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In vitro antiparasitic and antibacterial evaluation of organic extracts of Salvadoran flora

[Evaluación antiparasitaria y antibacterial *in vitro* de extractos orgánicos de la flora salvadoreña]

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Abstract: Currently, in developing countries, parasitic and bacterial diseases as amebiasis, giardiasis, trichonomiasis, leishmaniasis, trypanosomiasis, tuberculosis, and nocardiasis are a public health problem. The pharmacological treatment for these diseases is not completely effective and causes several side effects in patients. Therefore, the search for new compounds with biological activity is very important to develop new drugs safely and more efficiently. In this study, different organic extracts obtained from thirty-seven species of the Salvadoran flora were evaluated in several *in vitro* models to determine their potential activity against five protozoa (*Entamoeba histolytica*, *Giardia lamblia*, *Trichomonas vaginalis*, *Leishmania mexicana*, and *Trypanosoma cruzi*) and three bacteria (*Acinetobacter baumanni*, *Mycobacterium tuberculosis*, and *Nocardia brasiliensis*). The results showed the activity of eight extracts with IC_{50} values of less than 100 µg/mL against *L. mexicana* and five extracts with MICs values less than <50 µg/mL against *M. tuberculosis*. Besides, seven plant species showed MICs ≤3.125 µg/mL against *N. brasiliensis*. Additionally, secondary metabolites (flavonoids and monoterpene oxygenate) previously reported as active were fingerprint by UPLC-MS to establish a potential correlation with the biological activity showed.

Keywords: Protozoa; Tuberculosis; Micetoma; Infectious diseases; Secondary metabolites

Resumen: Actualmente, en los países en vías de desarrollo, enfermedades parasitarias y bacterianas como la amebiasis, giardiasis, trichonomiasis, leishmaniasis, tripanosomiasis, tuberculosis y nocardiasis son un problema de salud pública. El tratamiento farmacológico de estas enfermedades no es del todo eficaz y provoca varios efectos secundarios en los pacientes. Por lo tanto, la búsqueda de nuevos compuestos con actividad biológica es muy importante para desarrollar nuevos fármacos, seguros y eficaces. En este estudio se evaluaron diferentes extractos orgánicos obtenidos de treinta y siete especies de la flora salvadoreña en varios modelos *in vitro* para determinar su actividad potencial contra cinco parásitos (*Entamoeba histolytica*, *Giardia lamblia*, *Trichomonas vaginalis*, *Leishmania mexicana* y *Trypanosoma cruzi*) y tres bacterias (*Acinetobacter baumanni*, *Mycobacterium tuberculosis* y *Nocardia brasiliensis*). Los resultados mostraron la actividad de ocho extractos con valores de CI_{50} menores a 100 µg/mL contra *L. mexicana* y cinco extractos con valores de CIMs<50 µg/mL contra *M. tuberculosis*. Además, siete especies de plantas presentaron CIM≤3,125 µg/mL frente a *N. brasilienses*. Finalmente, los metabolitos secundarios (flavonoides y monoterpenos oxigenados) previamente reportados como activos fueron determinados por UPLC-MS para establecer una posible correlación con la actividad biológica mostrada.

Palabras clave: Parásitos; Tuberculosis; Micetoma; Enfermedades infecciosas; Metabolitos secundarios

INTRODUCTION

Despite advances in the development of new drugs of synthetic origin, the plants and their parts continue to be a viable and frequently used option in various countries or regions of the world, to treat different diseases or to have diverse applications in the health, industry, agriculture, among others (Carneiro *et al*., 2012).

Additionally, approximately 60% of the drugs in use have been generated from the secondary metabolites of the plants identified as active with a specific biological activity (Balunas & Kinghorn, 2005, Jiménez-Arellanes *et al*., 2014). Therefore, it is important to continue with isolation, identification, and evaluation of secondary metabolites from medicinal plants. For example, Avila-Blanco *et al*., reported ascaridole a metabolite obtained from the essential oil of *Chenopodium ambrosioides* with antiprotozoal activity (Ávila-Blanco *et al*., 2014). Recently, Blaschke *et al*. (2017), reported 15 isolated sesquiterpenes with *in vitro* cytotoxic activity (anticancer effect) and anti-invasive effects (anti-inflammatory). Moreover, several research groups have evaluated extracts and/or secondary metabolites of plants against protozoa (García *et al*., 2011, Eltayeb & Ibrahim, 2012, Ulloá *et al*., 2017, Grecco *et al*., 2017), bacteria (Ghasemin *et al*., 2019, Xu *et al*., 2019; Bauthong *et al*., 2019; Chabán *et al*., 2019), or even in the current pandemic of COVID-19, medicinal plants are considerer as a potential source of molecules for the treatment or prophylaxis of the severe acute respiratory syndrome produced by coronavirus-2 (Benarba & Pediella, 2020). All this evidence demonstrates that search from plant-derived active metabolites is a viable strategy to develop new antimicrobial agents.

On the other hand, parasitic and bacterial diseases continue to be recurrent public health problems in the world, mainly in developing countries.

