bacillus clausii*. The molecular docking analysis showed strong interactions (binding free energies between −6.5 and −9.5 kcal/mol) of sesaminol 2-O-triglucoside, pinoresinol dihexoside, isoverbascoside, and apigenin with the arginase, leishmanolysin, cysteine peptidase B, and pyruvate kinase leishmanial enzymes. Furthermore, almost all phenolic compounds interacted with the active site residues of *L. mexicana* enzymes. *In vitro*, the *B. clausii*-biostimulated sprout phenolic extracts and apigenin inhibited the growth of promastigotes with IC_{50} values of 0.08 mg gallic acid equivalent/mL and 6.42 μM (0.0017 mg/mL), respectively. Additionally, in the macrophage infection model, cells treated with *B. clausii*-biostimulated sprout phenolic extracts and infected with *L. mexicana* exhibited significantly ($P < 0.05$) reduced nitric oxide production and decreased parasite burden. Altogether, our study provides important data related to high efficacy and less toxic natural antileishmanial candidates against promastigotes of *L. mexicana*.

Impact Statement The evaluation of the effectiveness of plant-derived extracts as alternative candidate products in the treatment of neglected tropical diseases, such as leishmaniasis, is essential. This study explored the *in vitro* and *in silico* leishmanicidal activity of a *Bacillus clausii*-biostimulated-black sesame sprout phenolic extract against *Leishmania mexicana*. *In vitro* studies revealed the leishmanicidal potential of the biostimulated-extract or individual compounds against promastigotes and their microbicidal effect in infected macrophages. Bioinformatic analysis of the main phenolic compounds of the biostimulated sesame sprout extract showed high binding free energies and strong interactions with essential enzymes for the survival and proliferation of *L. mexicana*.

Keywords: *Leishmania mexicana*, *Bacillus*, sesame sprouts, phenolic compounds, macrophage, molecular docking

Introduction

Leishmaniasis is a parasitosis transmitted through female phlebotomine sandflies caused by protozoa of the *Leishmania* genus. Cutaneous leishmaniasis (CL) is the most common form of the disease (Gabriel et al. 2019). In 2021, >220 000 cases of CL were reported worldwide (WHO 2023). *Leishmania mexicana* causes CL, characterized by the formation of granulomas at the parasite inoculation site and cutaneous nodules with parasitized macrophages, the target cell of this protozoan (Jafarzadeh et al. 2019). Current treatment of leishmaniasis relies on chemotherapeutic drugs such as miltefosine, amphotericin B, pentamidine, paromomycin, and pentavalent antimonials. However, their use is limited due to the toxic side effects and increasing resistance of *Leishmania* species; unfortunately, no prophylactic vaccine is available (Aronson and Joya 2019).

Recently, compounds derived from natural sources have exhibited antiparasitic potential, including phenolic compounds (PCs). Reports have demonstrated the leishmanicidal potential of PC extracts from different food matrices (Ogungbe et al. 2014, Kheirandish et al. 2018). Sesame (*Sesamum indicum* L.) is an oilseed containing a PC profile with beneficial effects on human health (Moazzami et al. 2007). Interestingly, this profile can be improved by tools such as the natural biological phenomenon of germination (Zhu et al. 2005, Shi et al. 2010) and biostimulant treatments, including various bacterial genera (Złotek et al. 2019, Jiménez-Gómez et al. 2020). The genus *Bacillus* has been used as an inducer of PC synthesis; specifically, *Bacillus clausii* has influenced the phenolic profile in biostimulated black sesame sprouts, including increased synthesis of pinoresinol dihexoside, sesaminol 2-O-triglucoside, cinnamic acid, and apigenin (Garduño-Félix et al. 2021).

During *Leishmania* intracellular infection, macrophages, a major cell target, activate different microbicidal mechanisms, mainly nitric oxide (NO) production, to promote parasite death (da Silva and Floeter-Winter 2014). However, *Leishmania* evades all attacks characterized by decreased NO and activation of vital enzymes for survival and proliferation (Tiwari et al. 2017). Therefore, exploring promising compounds with an inhibitory effect on these vital enzymes is crucial. Furthermore, some PCs have been reported to have antileishmanial activity (Soto-Sánchez 2022). To this end, bioinformatics tools, such as molecular docking, have been extensively used to obtain binding free energies and interaction types between candidate molecules with enzymes (Raj et al. 2020). Therefore, this study aimed to analyze *in silico*, the PC

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identified in a greater proportion in *B. clausii*-biostimulated sesame sprouts with enzymes related to metabolism pathways and virulence mechanisms of *L. mexicana*. In addition, the *in vitro* effect of extracts and PC of sesame sprouts biostimulated with *B. clausii* on *L. mexicana* promastigotes and infected macrophages was evaluated.

