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IN VIVO AND *IN VITRO* ANTILEISHMANIAL EFFECTS OF METHANOLIC EXTRACT FROM BARK OF *BURSERA APTERA*

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Abstract

Background: Cutaneous leishmaniasis lacks effective and well-tolerated treatments. The current therapies mainly rely on antimonial drugs that are inadequate because of their poor efficacy. Traditional medicine offers a complementary alternative for the treatment of various diseases. Additionally, several plants have shown success as anti-leishmanial agents. Therefore, we sought to evaluate the *in vitro* and *in vivo* activity of MEBA against *Leishmania mexicana*.
Materials and Methods: Methanolic extract of *B. aptera* was obtained by macetration, after we determined *in vitro* anti-leishmanial activity of MEBA by MTT assay and the induced apoptosis in promastigotes by flow cytometry. To analyze the *in vivo* anti-leishmanial activity, we used infected mice that were treated and not treated with MEBA and we determined the levels of cytokines using ELISA. The phytochemical properties were determined by CG-MS and DPPH assay.
Results: We determined of LC_{50} of 0.408 mg/mL of MEBA for *in vitro* anti-leishmanial activity. MEBA induced apoptosis in promastigotes (15.3% ± 0.86). Treated mice exhibited smaller lesions and contained significantly fewer parasites than did untreated mice; in addition, we found that IFN-γ and TNF-α increased in the sera of MEBA-treated mice. GC-MS analysis showed that podophyllotoxin was the most abundant compound. Evaluation of the activity by DPPH assay demonstrated an SC_{50} of 11.72 µg/mL.
Conclusion: Based on the above data, it was concluded that MEBA is a good candidate in the search for new anti-leishmanial agents.

Key words: *Leishmania, cutaneous leishmaniasis, traditional medicine*.

Introduction

Leishmaniasis is an important public health problem in 98 endemic countries, with more than 350 million people at risk (Reveiz et al., 2013; World Health, 2010). Leishmaniasis has traditionally been classified into three major forms based on clinical symptoms (Handman, 2001). The deadliest form is visceral leishmaniasis (VL), which, if left untreated, leads to death. A number of other species of *Leishmania* cause cutaneous (CL) and mucocutaneous (MCL) leishmaniasis, which, if not fatal, are still responsible for the considerable morbidity of a large number of people in endemic foci (Prasad, 1999). The WHO estimated an incidence of 2 million new cases per year (0.5 million of VL and l.5 million of CL (World Health, 2010). Pentavalent antimony, which was discovered nearly one hundred years ago, continues to be the first line of treatment against leishmaniasis. However, toxic side effects, the long hospitalization required for parenteral administration, and the emergence of drug resistance are obstacles in antimony therapy (Chakravarty and Sundar, 2010). In recent years, an unprecedented increase in the unresponsiveness to sodium antimony gluconate, the first line of treatment, has necessitated the use of other toxic drugs, such as amphotericin B, pentamidine, paromomycin, and allopurinol (Chakravarty and Sundar, 2010; Mishra et al., 2007). In the ongoing search for better leishmanicidal compounds, many candidates have been evaluated (Akendengue et al., 1999; Kayser et al., 2003). We focused on studying the biological and phytochemical

properties of plants used in traditional Mexican medicine, such as *Bursera aptera* Ramirez, which is endemic to Mexico and has a wide distribution (Guerrero, Morelos, Oaxaca, Puebla,) (Rzedowski, 2005).

Recently, our team found that *B. aptera* has antibacterial, antifungal and antiprotozoal activities (Rodriguez-Canales, 2014; Rodriguez-Lopez, 2014) and may possess antileishmanial activity. Among the various mechanisms for mediating parasiticidal activity, programmed cell death (PCD) appears to be the most appropriate because this is the most studied mechanism in kinetoplastids in response to various stimuli chemotherapeutic agents (Szallies et al., 2002). Therefore, the aim of this work was to evaluate the antileishmanial activity of the methanolic extract of *B. aptera* and to characterize the mechanism of cell death observed.

Methods

Plant material

B. aptera was collected in October 2013 in San Rafael, Coxcatlan, Puebla. A voucher specimen was deposited at the Izta Herbarium at Facultad de Estudios Superiores Iztacala (IZTA HCM46), and the botanical authentication of the specimen was performed by M. D. Maria Edith Lopez Villafranco (curator at the IZTA Herbarium).