Amebiasis is considered also a tropical neglected disease that caused 55,500 deaths per year worldwide, so far, metronidazole is the only drug to choose (Debnath *et al*., 2019). Giardiasis is caused by *Giardia lamblia* however, the resistance to common (metronidazole, tinidazole, and nitazoxanide) antigiardial drugs has increased in recent years (Leung *et al*., 2019). Trichomoniasis is the most prevalent non-viral sexually transmitted disease worldwide (Edwards *et al*., 2016). Leishmaniasis and Chagas disease are tropical diseases caused by protozoan parasites without an effective pharmacological treatment (WHO, 2020). The drugs usually used for the treatment of leishmaniasis are first-line drugs such as sodium stibogluconate, the antimoniate of *N*-methylglucamine and pentamidine which are also administrated in combination with second-line drugs such as pentamidine isothionate, amphotericin B, miltefosine, and paromomycin sulfate, but even so, the efficacy is not optimal (Murray, 2001; Akendengue *et al*., 2002, Getti *et al*., 2009). In addition, these drugs cause severe side effects in patients such as acute pancreatitis, myalgia, peripheral neuropathy, hepatic and cardiac toxicity (Gutiérrez-Rebolledo *et al*., 2017). The drugs used to treat Chagas disease are Nifurtimox (Nfx) and Benznidazole (Bzn) which are not completely effective in the chronic phase of the disease, and present several adverse effects (Rivera *et al*., 2014, Kashif *et al*., 2017).

On the other hand, infectious diseases due to emergence and spread of drug-resistant bacteria are a worldwide problem in public health, for instance, infections caused by *Acinetobacter baumannii* (*A. baumannii*) are a major problem in hospitals all over the world, being associated with nosocomial drug-resistant infections (Nasr, 2019), and *Mycobacterium tuberculosis* (*M. tuberculosis*) is the etiological agent of human tuberculosis, the most important mycobacterial pathogen in terms of global patient numbers and gravity of disease; however, its permanence in the host and the acquired drug-resistance makes its pharmacological treatment extremely long, highly toxic and often inefficient (Le Chevalier *et al*., 2014). Additionally, micetoma caused by *Nocardia brasiliensis* (*N. brasiliensis*) is an opportunistic occupational infectious disease, extremely difficult to treat, that is developed by immune-competent or more frequently by immunocompromised patients. *N. brasiliensis* is a bacteria phylogenetic relational with *Mycobacterium* (Mehrabadi *et al*., 2020).

In El Salvador, approximately half a million people have suffered from infectious diseases associated with parasites and bacteria in 2020 (Ministerio de Salud [MINSAL], 2020).

Based on the foregoing, in this project, thirty-seven commonly used species from Salvadoran flora for treatment of infectious diseases (see annex No. 1), were screened to determine their potential biological activity as antiparasitic and antibacterial. Different plants parts (steam bark, leaves, seed, and whole plant) were used to obtain one hundred fifty-three organic extracts with different solvents like hexane, dichloromethane (DCM), ethyl acetate (AcOEt), and methanol (MeOH) which were tested against five

protozoa: *T. vaginalis, E. histolytica, G. lamblia, L. mexicana* and *T. cruzi*; and against three bacteria: *A. baummani, M. tuberculosis and N. brasiliensis.* From some plants studied in this project, their main metabolites have been previously reported; therefore, a fingerprint Ultra-Performance Liquid Chromatography-Mass Spectrometer (UPLC-MS) analysis was done to determine potentially related metabolites with the showed biological activity.

MATERIALS AND METHODS

Vegetal material samples

Thirty-seven species used in the traditional medicine of El Salvador were collected (see annex No. 1) from June to August 2017. The species were identified by Jenny Menjivar, curator of the Herbarium at the Museo de Historia Natural de El Salvador (MUHNES) and a voucher specimen has been deposited in the Herbarium at the MUHNES.

Preparation of plant extracts

One hundred fifty-three plant part extracts were prepared with 200 g of ground and dry material of each species, separately using 400 mL of hexane, DCM, AcOEt, and MeOH by assisted-ultrasonic extraction in a magnetic stirrer ultrasonic (VWR, USA, model 97043-988, operating frequency at 35 kHz) for 90 minutes at 25°C. Each extract was concentrated under reduced pressure at 40°C to obtain the crude residues.

Giardicidal activity

The strain of *G. intestinalis* IMSS: 0696: 1 was grown in modified medium TYI-S-33, supplemented with 10% calf serum and bovine bile under axenic conditions. *In vitro* susceptibility tests were performed using a previously described method (Cedillo-Rivera & Munoz, 1992, Cedillo-Rivera *et al*., 2002). 4×10^4 trophozoites of *G. lamblia* were used, incubated for 48 h at 37°C with increasing concentrations (20, 10, 5, 2.5, and 1.25 μg/mL) of the extracts and Bzn. As a negative control, the trophozoites were incubated with dimethyl sulfoxide (DMSO) used in the experiments. After incubation, the trophozoites were washed and subcultured for another 48 h in fresh medium alone. At the end of this period, the trophozoites were counted and the half-maximal inhibitory concentration (IC_{50}) was calculated by Probit analysis. The experiments were carried out in triplicate and repeated at least twice.