Materials and methods

Molecular docking

The main PCs identified within the phenolic extracts of *B. clausii*-biostimulated sesame sprouts and control sprouts nonbiostimulated (henceforth referred to as BS and CS, respectively; Supplementary Material S1) (Garduño-Félix et al. 2021) were used for molecular docking with the enzymes of *L. mexicana*: arginase, leishmanolysin, cysteine peptidase B (CPB), glycerol-3-phosphate dehydrogenase (GPDH), and pyruvate kinase (PK) (Supplementary Material S2). Amphotericin B was used as the cytotoxic control (Supplementary Material S1). Before molecular docking, the PCs were subjected to an energy reduction to the Merck Molecular Force Format (MMFF94) using the Avogadro v 1.2.0 software. The AutoDock Vina program (78% accuracy) determined the molecular docking through the CB-Dock web server, a free online access platform. (https://cadd.labshare.cn/cb-dock2/php/index.php), which predicts the binding sites of a protein, determines, and calculates their centers and range of detection of cavities (Liu et al. 2020). The 2D visualization of the interactions was obtained by the Discovery Studio v21.1.0 program.

Bacillus clausii-biostimulated sesame sprout extracts

Bacillus clausii (ATCC® 700160™) was used to biostimulate black sesame seeds. The strain was reactivated in trypticase soy broth (TSB; Difco, Mexico) at 37°C for 24 h, and the bacterial concentration was calculated using the McFarland scale. For cryopreservation, *B. clausii* was stored at −80°C in TSB with 15% (v/v) glycerol. Biostimulated sprouts were obtained as previously described by Garduño-Félix et al. (2021). Briefly, 15 mL of *B. clausii* (1×10^3 CFU/mL) culture in TSB was incubated with 5 g of disinfected and 24 h-hydrated (25°C, dark) black sesame seeds for 1 h in a rotator at 20 rpm/25°C. Then, bacterial-biostimulated and control seeds germinated for 3 days in the dark at 25°C. The sprouts were sprayed with 10 mL of sterilized water and drained every 12 h. After germination, sprouts were stored at −20°C prior to phenol extraction. Frozen sprouts (1.0 ± 0.1 g) were homogenized with 10 mL of an ethanol and water (80:20 v/v) cold solution in a rotator at 50 rpm/25°C for 1 h, then centrifuged at 3000 × *g* for 10 min. The supernatant (phenolic extract) was filtered through 0.22-μm syringe filters, protected from light, and stored at −20°C for later use (Mora-Rochin et al. 2010).

Leishmania mexicana

Leishmania mexicana promastigotes (MHOM/MX/92/UAY68) were kindly provided by Dr. López-Moreno (UAS, Mexico). They were cultured in 10 mL of RPMI-10 [RPMI-1640 (Sigma–Aldrich, UK) with 10% fetal bovine serum (FBS) and 50 μg/mL of gentamicin] in 25 cm^2 cell culture bottles and incubated at 25°C for 72 h before all assays (Enciso et al. 2016). *Leishmania* promastigotes were cryopreserved at −80°C in 90% FBS with 10% Dimethyl Sulfoxide (DMSO) (Hybri-Max™, Sigma–Aldrich, USA). Cell viability was calculated using the trypan blue exclusion test (Strober 2015). The promastigote viability percentage considered for all assays was >90%.

In vitro leishmanicidal activity

For the inhibition assay, 2.5×10^5 promastigotes/mL were placed in a microtube with different concentrations of BS and CS phenolic extracts [0.04–3.00 mg of gallic acid equivalent (GAE)/mL], individual PCs (0.08–4000 μM) such as apigenin (Sigma–Aldrich, USA), cinnamic acid (USP, USA), and protocatechuic acid (Sigma–Aldrich, USA) for 48 h as previously reported (Fonseca-Silva et al. 2011, Enciso et al. 2016); all diluted in RPMI-10 to a total volume of 200 μL. Isoverbascoside, sesaminol 2-O-triglucoside, and pinoresinol dihexoside individual compounds were unavailable for further studies. Controls included nontreated cells, cells treated with amphotericin B (Gibco, USA) and DMSO (10% final concentration; Sigma–Aldrich, USA). Viable cells were determined by the trypan blue exclusion test. The Methyl Thiazolyl Tetrazolium (MTT) assay was excluded due to optical interference with the phenolic extracts. The assay was performed in triplicates. The following formula was used to determine the percentage (%) inhibition: [100 − (treated cells/nontreated cells)] × 100. The results were expressed in IC_{50} and calculated through a nonlinear regression using the GraphPad Prism 8.0 software.