Preparation of methanolic extract from *B. aptera* (MEBA)

The bark extract was obtained by maceration. *B. aptera* bark (2.516 kg) was placed in a flask with methanol and exhaustive extraction was performed. The extract was filtered and then distilled under reduced pressure in a rotary evaporator. The extract was placed in glass containers until the evaporation of the solvent was complete (Domínguez, 1973).

Parasite culture and maintenance

L. mexicana infection (MNYC/BZ/62/M379) was maintained by passage in BALB/c mice. Amastigotes were isolated, differentiated to promastigotes and maintained in Schneider's medium, pH 7.4, supplemented with gentamicin (GIBCO) (100 mg/mL) and 10 % heat-inactivated foetal bovine serum (FBS).

Animals

In the present study, female BALB/c mice (6–8 weeks of age) were used. The mice used throughout this study were maintained in controlled environmental conditions and light–dark cycles (12:12 h) in filter-topped cages, provided with sterile food and water ad libitum and cared for according to the guidelines of the Federal Regulations for Animal Experimentation and Care (NOM-062-ZOO-1999, Ministry of Agriculture, Mexico), which were approved by the Institutional Ethics Committee of the Universidad Nacional Autonoma de Mexico – Facultad de Estudios Superiores Iztacala.

Evaluation of the anti-promastigote potential of MEBA

To evaluate MEBA's effects on the promastigotes of *L. mexicana*, colorimetric cell viability MTT assay was used as described elsewhere (Dutta et al., 2005). Briefly, promastigotes (2.5×10^4/100 µL/well) harvested from the logarithmic growth phase were added to a tissue culture plate. Then, 100 µL of various concentrations of MEBA, sodium stibogluconate (Stb) (Albert David Limited) (4-0.01 mg/mL) and amphotericin B (AmpB) (Sigma) (60-0.29 µg/mL) was added to each well and incubated at 25°C for 48 h. After incubation, 50 µL of MTT solution (2 mg/mL) was added to each well and incubated at 37°C for 4 h. Then, the plates were centrifuged (2000 rpm×5 min, 25°C). The supernatant was removed, and cold DMSO (200 µL) was added as a solvent for the formazan crystals to produce a purple colour. The absorbance was measured for each well at 492 nm using a Multiskan Spectrum Microplate reader (Thermo Scientific). All experiments were carried out in triplicate. The 50 % lethal concentrations (LC_{50} values) were determined by graphical extrapolation.

Externalized phosphatidylserine in *L. mexicana* promastigotes

The 2×10^6 exponential-phase *L. mexicana* promastigotes were incubated with MEBA (0.408 mg/mL, 1 h). After being pipetted gently, the cells were centrifuged for 5 min at 2500 rpm. The supernatant was removed, and cells were stained with Annexin V-FITC and propidium iodide (PI) according to the manufacturer's instructions (Molecular Probes). If the membrane integrity was altered (due to the externalization of phosphatidylserine), annexin V detected both early- and

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late-apoptotic cells. Therefore, the simultaneous addition of PI, which does not enter healthy cells with an intact plasma membrane, discriminates between early-apoptotic (annexin V-positive and PI-negative), late-apoptotic (both annexin V and PI-positive), necrotic (PI-positive) and live (both annexin V and PI- negative) cells (Kaur et al., 2009). Untreated promastigotes were used as controls for double staining. Data acquisition was performed on a FACS Calibur flow cytometer (BD) and analyzed using the WinMDI 2.9 software. For each measurement, at least 50,000 cells were counted. Statistical analysis was carried out on an Apple Macintosh computer using Prism 6 (GraphPad Software, San Diego, CA, USA). The significance of differences was determined with the unpaired *t* test (two-tailed) with Welch's correction, and coefficients of correlation were calculated by Spearman's rank-correlation analysis. $P < 0.05$ was considered statistically significant.

Measurement of Mitochondrial Membrane Potential

JC-1 is a cationic, positively charged fluorescent dye that exhibits potential-dependent accumulation in mitochondria, as indicated by a fluorescence emission shift from green (525 nm) to red (590 nm). Consequently, mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio (Raju et al., 2013). The assay was performed following the manufacturer's instructions (Molecular Probes). Promastigotes were treated for 1 h with MEBA (0.408 mg/mL) after the cells were collected and incubated for 10 min with 10 µM of JC-1 dye in 1 mL PBS at 37ºC. Cells were immediately analyzed by a flow cytometer (FACS Calibur and WinMDI 2.9 software) and were gated using the appropriate settings in FL1 (green) and FL2 (red) channels. As a positive control, cells were treated with the mitochondrial membrane disrupter CCCP (carbonyl cyanide 3-chlorophenylhydrazone) (50 mM) for 5 min at 37°C. For each treatment condition, at least 50,000 cells were counted and statistically analyzed.