Trichonomicidal activity

The strain of *T. vaginalis* GT3 was grown in TYI-S-33 medium supplemented with 10% bovine serum, under axenic conditions. *In vitro* susceptibility tests were performed using a previously described method (Cedillo-Rivera *et al*., 2002). 4×10^4 trophozoites of *T. vaginalis* were incubated for 48 h at 37°C with increasing concentrations (20, 10, 5, 2.5, and 1.25 μg/mL) of the extracts, and metronidazole. As a negative control, the trophozoites were incubated with DMSO used in the experiments. After incubation, the trophozoites were washed and subcultured for another 48 h in fresh medium alone. At the end of this period, the trophozoites were counted and the IC_{50} was calculated by Probit analysis. The experiments were carried out in triplicate.

Ameobicidal activity

E. histolytica HM1-IMSS was cultured in axenic conditions in TYIS-33 modified medium, supplemented with 10% bovine bile. *In vitro* susceptibility assays were performed using a method previously described (Hernandez-Nuñez *et al*., 2009). Briefly: 4 x 10^4 trophozoites of *E. histolytica* were incubated for 48 h at 37°C with increasing concentrations (20, 10, 5, 2.5, and 1.25 μg/mL) of extracts. After the incubation, the trophozoites were counted and the IC_{50} was calculated by equation sigmoidal dose-response (variable slope) by GraphPad Prim 4 software. Experiments were carried out in triplicate. As the negative control, trophozoites were incubated with DMSO used in the experiments, and metronidazole was used as a positive control.

Leishmanicidal and trypanocidal activity

The growth inhibition test was performed on promastigotes of *L. mexicana* (MHOM/MX/ISETGS; clinical strain originally isolated from a patient with leishmaniasis cutaneous diffuse) and epimastigotes of *T. cruzi* (MHOM/MX/1994/NINOA, clinical strain originally isolated from a patient with the disease in acute phase). The protozoa were cultured in Drosophila Schneider's medium, supplemented with 10% fetal bovine serum, penicillin (100 IU/mL) and streptomycin (100 μg/mL) at 26°C. Biological assays were performed on 96-well plates. All extracts were evaluated in triplicate. The extracts were solubilized in DMSO and diluted to reach concentrations of 20, 10, 5, 2.5, and 1.25 μg/mL, aliquots of 100 μL of diluted extracts were added to 100 μL of culture medium containing 10000 promastigotes of *L.*

mexicana or 20000 epimastigotes of *T. cruzi*. Bzn and miltefosine were used as a positive control for *T. cruzi* and *L. mexicana*, respectively. Control cultures were setting with protozoa only. The plates were incubated at 26°C for 72 h and the antiprotozoal activity of the extracts was determined by the direct count of protozoa in a Neubauer chamber. IC_{50} values were calculated by probit analysis.

Antibacterial activity

The evaluation of antibacterial activity was carried out in three bacteria: *A. baumannii*, *M. tuberculosis*, and *N. brasiliensis.*

To evaluate activity against *A. baumannii* minimal inhibitory concentrations (MIC) values were determined using Broth Microdilution Method as described by M07-Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically 11th (CLSI, 2011). In brief, a direct broth Müeller Hinton suspension was prepared from isolated colonies selected from 18 to 24 h agar Müeller Hinton plate culture. The suspension was adjusted to achieve turbidity equivalent to a 0.5 McFarland standard. The plant extracts concentrations tested were 250, 125, 62.5, 31.25, 15.62, 3.9, 0.97, and 0.22 µg/mL. The MIC values were read as the lowest concentration of antimicrobial agent that completely inhibits organism growth in microdilution wells as detected by the unaided eye. To determine bactericidal o bacteriostatic effects 10 µL of the well where MIC was observed was culture on 5% Sheep Blood Agar plates. After 24 h incubation at 37ºC, the absence of growth was considered as a bactericidal effect and if growth was present, the effect of the extract was considered as bacteriostatic. Two reference strains (ATCC-BAA-747 and BAA-1605) and 22 clinical multidrug-resistant isolates were included in the assays. All clinical strains were isolated from healthcare-associated infections (HAIs) and identify by means of Vitek MS MALDI-TOF instrument (BioMérieux SA, Marcy l'Etoile France) while the antimicrobial resistance was determined by Vitek 2 AST Cards (bioMérieux SA, Marcy l'Etoile France).

The biological activity against *M. tuberculosis* was evaluated *in vitro* against the strain H37Rv ATCC 27294 of *M. tuberculosis*, according to a modified microplate assay of Alamar blue (MABA) (Franzblau *et al*., 1998; Jimenez-Arellanes *et al*., 2003). The tests were performed in triplicate in independent experiments. The reference strain of *M. tuberculosis* H37Rv was tested with the reference drugs rifampicin and isoniazid. The lowest extract dilution that produced a 99% inhibition of the growth, was considered as the MIC. A similar methodology was followed to evaluate the antinocardia activity of the plant extracts, but for this bacteria, the grown was in Muller Hinton broth. Two reference strains of *N. brasiliensis* (CECT-3052 and ATTC-19296) and two clinical isolates (NB-300 and NB-700) from Mexican patients with micetoma were included in the study.