Macrophage cytotoxicity

RAW 264.7 (ATCC® TIB-71™) macrophages were cultured at a density of 9×10^3 cells/well in 96-well plates at a capacity of 100 μL with DMEM-10 without phenol red medium [DMEM (Sigma–Aldrich, UK) with 10% FBS and 50 μg/mL of gentamicin]. First, cells were incubated for 1 h at 37°C and 5% CO_2 to allow macrophage adhesion. Then, 180 μL of DMEM-10 medium and 20 μL of BS (2.0 mg GAE/mL), CS (6.0 mg GAE/mL), apigenin (2.3 mg/mL, 8516.94 μM), and amphotericin B (2.5 μg/mL, 2.70 μM) were added and incubated for 24 h at 37°C and 5% CO_2. Apigenin was the individual compound evaluated in macrophages due to its efficacy in the leishmanicidal assay. Controls included mock-treated cells or treated with 10% DMSO at room temperature (cytotoxicity control). After incubation, the supernatant was discarded, 100 μL of cold PBS-EDTA was added, and cells were incubated for 5 min. Viable cells were determined by the trypan blue exclusion test in three replicates. Cellular cytotoxicity was expressed as a percentage (%) with the following formula: [100 − (treated cells/nontreated cells)] ×100.

Macrophage infection assay and NO measurements

In a 96-well flat-bottom plate, 9×10^3 RAW 264.7 macrophages were cultured in 100 μL of DMEM-10 without phenol red and incubated for 1 h at 37°C and 5% CO_2. After incubation, the medium was discarded, 100 μL of fresh DMEM-10 and 20 μL of phenolic extracts were added as described above, and incubated for 24 h at 37°C and 5% CO_2. Cultures were washed with phosphate-buffered saline, and 100 μL of *L. mexicana* promastigotes in RPMI-10 at an infection ratio of 1:10 (macrophage: parasite) were incubated for 6 h at 37°C and 5% CO_2. Control treatments included: noninfected cells without phenolic extracts (mock-treated noninfected cells), infected cells without phenolic extracts

Table 1. Binding free energies (kcal/mol) in PC–*L. mexicana* enzyme interactions.

	Leishmania mexicana enzyme				
	Arginase [L-arginine]	**Leishmanolysin [Ser-Phe]**	**CPB [Arg-Phe]**	**GPDH [G3P]**	**PK [Phosphoenol-pyruvate]**
PC					
Apigenin	−6.5	−7.1	−6.9	−9.5	−7.9
Cinnamic acid	−6.0	−5.3	−5.0	−6.4	−5.6
Isoverbascoside	−6.9	−8.2	−7.6	−9.1	−9.4
Pinoresinol dihexoside	−7.3	−8.7	−8.7	−9.1	−9.1
Protocatechuic acid	−5.9	−5.4	−4.8	−6.4	−6.0
Sesaminol 2-O-triglucoside	−7.5	−8.2	−8.5	−8.7	−9.4
Control					
Amphotericin B	−7.7	−8.0	−6.8	−6.2	+1.0
Substrate	−6.4	−5.9	−4.8	−5.8	−5.6

[]: Enzyme substrate; PC: Phenolic compounds; CPB: Cysteine peptidase B; GPDH: Glycerol-3-P-dehydrogenase; PK: Pyruvate kinase; G3P: Gycerol-3-phosphate.

(mock-treated infected cells), noninfected cells treated with phenolic extracts (mock-infected treated cells), and infected and noninfected cells treated with 1 μg/mL of LPS (Sigma–Aldrich, USA). The plate was centrifugated (1500 × *g*/5 min), and cell-free supernatants were used for NO release assay according to manufacturer's instructions using the Griess reagent (Sigma–Aldrich, USA). In a 96-well flat-bottom plate, 100 µL of the supernatant, 10 µL of the Griess I reagent, 10 µL of the Griess II reagent, and 80 µL of nitrite assay were mixed. After 10 min of incubation at 25°C, the absorbance at 540 nm was measured, and the NO concentration was determined as nitrites (NO_2) with a standard curve of sodium nitrite ($NaNO_2$) (Kheirandish et al. 2018, Rochín-Medina et al. 2021). The assay was performed as two independent experiments.

Parasite load

Sterile coverslips were placed in flat-bottom 24-well plates, and 1 × 10^4 RAW 264.7 macrophages were seeded in 500 µL of DMEM-10 medium, stimulated, and infected as previously described. After infection, infected cells were washed twice with PBS, fixed with 300 µL of methanol for 15 min, and stained with Giemsa. The percentage of infected macrophages was determined using light microscopy by counting cells in three random microscope fields on each coverslip (40 ± 5 cells/coverslip) from three independent experiments.