***In vivo* anti-leishmanial effect on experimental cutaneous leishmaniasis**

Activity of MEBA on lesion size. BALB/c mice received subcutaneous injections in the back rump with 5×10^6 stationary-phase *L. mexicana* promastigotes and were randomly divided into three groups of six mice each. The groups were as follows: 1, untreated; 2, treated with MEBA (200 µL) dissolved in a surgical gel at a concentration of 0.408 mg/mL; and 3, treated with 200 µL of surgical gel without MEBA (vehicle). The development of the lesion was monitored and measured with a digital caliper (Mitutoyo, Kanagawa, Japan) weekly during the course of infection (8 weeks). The significance of differences was determined with multiple *t* tests (one per row). $P < 0.05$ was considered statistically significant.

Parasite burden by limiting dilution assay. At the eighth week after infection, the animals in each group were killed by cervical dislocation to assess the parasite number in each lesion using the culture microtitration method. Forty-eight days' post-infection mice from each group were picked randomly to assess the parasite number in each lesion. Parasite numbers in the lesions were determined by limiting dilution assay, as described previously (Wong et al., 2014). Under sterile conditions, the lesion-bearing back rump was removed, cut into small pieces, and re-suspended in supplemented Schneider's medium. The area of injury was homogenized in a glass homogenizer. The cell suspensions were serially diluted in 10-fold dilutions (from 1:10 to 1:1 × 10) in a 96-well flat-bottomed tissue culture plate in triplicate. The plate was incubated at 27°C for 48 h and then examined under an inverted microscope to determine the presence or absence of mobile promastigotes. The final titer was defined as the last dilution at which at least one well contained no parasite and was expressed as log parasite titers ± SD. Statistical analysis was carried out using Prism 6. The significance of differences was determined with unpaired *t* tests (two-tailed) with Welch's correction, and $P < 0.05$ was considered statistically significant.

Determination of cytokine levels in serum from mice infected with *L. mexicana*

The serum of infected mice with *L. mexicana* treated and not treated with MEBA were obtained at days 0, 15, 30, 45 and 60 after infection. The levels of IFN-γ, TNF-α, IL-4 and IL-10 in serum were determined by sandwich ELISA according to the manufacturer's instructions (PEPROTECH). Briefly, 96-well flat-bottom plates (Nunc, Thermo Scientific) were coated with primary capture antibody for 2 hours at room temperature. Then, plates were washed with PBS-Tween (0.05 % Tween 20 in PBS, pH 7.4) and blocking buffer (1 % BSA in PBS) was for 2 hours at room temperature. The plates were then incubated with 50 µL of serum (in triplicate) or serially diluted recombinant cytokine (in duplicate) for 2 hours at room temperature. The plates were washed and incubated with biotinylated detection antibody against specific cytokines for 2 hours at room temperature and were then washed and incubated with avidin-HRP conjugate for 30 minutes at room temperature in the dark. Finally, plates were washed before the addition of ABTS. Absorbance was read at 405 nm using a Bio-Tek EL800 plate reader (Bio-Tek), and the cytokine levels were quantified against a standard curve.

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Antioxidant Activity (DPPH decolouration assay)

The antioxidant activity was determined according to the method described by (Okusa et al., 2007). Ninety-six-well ELISA plates were filled with extract concentrations ranging from 1-100 µg/mL. HPLC-grade methanol served as a blank sample, and a 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution (100 µM) served as a control. The plates were incubated for 30 min at 37°C, and the absorbance values were determined at 540 nm with an ELISA plate reader. The antioxidant activity value was resolved according to the following equation: % inhibition = [(absorbance of control-absorbance of sample)/ absorbance of control] *100. The concentration leading to a scavenging activity 50 % (SC_{50}) was determined graphically. Quercetin was used as a standard (positive control).

Total Phenolic Content

The total phenolic (TPC) content was determined by Folin–Ciocalteu reagent (Lobo et al., 2011). Briefly, 75 µL of diluted extract and 425 µL of distilled water were added to 500 µL Folin–Ciocalteu reagent and 500 µL of Na_2CO_3 (10 % w/v). The mixture was mixed and incubated for 1 h in the dark at room temperature. After incubation, the absorbance was measured at 760 nm using a UV–Vis spectrophotometer (DU 640 Spectrophotometer, Beckman, Brea). The total phenolic content was expressed as mg gallic acid equivalent (GAE)/ g of extract.