Identification of active metabolites by Ultra-Performance Liquid Chromatography-Mass Spectrometer

For the identification of the secondary metabolites (fingerprint) from the active extracts, 1 mg of extract was dissolved in 1 mL of MeOH. Then, 0.1 mL to 0.9 mL of MeOH was added for analysis by UPLC with a Waters ACQUITY QDa Mass detector (Milford, MA, USA). The following conditions were used: column: ACQUITY UPLC® CORTECS® C_{18} 1.6 µm, 3.0×100 mm; mobile phase A (0.1% formic acid in water) mobile phase B (0.1% formic acid in acetonitrile) in a flow gradient (0.5 min, A: 95% B: 5%; 2.0 min, A: 75% B: 25%; 3.5 min, A: 60% B: 40%; 5.0 min, A: 50% B: 50%; 6.5 min, A: 25% B: 75%; 9.0 min, A: 5% B: 95 %; 15.0 min, A: 95% B: 5%); total execution time: 15 min; flow rate: 0.3 mL/min; injection volume: 3 µL; Temperature column: 40°C.

RESULTS AND DISCUSSION

Medicinal plants are an important source of active compounds, some of them have been used for the treatment of different diseases. The plant products have shown potential as a source of antiprotozoal and antibacterial drugs are considered a promising approach (Buathong *et al*., 2019). In this sense, a screening of Salvadoran flora was performed. The yields of the extract ranged between 0.13-0.80%, 0.20-1.52%, 0.30-6.23%, and 3.73-21.20% for Hexane, DCM, AcOEt, and MeOH extracts, respectively.

Antiprotozoal activity

In the present work, none of the extracts from Salvadoran flora showed biological effects against the protozoa, *T. vaginalis*, *G. lamblia*, *E. histolytica*, and *T. cruzi*. Only eight plants showed a biological activity against promastigotes of *L. mexicana*. The results are shown in Table No. 1.

Table No. 1
Extracts of Salvadoran flora with antiprotozoal activity against *L. mexicana*

Species	Plant parts used	Extract solvent	*L. mexicana* IC_{50} µg/mL
Petiveria alliacea	Roots	DCM	14.69
		AcOEt	28.48
Persea americana	Aerial parts	AcOEt	25.57
	Steam bark	AcOEt	28.06
Ruta graveolens	Aerial parts	Hexane	33.92
Tridax procumbens	Whole plant	Hexane	1.28
		DCM	0.90
Bursera simaruba	Steam bark	Hexane	100
		DCM	100
Pluchea odorata	Leaves	DCM	41.00
Lippia graveolens	Leaves	DCM	100
Dysphania ambrosioides	Buds	AcOEt	100
Miltefosine	-	-	**1.28**

Dichloromethane and hexane extracts of whole *Tridax procumbens* showed the best leishmanicidal activity with values of IC_{50} 0.90 and 1.28 µg/mL, respectively. These values are better or equal to that miltefosine drug reference. Previously, extracts of *T. procumbens* (whole plant) have been reported with anti-leishmanial activity, where toxicity test results showed that the limit dose of 2000 mg/kg was safe for mice, and according to these results, the half-maximal lethal dose (LD_{50}) of *T. procumbens* was found above 2000 mg/kg of body weight; in addition, the simultaneous application of a mixed extract (*T. procumbens* and *Allium sativum*) prevented the development of a lesion due to *L. mexicana*. On the other hand, another study evaluated a MeOH extract from *T. procumbens,* which inhibited the growth of promastigotes of *L. mexicana* with an IC_{50} of 3 µg/mL (Martin-Quintal *et al*., 2009; Gamboa-Leon *et al*., 2014).

Previously, Kaempferol and luteolin flavonoids have shown moderate leishmanial activity against *L. amazonensis* amastigotes (Schinor *et al.*, 2007). These flavonoids have been identified in the whole plant hexane extract from *T. procumbens* by UPLC-MS (Table No. 5).

Dichloromethane and ethyl acetate extracts of roots from *Petiveria alliacea* showed activity against *L. mexicana with* IC_{50} of 14.69 µg/mL and 28.48 µg/mL respectively. *P. alliaceae* also has been previously reported with leishmanicidal activity (Revelo-Díaz, 1989), and also some compounds have been isolated from its leaves with leishmanicidal activity including flavonoids, triterpenes, and acetogenins (Lemos da Silva *et al*., 2019), therefore, in this sense, it can be assumed that *P. alliaceae* is a source of active molecules that could help in the treatment of leishmaniasis.

Ethyl acetate extract of aerial parts and steam bark of *Persea americana*, also presented a high leishmanicidal activity with IC_{50} values of 25.57 and 28.06 µg/mL, respectively. A similar effect (IC_{50} = 33.92 µg/mL) was obtained with hexane extract of *Ruta graveolens*, *P. americana* and *Lippia graveolens* have both been previously reported with some antiprotozoal compounds, from *P. americana*, four acetogenins were tested against *L. amazonensis* where only heptadec-16-yne-1,2,4-triol and heptadec-16-ene-1,2,4-triolonly compounds showed low activity (Lemos da silva *et al*., 2019). The antiprotozoal activity of *R. graveolens* has been tested against *Trypanosoma brusei* showing good biological activity (IC_{50} = 2.5 µg/mL) (Salomé-Gachet *et al*., 2010). Also, Malik *et al*. (2017), showed that this plant has activity against promastigotes and amastigotes of a species from gender *Leishmania*. Leaves extracts of *Pluchea odorata* show good IC_{50} (41.0 µg/mL) values against promastigotes of *L. mexicana*.