Statistical analysis

All results were compared using GraphPad Prism version 8.0.0 (GraphPad Software, USA) with one-way or two-way analysis of variance (ANOVA), followed by Dunnett's multiple comparisons test or confirmed with a *t*-test. Differences with $P < 0.05$ were considered significant, with a confidence interval of 95%.

Results and discussion

Molecular docking between PC of sesame sprouts and enzymes of *L. mexicana*

The treatment of leishmaniasis is challenging; although current drugs are effective, toxic effects and resistance are present (Aronson and Joya 2019). This opens an opportunity to explore alternatives; therefore, we evaluated the leishmanicidal capacity of extracts obtained from *B. clausii*-stimulated sesame sprouts. First, we analyzed the interaction of *L. mexicana* enzymes by molecular docking and recognized drug targets with the most abundant components of the stimulated sesame and control sprout extracts. The main phenolics identified in BS extracts were pinoresinol dihexoside (30.29%) followed by sesaminol 2-O-triglucoside (20.22%), cinnamic acid (12.84%), isoverbascoside (5.04%), and apigenin (4.62%), whereas CS extracts contained predominantly sesaminol 2-O-triglucoside (42.20%), isoverbascoside (20.72%), and the protocatechuic acid (5.27%) (Garduño-Félix et al. 2021). Amphotericin B and the substrates for each enzyme were used as controls. The results included binding free energies (Table 1), total hydrogen bonds, other interactions (van der Waals forces, pi-cation, alkyl, pi-alkyl, pi–pi, pi-sulfide, and covalent), and interactions with active site amino acids (Table 2, Supplementary Material S3–S7).

PC and Arginase

Arginase is the enzyme involved in the biosynthesis of polyamines and necessary for the growth and proliferation of the promastigote (Colotti and Ilari 2011, Muxel et al. 2018). Molecular docking of the PC with the arginase of *L. mexicana* showed that sesaminol 2-O-triglucoside and pinoresinol dihexoside had the best binding free energies, −7.5 and −7.3 kcal/mol, respectively, even stronger than the substrate L-arginine (−6.4 kcal/mol) (Table 1). Five of the six PC exhibited interactions with amino acids (Thr257, His114, Asp141, and His154) from the arginase active site directly involved in the catalytic mechanism of the enzyme. Cinnamic acid interacted with almost all of them including His114, Asp137, Ala138, His139, Asp141, His154, Asp243, Asp245, Thr257, and Glu288 (Table 2, Supplementary Material S2 and S3). Different PC from fruits and vegetables have been reported as enzymatic inhibitors of *Leishmania* arginase (Manjolin et al. 2013). Also, this enzyme has demonstrated high binding free energies with flavonoids and benzimidazole-derived compounds (Méndez-Cuesta et al. 2012, Glisic et al. 2016).

PC and Leishmanolysin

Another important target is leishmanolysin, *Leishmania* promastigotes' most abundant surface protein, localized in structures such as the flagellum and the flagellar pocket (Hallé et al. 2009). This enzyme protects the parasite against the cytolytic effects of antimicrobial peptides that induce disruption of surface-membrane permeability and apoptosis (Kulkarni

Table 2. PC-*L. mexicana* enzymes interactions.

	Arginase [L-arginine]				Leishmanolysin [Ser92-Phe93]				CPB [Arg-Phe]				GPDH[G3P]				PK [Phosphoenol-pyruvate]			
Compound	**HB**	**ASHB**	**OI**	**ASOI**	**HB**	**ASHB**	**OI**	**ASOI**	**HB**	**ASHB**	**ASOI**	**ASOI**	**HB**	**ASHB**	**OI**	**ASOI**	**HB**	**ASHB**	**OI**	**ASOI**
Apigenin	2	Asp141	11	His139 His154 Thr257	2	–	12	–	0	–	10	Cys26 His164	5	Lys125 Thr267	13	Lys210(2) Asp263	2	–	13	–
Cinnamic acid	3	Asp141 His154 Thr257	8	His114 Asp137 His139 Asp141 His154 Asp243 Asp245 Glu288	1	–	8	His264	1	Cys26	9	His164	1	–	11	Lys125 Lys210 Thr267	2	–	10	–
Isoverbascoside	7	–	16	His139 His154 Thr257	4	–	15	His264 His268 His334	3	–	17	Cys26 His164	1	–	23	Lys125	7	–	22	Asp264
Pinoresinol dihexoside	4	–	12	–	2	–	19	His264 His268 His334	5	–	11	Cys26 His164	3	Lys125 Lys210	24	Asp263 Thr267	3	–	20	Asp264
Protocatechuic acid	2	Asp141 Thr257	6	His139 His154 Asp245	3	His264	8	–	3	Cys26 His164(2)	8	–	3	Asp263	9	Lys125 Thr267	5	–	5	–
Sesaminol 2-O-triglucoside	5	His139	14	Asp141 His154 Thr257	3	–	19	His264 His268	5	Cys26 His164	16	–	3	–	17	–	5	–	21	Met50 Gln297
Control Amphotericin B	4	–	17	His139 Asp141 His154 Thr257	2	–	18	His264	4	–	13	Cys26 His164	0	–	13	–	4	–	21	Gln297
Substrate	5	Asp141 His154 Thr257	10	His114 Asp137 His139 Asp141 Asp243 Asp245 Glu288	4	His268 His334	10	His264	4	Cys26(2) His164	12	–	6	Lys125 Lys210	7	Asp263 Thr267	7	Asp264(3)	9	Asp264