Total Flavonoid Content

The Dowd method, as adapted by (Ramamoorthy and Bono, 2007), was used to determine the flavonoid content. A solution of 2 % aluminum trichloride ($AlCl_3$) in HPLC-grade methanol and an extract concentration of 0.2 mg/mL were used. Readings at 415 nm using a spectrophotometer were taken after 10 minutes against a blank sample (5 mL extract solution and 5 mL methanol without $AlCl_3$). A quercetin (1-100 µg/mL) calibration curve was used as the standard. The mean of three readings was expressed as mg of quercetin equivalent QE/g of extract.

Analysis of the Chemical Composition by GC-MS

The chemical components of the MEBA were identified with an HP 6890 gas chromatograph using an HP 5973 mass selective detector equipped with Chemstation and Wiley 275 Spectra Data software. An HP-5 5 % Phenyl Methyl Siloxane capillary column (30 m x 0.32 mm; 0.25 µm thickness) was used. The chromatographic conditions were as follows: column temperature 70°C (0.5 min), 70-150°C (15°C/min), 150-220°C (20°C/min), 220-310°C (25°C/min) 310°C (5 min), interface 180°C; split ratio 1:10 carrier gas, He (3.43 Psi), flow rate 1.6 mL/min; ionization energy 70 eV; mass range 30-550; volume injected 2.0 µL. Identification of individual components in MEBA was done using the GC-MS (NIST 2008 and Wiley 8NO8) spectral libraries.
Separated compounds were identified using NIST chemical library.

Results

Of the total weight of bark (2.516 Kg), the extraction yield of *Bursera aptera* bark was 2.37% (59.78 g) of dry extract. MEBA was dissolved in Schneider's medium (Sigma) for the *in vitro* interactions.

***In vitro* effect of MEBA on *L. mexicana* promastigotes.**

The treatment of promastigotes with MEBA demonstrated a dose-dependent inhibition of parasite growth and an LC_{50} value in promastigotes of 0.408 mg/mL, whereas the values of the reference drugs Stb and AmpB were evaluated as 0.203 mg/mL and 0.133 µg/mL, respectively.

MEBA-treated promastigotes show both annexin V and PI binding

Flow cytometric evaluation of the externalization of phosphatidylserine, a membrane-associated phospholipid, using Annexin-V/FITC and PI, was presented in Fig. 1. The percentage of early- and late-apoptotic cells increased from 0.64 % ± 0.08 in the untreated group to 15.3 % ± 0.86 in MEBA-treated group (0.408 mg/mL) for 1 h; similarly, the percentage of necrotic cells increased from 0.43 % ± 0.02 in the untreated group to 17.2 % ± 0.59 in the MEBA-treated group.

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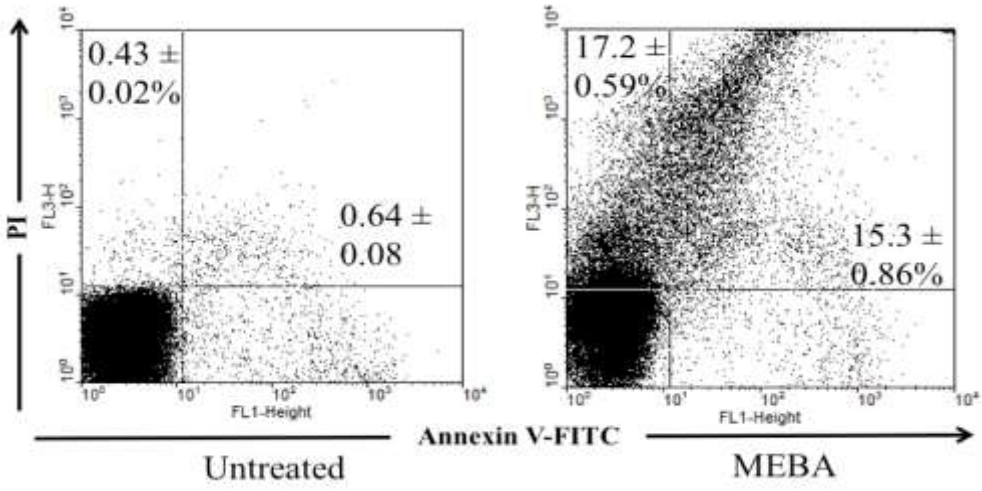