Ethyl acetate extract of buds of *Dysphania ambrosioides* showed low inhibitory activity (IC_{50} = 100 µg/mL) on promastigotes growth from *L. mexicana*. This biological behavior was presented by the extracts of *Bursera simaruba* and *L. graveolens* Kunth. According to Patrício *et al*. (2008), *D. ambrosioides* has shown *in vitro* and *in vivo* activity against leishmaniasis, and also it seems to have a

direct leishmanicidal effect since in the evaluation of the effect of *D. ambrosioides* extract on the growth of the lesion with oral treatment (5 mg/kg) decreased the thickness of the mouse leg compared to the control group. In the same way, Monzote *et al*. (2014), have demonstrated that the essential oil has excellent activity (IC_{50} = 58.2 µg/mL) against cutaneous leishmaniasis, and isolated compounds as ascaridole, isoascaridole, and other oxygenated monoterpenes showed *in vitro* antileishmanial activity. Ascoridole and iso-ascaridole have been identified in the aerial parts ethyl acetate extract from *D. ambrosioides* by UPLC-MS (Table No. 5).

Antibacterial activity

Annex No. 2 shows the antibacterial activity against *A. baumanni* of all extracts from Salvadoran species. In particular, excels, 7TRA-H extract (AcOEt extract of *Tabebuia rosea* leaves) with a MIC value of 50 µg/mL. Additionally, Table No. 2 showed the extracts with a bacteriostatic effect against *A. baumanni* at 250 µg/mL.

Table No. 2
Extracts of Salvadoran flora with bacteriostatic activity against *A. baumannii* at 250 µg/mL

Species	Plant parts used	Extract solvent
Cymbopogon citratus	Leaves	Hexane
		DCM
Ruta graveolens	Buds	Hexane
Coutarea hexandra	Steam bark	DCM
		MeOH
Guazuma ulmifolia	Steam bark	MeOH
		Hexane
		AcOEt
		DCM
Lippia graveolens	Leaves	AcOEt
		DCM
		Hexane
		MeOH
Eucalyptus globulus	Leaves	AcOEt
Hamelia patens	Leaves	Hexane
Cecropia obtusifolia	Leaves	MeOH
		Hexane
Jatropha curcas	Leaves	DCM
Punica granatum	Steam bark	Hexane
Cordia allidora	Steam bark	MeOH

The Table No. 3 shows the best results of the antimycobacterial activity of the evaluated extracts. Only four plants presented MIC values <50 µg/mL (*Tabebuia rosea*, *Bursera simaruba*, *Buddleja americana*, and *Petiveria alliacea*). So far, very few studies have analyzed the antimycobacterial activity of these plants, for instance, Frame *et al*. (1998), reported that ethanolic extracts of *Bursera simaruba* and *Petiveria alliacea* were not active against *M. smegmatis*, but they did not analyze the activity against *M. tuberculosis*. There are not reports on the antituberculosis activity of *Tabebuia rosea*, however, it has been reported that other two species of *Tabebuia* genus, *Tabebuia aurea,* and *Tabebuia ovellanedae* are active against *M. tuberculosis* (Oliveira *et al*., 2007; Jimenez-Gonzalez *et al*., 2013; Agarwal & Chauhan, 2015). Interestingly, in the study of Oliveira *et al*. (2007), hydroalcoholic beverage of *T. ovellanedae* was analyzed and showed an important reduction in the colony forming units (CFU) of *M. tuberculosis* after 30 min of contact with the bacteria, and after one hour they did not recover any viable bacteria. Finally, the ethanolic extract of *Buddleja americana*, was tested against *Mycobacterium intracellulare*, showing no activity against this mycobacterium (Lentz *et al*., 1998), no

further studies of this plant have been reported against *M. tuberculosis*.

Table No. 3
Extracts of Salvadoran flora with best antibacterial activity against *M. tuberculosis*

Specie	Plant parts used	Extract solvent	MIC µg/mL
Tabebuia rosea	Leaves	AcOEt	<50
Bursera simaruba	Steam bark	AcOEt	100
		DCM	100
		Hexane	<50
Buddleja americana	Leaves	AcOEt	100
		DCM	<50
Erythrina berteroana	Steam bark	Hexane	100
Menta citrata	Leaves	DCM	100
Eucalyptus globulus	Leaves	Hexane	100
Petiveria alliacea	Root	AcOEt	<50
		DCM	<50
Ruta graveolens	Buds	DCM	100
		Hexane	100
Coutarea hexandra	Steam bark	Hexane	100
Guazuma ulmifolia	Steam bark	Hexane	100
Lippia graveolens	Leaves	DCM	100
		MeOH	100
Rifampicin	**-**	**-**	**0.03**
Isoniazid	**-**	**-**	**0.12**