PC: Phenolic compounds; HB: Total hydrogen bonds; ASHB: Active site amino acids interaction with hydrogen bond; OI: Other interactions; ASOI: Active site amino acids with other interactions; (): Interactions with the same amino acid; []: Enzyme substrate; CPB: Cysteine peptidase B; GPDH: Glycerol-3-P-dehydrogenase; PK: Pyruvate kinase; G3P: Gycerol-3-phosphate;—: without ASHB interactions.

Table 3. The leishmanicidal activity of phenolic extracts and identified PC of sesame sprouts.

Treatment	IC_{50}	
BS extract	0.08 ± 0.01	mg GAE/mL
CS extract	0.37 ± 0.08	mg GAE/mL
Apigenin	6.42 ± 0.68	μM
Cinnamic acid	382.22 ± 80.7	μM
Protocatechuic acid	>973.26 ± 90.0	μM
Amphotericin B	0.02 ± 0.01	μM

BS: Biostimulated sesame sprouts; CS: Control sesame sprouts; GAE: Gallic acid equivalent.

et al. 2006). The inhibition of leishmanolysin may contribute to a general perturbation of *Leishmania*, making the parasite more vulnerable. The bioinformatics analysis for leishmanolysin and PC showed pinoresinol dihexoside, sesaminol 2-O-triglucoside, isoverbascoside, and apigenin as the PC with higher binding free energies than the leishmanolysin substrate (Table 1). Furthermore, protocatechuic acid was the only PC that showed a hydrogen bond with an amino acid of the active site (His264), whereas pinoresinol dihexoside and isoverbascoside presented interactions with three of the four active site residues (Table 2, Supplementary Material S2 and S4).

PC and CPB

CPB is a zinc-dependent metalloprotease found on the surface of *Leishmania* promastigotes, responsible for positively regulating the expression of leishmanolysin (Casgrain et al. 2016, Mercado-Camargo et al. 2020). Pinoresinol dihexoside and sesaminol 2-O-triglucoside presented the strongest binding free energy values with CPB, −8.7 and −8.5 kcal/mol, respectively; all the PC showed higher or the same value than the CBP substrate (−4.8 kcal/mol) (Table 1). The six PC evaluated interacted with Cys26 and His164, residues of the enzyme active site; however, only sesaminol 2-O-triglucoside and the protocatechuic acid interactions were through hydrogen bonds (Table 2, Supplementary Material S2 and S5). These findings are consistent with the study of the inhibitory enzymatic effect of compounds derived from thiosemicarbazone against *L. mexicana* CPB, where this effect was related to the interaction with the residue Cys26 (Schröder et al. 2013). In our analysis, all the PC interacted with this amino acid with powerful binding-free energies, profiling the potential of BS and CS extracts as structural disruptors of *L. mexicana* and potential inhibitor candidates for leishmanolysin expression.

PC and GPDH

During parasite glycolysis, NADH is generated in the cytosol by the oxidation of glycerol-3-phosphate (G3P). Although the mitochondrial membrane is impermeable to NADH, this molecule is needed to be introduced through the G3P shuttle; the reaction involved in this process is catalyzed by GPDH (Marché et al. 2000). The molecular docking results showed that all PC obtained higher binding free energies with GPDH than amphotericin B (−6.2 kcal/mol) and the GPDH-enzyme substrate (−5.8 kcal/mol) (Table 1). Apigenin obtained the highest binding free energy (−9.5 kcal/mol), with a strong docking with all the active site amino acids, two hydrogen bonds with Lys125 and Thr267, and other interactions with Lys210 and Asp263 (Table 2, Supplementary Material S2 and S6); followed by pinoresinol dihexoside with −9.1 kcal/mol. Furthermore, five PC interacted with active site amino acids of GPDH (Table 2). An analysis of 352 PC with GPDH of *L. mexicana* demonstrated the value of this enzyme in antileishmanial activity (Ogungbe et al. 2014), also; similar results were reported by Ogungbe et al. (2013), where the alkaloid 13-oxocardiopetamine docked with *L. mexicana* GPDH with a hydrophobic interaction with active site amino acid Lys125.