Figure 1: Analysis of externalization of phosphatidylserine in promastigotes treated with MEBA. *L. mexicana* promastigotes were incubated with MEBA (0.408 mg/mL) for 1 h, stained with PI and annexin V–FITC and analyzed by flow cytometry. As observed, MEBA promotes the externalization of phosphatidylserine in treated promastigotes, which indicated that the leishmanicidal activity of MEBA occurs primarily via apoptosis. Dotplots shown are from representative experiments. Numbers represent the mean percentage values of expression ± SE of five independent experiments. $P < 0.05$, unpaired *t* test with Welch's correction.

MEBA induces the depolarization of mitochondrial transmembrane potential in promastigotes

Treatment with 0.408 mg/mL (1 h) resulted in the reduction of mitochondrial membrane potential in relation to the control, which may be due to imperfect mitochondrial function (Fig. 2).

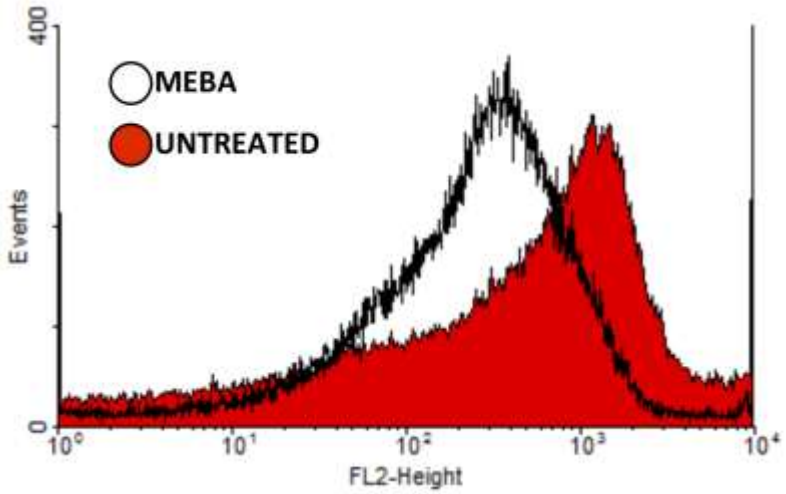

Figure 2: Analysis of changes in the mitochondrial membrane potential of *L. mexicana* promastigotes treated with MEBA for 1 h, followed by staining with JC-1 and flow cytometry analysis. Histogram showing a reduction in JC-1 red staining in the parasite population treated with MEBA compared to that in untreated parasites. Treatment with MEBA caused a loss of the membrane potential, which reduced the red staining of the parasite. Histograms shown are from representative experiments.

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Activity of MEBA in the lesion size and quantitation of parasite burden in experimental cutaneous leishmaniasis

To evaluate the *in vivo* effects of MEBA in cutaneous leishmaniasis, BALB/c mice were infected with *L. mexicana* in the back rump and treated daily with MEBA (topical administration) or vehicle. MEBA-treatment mice did not exhibit an increase in lesion size compared with untreated mice (Figure 3). MEBA treatment also caused an important reduction in the number of parasites compared with untreated mice, and the infected lesions from untreated mice contained significantly more parasites (9.6 ± 0.175) than those from treated mice (6.8 ± 0.247) (Fig. 4). Taken together, these findings indicate that MEBA can serve as an anti-leishmanial agent.

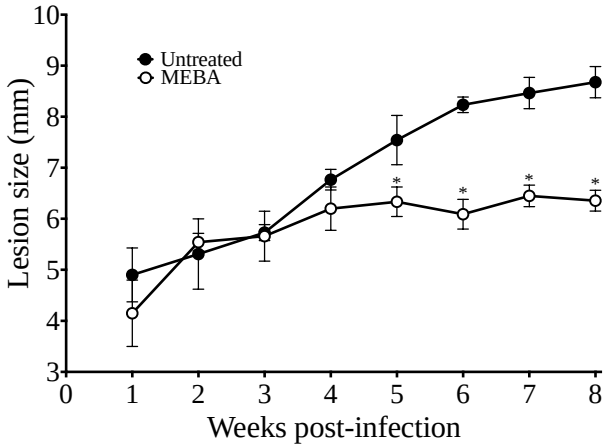

Figure 3: Evaluation of the effect of MEBA on lesion size. Untreated mice developed large lesions (●), while MEBA-treated mice showed a significant absence of the increase in the size of the lesions (○). From the fourth week, the size of lesions in MEBA-treated mice decreased significantly in relation to the control. The significance of differences was determined with multiple *t* tests (one per row). * $P < 0.05$ was considered statistically significant.