The highest antibacterial activity from the group of evaluated plants in this study was against the *N. brasiliensis* group, these bacteria has a phylogenetic relationship with *M. tuberculosis*. Table No. 4 shows the results of the most active extracts against two reference strains of (CECT-3052 and ATTC-19296) and clinical isolates (NB-300 and NB-700). Some of the plant extracts were very active, with MICs values $\leq$3.125 µg/mL, plants with this high activity were *Petiveria alliacea, Aloe vera, Gliricidia sepium, Lippia graveolens, Ruta graveolens, Erythrina berteroana,* and *Sansevieria trifasciata.* To the best of our knowledge, none of these plants have been evaluated *in vitro* or *in vivo* against *N. brasiliensis.*

Identification of active metabolites by Ultra-Performance Liquid Chromatography-Mass Spectrometer

After carrying out the biological evaluation, the active extracts were analyzed using UPLC-MS to identify which metabolites were present in the samples previously reported with biological activity against *L. mexicana*. The results (Table No. 5) showed the potential metabolites in the plant extracts from *T. procumbens* and *D. ambrosioides* were flavonoids (Ikewuchi *et al.*, 2012) and monoterpenes oxygenate (Ávila-Blanco *et al.*, 2014; Mwanauta *et al.*, 2014; Soares *et al.*, 2017; Arena *et al.*, 2018), respectively, and that previously have been reported in the literature with leishmanicidal activity (Schinor *et al.*, 2007; Monzote *et al.*, 2014). Therefore, the results have shown a correlation with the biological effects.

Table No. 4
Extracts of Salvadoran flora with biological activity against *N. brasiliensis* and clinical isolates

			N. brasiliensis **MIC (µg/mL)**			
Specie	**Plant Part Used**	**Extract solvent**	**CECT-3052**	**ATTC-19296**	**NB-300**	**NB-700**
Terminalia catappa	Seed	DCM	200	200	200	100
Erythrina berteroana	Steam bark	DCM	6.25	3.125	3.125	12.5
	Leaves	MeOH	200	>200	100	50
	Steam bark	Hexane	200	6.25	6.25	25
Hymenaea courbaril	Steam bark	Hexane	50	200	25	25
		DCM	50	200	25	25
Gliricidia sepium	Leaves	Hexane	<3.125	200	100	200
		DCM	25	<3.125	3.125	100
Aloe vera	Leaves	DCM	<3.125	<3.125	3.125	12.5
		AcOEt	3.125	<3.125	6.25	12.5
Sansevieria trifasciata	Leaves	AcOEt	<3.125	200	6.25	100
		DCM	100	200	6.25	200
Eucalyptus globulus	Leaves	DCM	12.5	25	3.125	25
Petiveria alliacea	Roots	AcOEt	6.25	3.125	<3.125	12.5
		Hexane	<3.125	12.5	<3.125	25
		DCM	<3.125	3.125	<3.125	6.25
Ruta graveolens	Buds	Hexane	25	3.125	<3.125	50
Coutarea hexandra	Steam bark	DCM	200	200	6.25	100
		Hexane	100	200	12.5	200
Guazuma ulmifolia	Steam bark	AcOEt	200	200	200	200
		Hexane	6.25	200	12.5	25
Lippia graveolens	Leaves	AcOEt	6.25	6.25	6.25	<3.125
		DCM	12.5	50	6.25	<3.125
		Hexane	25	100	6.25	6.25

Table No. 5
Secondary metabolites reported in two Salvadoran flora extracts by UPLC-MS

		T. procumbens (Whole plant, Hexane)	
Metabolites	**Reference**	**(mw reported)**	**(mw founded)**
Kaempferol	(Ikewuchi *et al*., 2012)	286.23 g/mol	286.84 g/mol
Luteolin	(Ikewuchi *et al*., 2012)	286.24 g/mol	286.84 g/mol
		D. ambrosioides (Aerial parts, AcOEt)	
		(mw reported)	(mw founded)
Ascaridole epoxide	(Ávila-Blanco *et al*., 2014)	184.23 g/mol	183.16 g/mol
Ascaridole	(Mwanauta *et al*., 2014; Soares *et al*., 2017; Arena *et al*., 2018)	168.23 g/mol	167.02 g/mol
Iso-ascaridole	(Mwanauta *et al*., 2014; Soares *et al*., 2017; Arena *et al*., 2018)	168.23 g/mol	167.02 g/mol
Cis-ascaridole	(Ávila-Blanco *et al*., 2014)	168.23 g/mol	167.02 g/mol

CONCLUSIONS

Different organic extracts obtained from the Salvadoran flora were evaluated in several *in vitro* models to identified the potential activity against *E. histolytica*, *G. lamblia*, *T. vaginalis*, *L. mexicana*, *T. cruzi*, *A. baumanni*, *M. tuberculosis*, and *N. brasiliensis*. Obtaining favorable results from eight organic extracts of five plant species (*Petiveria alliacea*, *Persea americana*, *Ruta graveolens*, *Tridax procumbens*, and *Puchea odorata*,) with IC_{50} values of less than 100 µg/mL against *L. mexicana*. Five extracts from *Tabebuia rosea*, *Bursera simaruba*, *Buddleja americana*, and *Petiveria alliacea* with MIC values <50 µg/mL against *M. tuberculosis* and seven plants with important antinocardia activity (MIC <3.25 µg/mL): *Petiveria alliacea*, *Aloe vera*, *Gliricidia sepium*, *Lippia graveolens*, *Ruta graveolens*, *Erythrina berteroana*, and *Sansevieria trifasciata*. In addition, two secondary metabolites of *T. procumbens* (kaempferol and luteolin) and four secondary metabolites of *Dysphania ambrosioides* (ascaridole epoxide, ascaridole, iso-ascaridole, and *cis*-ascaridole) were identified by UPLC-MS. Kaempferol, luteolin, ascaridole, and iso-ascaridole previously have been reported as active secondary metabolites against *Leishmania*; future work should be aimed at establishing the metabolites responsible for the antituberculosis and antinocardia activity observed from active plants extracts.