PC and PK

PK is an enzyme of the energy metabolism of *Leishmania*; in the last stage of the glycolytic pathway, PK transforms phosphoenol-pyruvate, and ADP into pyruvate and ATP, as glycolysis is the only source of energy generation, this enzyme is considered essential for the survival of these parasites (Chawla and Madhubala 2010, Amiri-Dashatan et al. 2021). All PC presented binding free energies with PK equal to or higher than phosphoenol-pyruvate (−5.6 kcal/mol) and amphotericin B (Table 1). Noteworthy, sesaminol 2-O-triglucoside and the isoverbascoside presented the highest binding free energy values (−9.4 kcal/mol) because of the interaction with active site amino acids of PK, Met50, and Gln297; and Asp264, respectively (Table 2, Supplementary Material S2 and S7).

Altogether, molecular docking analysis showed apigenin, isoverbascoside, pinoresinol dihexoside, and sesaminol 2-O-triglucoside as the PC with higher binding free energy values than their substrates for all enzymes evaluated, resulting in better stability for the possible complexes formed. In addition, pinoresinol dihexoside, sesaminol 2-O-triglucoside, and sometimes arginase showed higher binding free energies than the control leishmanicidal drug amphotericin B. Isoverbascoside was the only PC that interacted with the active site residues of all enzymes. Although the calculated binding free energies do not always reflect the biological response, the specific ligand-enzyme interactions of the PC contained in BS and CS could be used as a guideline for designing new molecules against leishmaniosis and pave the way for *in vitro* studies.

In vitro growth inhibition of *L. mexicana* promastigotes

The antileishmanial effect of BS, CS, apigenin, cinnamic acid, and protocatechuic acid was evaluated in treated *L. mexicana* promastigotes for 48 h. All treatments showed a reduction in proliferation compared to the negative inhibition control. No viable parasites were detected at 0.97 mg/mL (IC_{50} = 0.08 mg GAE/mL) and 3.02 mg/mL (IC_{50} = 0.37 mg GAE/mL) of BS and CS extracts (Table 3), respectively. The promastigotes treated with the BS extract were observed with a series of morphological alterations such as rounding and reduction of the parasite body, loss or shortening of the flagellum, and cell aggregation (Fig. 1a and b) as compared to control cultures (Fig. 1c). On the other hand, apigenin was the best individual compound with leishmanicidal activity with an IC_{50} value of 6.42 μM (Table 3), followed by cinnamic acid (IC_{50} = 382.22 μM). In contrast, higher protocatechuic acid concentrations were insufficient to eliminate at least 50% of parasites.

Both BS and CS extracts were able to eliminate the parasite; however, differences between IC_{50} values were observed that could be attributed to the distinct PC profile and individual compound quantity induced by the biostimulation and germination conditions. The research on the antileishmanial activity of plant extracts and phytochemicals

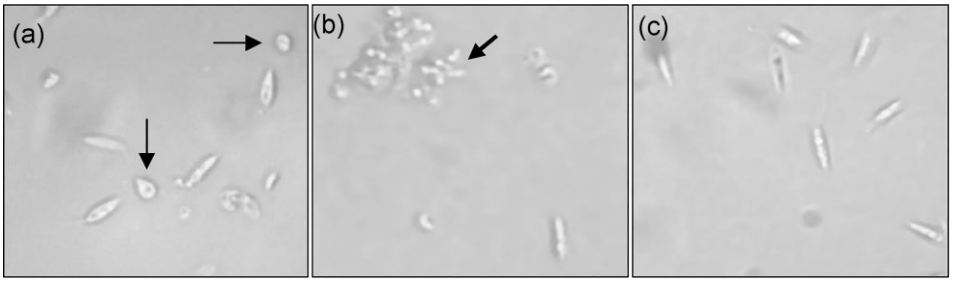

Figure 1. Effect of BS phenolic extracts on *L. mexicana*. Bright-field microscopic image of cells (40 × magnification) treated with BS extracts (3.02 mg of GAE/mL) after 48 h (a and b) and nontreated *Leishmania* (c). Arrows indicate morphology alterations such as reduction of the parasite body and flagellum loss (a) and cell aggregation (b).