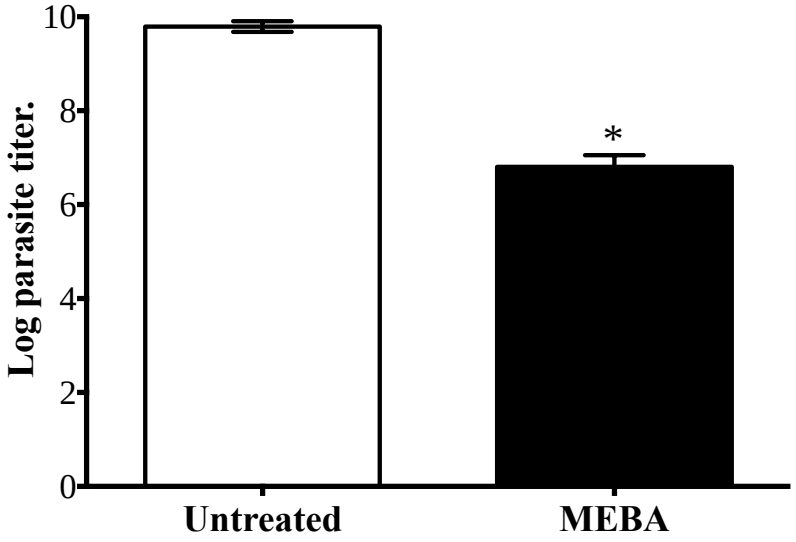

Figure 4: Parasite burden was determined at 8 weeks following infection with *L. mexicana* by limiting dilution. Data are expressed as the mean log dilution (n = 6). Significant differences in the number of parasites in the lesions of treated and untreated mice (6.8 ± 0.247 and 9.6 ± 0.175, respectively) were observed. As shown, these findings indicate that MEBA has anti-leishmanial activity against *L. mexicana*. Untreated mice developed large ulcerative lesions full of parasites,

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whereas the mice treated with MEBA developed small lesions with few parasites. The significance of differences was determined with unpaired *t* tests (two-tailed) with Welch's correction. *$P < 0.05$ was considered statistically significant.

Mice treated with MEBA during infection with *L. mexicana* showed an increase in levels of IFN-γ and TNF-α

The results obtained by ELISA showed that mice treated with MEBA developed a significant increase in the production of IFN-γ and TNF-α during infection with *L. mexicana* in relation to the untreated group. In contrast, the levels of IL-4 and IL-10 in mice treated with MEBA did not increase and remained constant during infection, but in the untreated group, a marked increase in the levels of these cytokines was recorded (Fig. 5).