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ANNEXES

Annex No. 1

List of Salvadoran plants collected to determine the antiparasitic and antimicrobial activity

Family/Scientific name	Vernacular name/Plant part used[a]	Voucher number	Ethnobotanical uses[b]
Aloaceae			
Aloe vera (L.) Burm. f.	"Sábila"/Le	J. Menjívar *et al*., 3698	Gastritis, internal infection $(Inf)_i$, acne $(Dc)_e$, skin condition $(Dc)_e$, inflammations $(Dc)_e$, antiulcer, antiparasitic $(Ing)_i$, digestive (Ing, $Dec)_i$
Anacardiaceae			
Anacardium occidentale L.	"Marañón"/Le	J. Menjívar *et al*., 4157	Vermifuge, ulcers, astringent, diarrhea $(Inf)_i$, paludism $(Inf)_i$
Anacardium occidentale L.	"Marañón"/St	J. Menjívar *et al*., 4157	Vermifuge, ulcers, astringent, mild diarrhea $(Inf)_i$ diarrhea $(Mac)_i$, paludism $(Inf)_i$
Mangifera indica L.	"Mango"/St	J. Menjívar *et al*., 4161	Stomachache $(Dec)_i$, diarrhea $(Dec)_i$
Spondias purpurea L.	"Jocote"/Le	J. Menjívar & M. Núñez 4595	Amebiasis, dysentery $(Dec)_i$
Spondias purpurea L.	"Jocote"/St	J. Menjívar & M. Núñez 4595	Amebiasis, dysentery $(Dec)_i$, diarrhea $(Dec)_i$
Arecaceae			
Cocos nucifera L.	"Coco"/T	J. Menjívar & M. Núñez 4265	Diarrhea, dysentery $(Dec)_i$
Asteraceae			
Pluchea odorata (L.) Cass.	"Siguapate"/Le	J. Menjívar & M. Núñez 4272	Anthelmintic, stomachache $(Dec)_i$ diarrhea $(Mac)_i$, dysentery $(Mac)_i$
Tridax procumbens L.	"Hierba del toro"/Wp	J. Menjívar *et al*., 4160	Dysentery $(Dec)_i$, paludism

			$(Mac)_i$, inflammatory disease $(Dec)_e$, against amebiasis $(Dec)_i$
Bignoniaceae			
Tabebuia rosea (Bertol.) DC.	"Maquilishuat"/Le	J. Menjívar *et al*., 4163	NR
Tabebuia rosea (Bertol.) DC.	"Maquilishuat"/St	J. Menjívar *et al*., 4163	Stomachache $(Dec)_i$
Bixaceae			
Bixa orellana L.	"Achiote"/Le	J. Menjívar & M. Núñez 4273	Digestive affection, tonsils, dysentery, hepatitis $(Dec)_i$
Boraginaceae			
Cordia alliodora (Ruiz & Pav.) Oken	"Laurel"/St	J. Menjívar & M. Núñez 4164	Dysentery $(Inf)_i$, diarrhea $(Inf)_i$
Brassicaceae			
Nasturtium officinale W.T. Aiton	"Berro"/Wp	J. Menjivar & U. Castillo 3411	Stomach pain $(Dec)_i$
Burseraceae			
Bursera simaruba (L.) Sarg.	"Jiote"/St	J. Menjívar *et al*., 4165	Digestive, diarrhea $(Inf)_i$, carminative $(Dec)_i$, diarrhea $(Dec)_i$
Chenopodiaceae			
Dysphania ambrosioides (L.) Mosyakin & Clemants	"Epazote"/Ap	J. Menjívar & M. Núñez 4282	Stomach pain, belly vermifuge $(Mac)_i$, fungicide $(Inf)_{e,i}$, $(Mac)_e$, digestive, anthelmintic, ascaricide $(Mac)_i$, antiparasitic $(Dec)_i$
Combretaceae			
Terminalia catappa L.	"Almendro"/S	J. Menjívar *et al*., 4166	Digestive affection, depurative, dysentery $(Inf)_i$ diarrhea $(Dec)_i$
Dracaenaceae			
Sansevieria trifasciata Prain	"Curarina"/Le	J. Menjívar *et al*., 4266	NR
Euphorbiaceae			
Cnidoscolus aconitifolius (Mill.) I.M. Johnst.	"Chaya"/Le	J. Menjívar *et al*., 4276	NR
Cnidoscolus aconitifolius (Mill.) I.M. Johnst.	"Chaya"/St	J. Menjívar *et al*., 4283	NR
Croton guatemalensis Lotsy	"Copalchí"/St	J. Menjívar & M. Núñez 4278	NR
Fabaceae			
Cassia grandis L. f.	"Carao"/St	J. Menjívar *et al*., 4168	Dysentery $(Dec)_i$
Erythrina berteroana Urb.	"Pito"/Le	J. Menjívar & M. Núñez 4279	Insomnia, nerves $(Dec)_i$, contusions $(Dc)_e$
Erythrina berteroana Urb.	"Pito"/St	J. Menjívar & M. Núñez 4279	Dysentery $(Inf)_1$