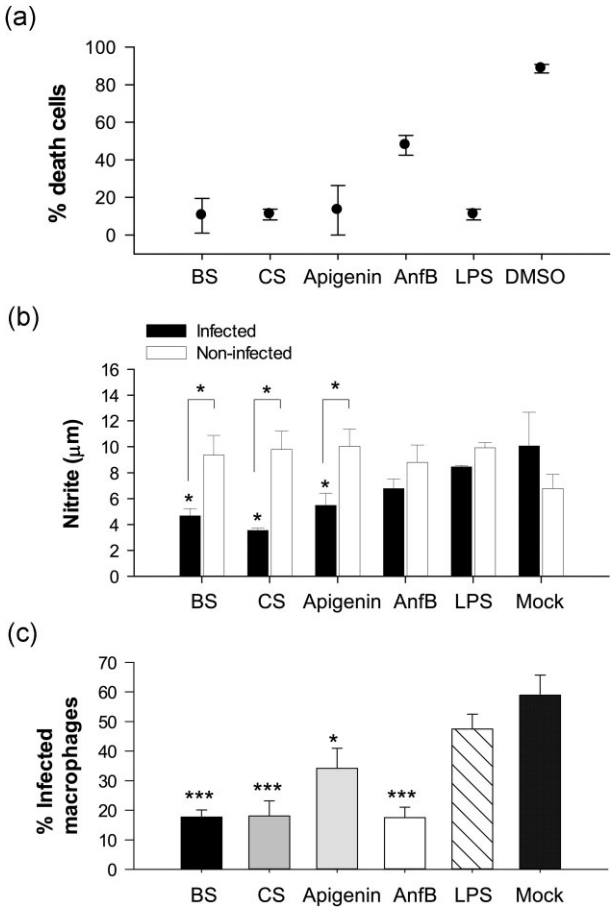

Figure 2. Effect of BS phenolic extracts on *L. mexicana*-infected macrophages. (a) Cytotoxic effect of phenolics extracts, apigenin, and control treatments on macrophages exposed for 24 h. Data represent the mean ± SE of three independent experiments. (b) Nitrite production of cells treated as described above and infected with *L. mexicana* during 6 h. Data represent the mean ± SE of two independent experiments. Infected (■), noninfected (□). (c) Percentage of *Leishmania*-infected macrophages for all treatments. The data represent the mean ± SE of three random microscopic fields/treatment of three independent experiments. $^{***}P < 0.001$, $^{*}P < 0.05$ compared to mock-treated cells or between groups. BS: biostimulated sprout extract; CS: control sprout extract; AnfB: amphotericin B; LPS: lipopolysaccharide; Mock: mock-treated cells.

has been extensive in species such as *Leishmania infantum*, *Leishmania tropica*, and *Leishmania major*, but to a lesser extent in *L. mexicana* (Chacón-Vargas et al. 2022, Hassan et al. 2022). Hence, comparisons between species and their effective compounds and concentrations could differ, in addition to *Leishmania* species-differential mechanisms of survival and immune evasion (Jafarzadeh et al. 2019). Antwi et al. (2019) analyzed the inhibitory effect of PC on *Leishmania donovani* promastigotes and determined an IC_{50} value of 22.77 ± 0.01 μM for apigenin, a higher concentration as compared to our study on *L. mexicana* promastigotes. However, they reported the inhibitory potential of protocatechuic acid (IC_{50} value of 72.54 μM); unlike our study, at a concentration of 973.26 μM, this acid showed a proliferation inhibition only of 3.27%. Tomiotto-Pellissier et al. (2021) evaluated the leishmanicidal effect of phenolic extracts from *Caryocar coriaceum* pulp and peel against *Leishmania amazonensis*. They attributed the antipromastigote effect to PC, including quercetin, which shares structural characteristics with apigenin. Also, they observed parasites with reduced cell length and flagellum loss, as we observed in our study.

The mechanism of action of most PCs on *Leishmania* has yet to be fully elucidated. The *in silico* data suggested the PC could interact with key targets of *Leishmania* related to their growth, proliferation, structure, leishmanolysin expression, and energetic metabolism. Individual compounds such as apigenin, only identified in BS extracts, have shown inhibition of parasite proliferation, distorted cell membrane, increased reactive oxygen species, *trans*-Golgi network rupture, cytoplasmic vacuolization, and mitochondrial damage that could be associated with apoptotic cell death in *L. donovani* and *L. amazonensis* (Fonseca-Silva et al. 2015, Antwi et al. 2019). Furthermore, cinnamic acid derivates, including isoverbascoside, inhibited *in vitro* recombinant *L. amazonensis* arginase and promastigotes with impaired cytokinesis and apoptosis (da Silva et al. 2019, Rodrigues et al. 2019).