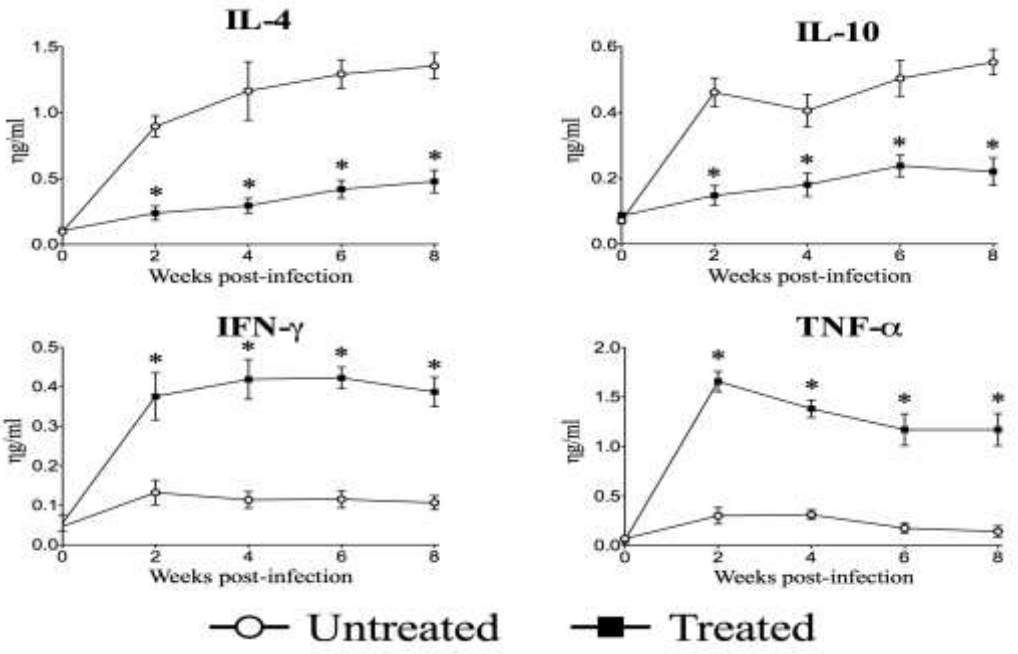

Figure 5: Cytokine levels in sera of mice infected with *L. mexicana* by ELISA sandwich assay. In treated mice, a significant increase in IFN-γ and TNF-α was observed compared with the untreated group, whereas IL-4 and IL-10 showed a significant decrease in relation to the untreated group. The significance of differences was determined with multiple *t* tests (one per row). * $P < 0.05$ was considered statistically significant.

Total polyphenolic and flavonoid content and antioxidant activity

The total polyphenolic content (TPC) of MEBA was determined by Folin–Ciocalteu assay. According to the literature, phenols are the main compounds responsible for the antioxidant activity; for this reason, MEBA was analyzed (Scherer and Godoy, 2014). The results showed that MEBA displayed a TPC of 61.2 mg GAE/g. MEBA exhibited 15 mg QE/g of flavonoids. *In vitro* antioxidant tests using free radical traps are relatively straightforward to perform. Among the free radical scavenging methods, the one involving DPPH is rapid, simple, highly reproducible and inexpensive in comparison to other test models. The results were expressed as AC_{50} (Table 1). According to the Al-Fatimi criteria (Ramamoorthy and Bono, 2007), MEBA showed a good DPPH scavenging activity (SC_{50} of 11.72 µg/mL), while quercetin (used as standard) showed an SC_{50} of 4.3 µg/mL.

Table 1: The total polyphenol content (TPC) and total flavonoid content (TFC) of MEBA.

	TPC **mg GAE/g**	**TFC** **mg QE/g**	**DPPH** **AC_{50}** µg/mL
MEBA	61.2	15	11.72

(GAE)/g = mg gallic acid equivalent per gram of dried extract; (QE)/g = mg of quercetin equivalent per gram of dried extract.

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GC-MS analysis

GC-MS analysis revealed the presence of 11 compounds in the methanolic extract of bark from *B. aptera*. The six main compounds were podophyllotoxin, methyl palmitate, oleic acid methyl ester, palmitic acid, clionasterol and kaurene (Table 2).

Table: Compounds in MEBA identified by GC-MS.

No.	Compound	Abundance (%)	Retention time (min)	CAS# of NIST
1	Podophyllotoxin	13.79	16.925	19186-35-7
2	Methyl palmitate	4.93	10.729	112-39-0
3	Oleic acid, methyl ester	4.93	12.183	112-62-9
4	Palmitic acid	4.76	10.960	57-10-3
5	Clionasterol	4.60	17.299	83-47-6
6	Kaurene	4.03	11.720	562-28-7
7	Methyl linoleate	3.59	11.892	112-63-0
8	Stearic acid	3.26	12.278	57-11-4
9	Pyranone	3.18	4.253	28564-83-2
10	Methyl stearate	2.42	12.070	112-61-8
11	Linoleic acid	1.34	12.124	60-33-3

Discussion

Plants of the *Bursera* genus are widely used in traditional medicine, and the anti-leishmanial activity of the methanolic extract of *B. aptera* was reported for the first time in this work. This finding is of great importance because the use of pentavalent antimony drugs, which is the main therapy used to treat leishmaniasis, has multiple toxicities and is becoming increasingly ineffective due to the development of parasite resistance (Roberts, 2005). Natural products are an important potential source of new drugs and alternative therapies (Sulsen et al., 2008).