Effect of BS extracts on macrophage viability

The cytotoxic effect of BS and CS extracts was evaluated in RAW264.7 macrophages for 24 h. The concentrations evaluated were selected to warrant leishmanicidal activity as determined by the *in vitro* inhibition assays. Cell viability was maintained at 90% with both extracts and mock-treated macrophages (Fig. 2a), suggesting these extracts were noncytotoxic. Meanwhile, apigenin-treated cells showed variability, but cell viability was above 75%. The control compound amphotericin B exhibited some level of cytotoxic effect.

Sesame extracts' effect on infected macrophages

The ability of sesame extracts and apigenin to help the macrophage to diminish or clear *Leishmania* infection was evaluated through NO production and parasite burden.

Sesame extracts-mock-infected cells showed no interference in NO production (Fig. 2b). BS-, CS-, and apigenin-treated and infected macrophages exhibited significant ($P < 0.05$) nitrite reduction (45%–50%) as compared to mock-treated infected macrophages (Fig. 2b). In contrast, the number of infected macrophages significantly decreased (Fig. 2c, $P < 0.05$). Meanwhile, infected macrophages treated with amphotericin B exhibited a nitrite reduction to a lesser extent ($P = 0.07$) but with a significant reduction ($P < 0.001$) in the number of infected cells. LPS-activated cells decreased NO production by 14% but exhibited a high number of infected macrophages (47.5%), apparently unable to efficiently control parasite infection compared to the extract-treated macrophages.

To establish infection and promote parasite survival, *Leishmania* alters different macrophage functions, including a reduction in NO production (Hassani et al. 2011). *Leishmania*-infected macrophages previously treated with BS-, CS-extracts, amphotericin B, and apigenin exhibited reduced nitrite production; therefore, parasite survival was expected. However, few and damaged parasites were present, suggesting *Leishmania* control over NO production was not sufficient to prevent the macrophage from being able to eliminate the parasites. Our results suggest that BS and CS extracts enhanced the macrophage leishmanicidal capacity similar to amphotericin B-treated cells. This activity could be related to other microbicidal mechanisms, including the activation of signaling pathways of innate immune responses, antimicrobial reactive oxygen, and proinflammatory cytokines (Liu and Uzonna 2012). Also, NO production for parasite elimination in our *in vitro* model could have been induced in the early stages, in addition to other factors such as extract composition, the timing of macrophage stimulation, and extract pinocytosis and accumulation in vacuoles that could interfere with diverse cell functions. Interestingly, we were not the first study to observe this phenomenon; the reduction of NO production in cells with high leishmanicidal activity and low parasite burden has also been detected in *L. mexicana*-infected murine dendritic cells treated with IFN-γ+LPS for 24 h (Wilkins-Rodríguez et al. 2010).

Several studies have been conducted on infected macrophages treated with apigenin. In a model of *L. amazonensis*-infected macrophages, apigenin induced reactive oxygen species production and increased macrophage autophagosomes. *In vivo*, apigenin was able to reduce parasitic load with no toxicity (Fonseca-Silva et al. 2016). In mice infected with *L. infantum*, apigenin demonstrated a clearance of 99.7% in liver parasite load with no toxicological alterations (Emiliano and Almeida-Amaral 2023).

Conclusion

In summary, the molecular docking data revealed that PCs from biostimulated sesame sprouts possibly inhibited essential survival and proliferation enzymes of *L. mexicana*. *In vitro* studies in promastigotes confirmed the extracts or individual PC leishmanicidal potential, and the macrophage model proved their strong effect on microbicidal activity. Future *in vivo* and *in vitro* studies must reveal a more detailed understanding of the mechanism of action of this leishmanicidal activity. This study has provided helpful evidence to provide a framework for further phytochemical investigations for alternative products that could be combined with existing drugs.

Supplementary data

Supplementary data is available at *LAMBIO Journal* online.

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Author contributions

Karime G. Garduño-Félix (Conceptualization [equal], Data curation [lead], Formal analysis [equal], Writing – original draft [equal]), Jesús J. Rochín-Medina (Conceptualization [equal], Formal analysis [equal], Software [lead], Validation [equal], Writing – original draft [equal], Writing – review & editing [equal]), Carolina C. Murua-López (Data curation [equal], Formal analysis [equal], Investigation [equal], Methodology [equal], Writing – original draft [equal]), Héctor S. López-Moreno (Conceptualization [equal], Supervision [equal], Validation [equal], Writing – original draft [equal], Writing – review & editing [supporting]), and Karina Ramírez (Conceptualization [lead], Formal analysis [lead], Funding acquisition [lead], Investigation [supporting], Methodology [equal], Project administration [lead], Supervision [lead], Writing – original draft [lead], Writing – review & editing [lead]).

Data availability statement

The data underlying this article will be shared on reasonable request to the corresponding author.

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