In this work, we demonstrate the *in vitro* anti-promastigote effect of MEBA on *L. mexicana*, using an MTT assay, wherein the conversion of MTT to formazan by mitochondrial enzymes served as an indicator of cell viability; thus, a decrease in formazan production indicated decreased cell viability (Dutta, et al., 2005), we recorded a LC_{50} value of 0.408 mg/mL. These results were to be expected because MEBA is a mixture of compounds and the drugs are pure compounds. The *in vitro* antileishmanial activity is consistent with previous reports using other species of this genus, such as *B. graveolens* (36.7 ± 4.7 µg/mL) and *B. simaruba* (163.3 ± 1.8 µg/mL) against amastigotes of *L. amazonensis*; however, these studies were tested with essential oil (Garcia et al., 2012) and ethanolic extract of the leaves of the plant (Monzote et al., 2012). Furthermore, it was found that ethanolic extract of leaves from *B. fagaroides* inhibits 50 % of the activity of ornithine decarboxylase (ODC) at a concentration of 8 mg/mL (Rosas-Arreguin et al., 2008). ODC catalyzes the decarboxylation of ornithine to produce putrescine and is essential for biosynthesis, polyamines and trypanothione of parasitic protozoa and may be a potential target for the development of new drugs against *Leishmania* (Yadav et al., 2015).

However, the analysis of the antioxidant activity and total polyphenolic and flavonoid content showed that there was a relationship between the chemical composition of the extract as detected by GC-MS and the biological activities evaluated. For example, the presence of dead cells by apoptosis and necrosis may be due to the compounds found in the extract, such as podophyllotoxin. This compound registered a greater abundance in the GC-MS. We found that the extract promotes the externalization of phosphatidylserine and a loss of mitochondrial membrane potential, these events are characteristic during programmed cell death (PCD) (Mehta and Shaha, 2004; Sen et al., 2007), and these findings are consistent with those reported by Velez et al. (2014), who reported that podophyllotoxin and its analogues have the ability to generate apoptosis in COLO-205 cells to promote the externalization of phosphatidylserine between 20 % and 50 %. This generates permeability of the mitochondrial membrane. Another compound found in the extract was palmitic acid, which induces mitochondrial pore opening and leads to a decrease of the mitochondrial membrane potential and then to cell

death (Belosludtsev et al., 2006). Additionally, podophyllotoxin has been widely used topically in ethanolic solution and creams in the treatment of skin infections (Gilson et al., 2009; Lacey et al., 2003). This may be related to the anti-leishmanial activity *in vivo*, as the extract prevents the growth of the lesion without causing irritation or skin damage. Furthermore, the presence of other phenolic compounds in the extract, especially flavonoids, is very important to explain the *in vivo* anti-leishmanial activity of MEBA. Our findings show that the size of the lesion and the parasitic load is smaller in MEBA-treated mice than untreated mice. It has been reported that flavonoids isolated from *Selaginella sellowi*, amentoflavone (0.1 µg/mL) and robustoflavone (2.8 µg/mL), have anti-leishmanial activity on amastigotes of *L. amazonensis* (Rizk et al., 2014). In addition, previous reports demonstrated the antileishmanial activity of quercetin, quercetrin and isoquercetrin, as they inhibit recombinant arginase from *L. amazonensis* at an LC_{50} of 4.3, 10.0 and 3.8 µM, respectively (Da Silva et al., 2012). This enzyme is involved in the synthesis of polyamines, which are essential for cell proliferation and the production of trypanothione, which is involved in reactive oxygen species detoxification (Colotti and Ilari, 2011). We found that MEBA inhibited lesion development and parasitic burden when it was administered topically at a 0.408 mg/mL. This is noteworthy because the flavonoid is a pure compound, whereas the extract is a mixture of compounds. In relation to the production of cytokines, it was found that treatment of mice with MEBA promotes the production of proinflammatory cytokines during infection with *L. mexicana*. Increased IFN-γ and TNF-α can also result from a high content of phenolic compounds and flavonoids in MEBA, as there are reports that phenolic compounds, such as tannins and proanthocyanidins, increase proinflammatory cytokine production and inducible nitric oxide synthase expression in RAW 264.7 macrophages infected with *Leishmania* (Kolodziej et al., 2005). Increased IFN-γ and TNF-α in mice treated with MEBA may also be directly related to the *in vivo* anti-leishmanial activity because in response to these cytokines, macrophages become activated and produce microbicidal free radical molecules, such as nitric oxide and reactive oxygen intermediates (Liu and Uzonna, 2012). Our results indicate that in addition to direct leishmanicidal activity against extracellular and intracellular *L. mexicana* parasites, MEBA displays immunomodulatory activities to eliminate intracellular parasites mediated mainly by phenolic compounds as flavonoids and podophyllitoxin. The activity of MEBA makes it a good candidate in the search for new antiprotozoal agents.

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