Gliricidia sepium (Jacq.) Kunth ex Walp.	"Madre cacao"/Le	J. Menjívar & M. Núñez 4280	Gastritis $(Inf)_i$, antibiotic $(Inf)_i$, inflammations $(Dec)_e$
Gliricidia sepium (Jacq.) Kunth ex Walp.	"Madre cacao"/St	J. Menjívar & M. Núñez 4280	Gastritis, antibiotic $(Inf)_i$, inflammations $(Dec)_e$
Hymenaea courbaril L.	"Copinol" /St	J. Menjívar & U. Castillo 4274	Excretory system $(Inf)_i$, dysentery $(Dec)_i$, diarrhea $(Mac)_i$
Laminaceae			
Mentha citrata Ehrh.	"Hierba buena"/Le	J. Menjívar & M. Núñez 4277	Dysentery, stomach pain, vomits $(Dec)_i$, vermifuge, colic $(Ing)_i$, antiparasitic (Dec, $Inf)_i$, antitussive $(Ing)_I$, dysentery $(Dec)_i$
Lauraceae			
Persea americana Mill.	"Aguacate"/St	J. Menjívar *et al*., 4162	Diarrhea, dysentery, anthelmintic $(Dec)_i$, dysentery, vermifuge $(Dec)_i$
Lythraceae			
Punica granatum L.	"Granada"/St	J. Menjívar & U. Castillo 4167	Diarrhea, dysentery anthelmintic $(Dec)_i$
Malvaceae			
Guazuma ulmifolia Lam.	"Caulote"/St	J. Menjívar & U. Castillo 4275	Dysentery $(Inf)_i$, indigestion $(Dec)_i$, diarrhea $(Inf)_i$, skin affectation $(Dec)_e$, depurative $(Dec)_i$, inflammations $(Dc)_i$, stomachache $(Dec)_i$
Myrtaceae			
Eucalyptus globulus Labill.	"Eucalipto"/Le	J. Menjívar *et al*., 4269	Fever $(Dec)_e$, dyspepsia, antipyretic, tuberculosis $(Inf)_i$
Nyctaginaceae			
Bougainvillea glabra Choisy	"Veranera"	J.Menjívar *et al*., 4267	Purgative $(Dec)_i$
Petiveriaceae			
Petiveria alliacea L.	"Epacina"/R	J. Menjívar & M. Núñez 4270	Toothache, fever $(Dec)_e$, diarrhea $(Dec)_i$
Poaceae			
Cymbopogon citratus (DC.) Stapf	"Zacate limón"/Le	J. Menjívar & M. Núñez 4285	Anti-flu, antipyretic $(Dec)_i$
Portulacaceae			
Portulaca oleracea L.	"Verdolaga"/Wp	J. Menjívar & M. Núñez 4284	Diuretic, against cystitis $(Inf)_i$
Rubiaceae			
Coutarea hexandra (Jacq.) K. Schum.	"Quina"/St	J. Menjívar *et al*., 4158	Wounds, inflammations $(Dec)_e$, stomach pain, belly pain, tetanus $(Dec)_i$, fevers

			$(Inf)_i$, contusions $(Dc)_e$, anti-infective, fungicide $(Dec)_{e)}$, skin condition $(Dec)_e$, paludism $(Inf)_i$
Hamelia patens Jacq.	"Chichipince"/Le	J. Menjívar *et al*., 4159	Skin conditions, wounds, inflammation $(Dec)_e$, colic, dysentery, stomach pain, belly pain, gastritis, urinary tract $(Dec)_i$, diuretic, stomach pain, belly pain $(Dec)_i$
Rutaceae			
Ruta graveolens L.	"Ruda"/Ap	J. Menjívar & M. Núñez 4286	Colic $(Mac)_i$, gastric ulcers, digestive affection $(Inf)_i$, anthelmintic $(Mac)_i$, anti-flatulent, antipyretic $(Dec)_i$, stomach pain $(Mac)_i$
Scrophulariaceae			
Buddleja americana L.	"Salviona"/Le	J. Menjívar & M. Núñez 4281	Fever, flu $(Dec)_e$ colic, stomachache, belly pain $(Dec)_i$, constipation $(Dc)_e$, stomachache $(Dec)_i$
Urticaceae			
Cecropia obtusifolia Bertol.	"Guarumo"/Le	J. Menjívar *et al*., 4268	Throat, wound infection $(Dec)_i$
Verbenaceae			
Lippia graveolens Kunth	"Orégano"/Le	J. Menjívar *et al.*, 4271	Skin conditions, wounds $(Dec)_e$, colic, stomach pain, indigestion $(Dec)_i$, digestive, constipation $(Inf)_i$, vaginal affection, contusions $(Dc)_i$, antiarthritic $(Dec)_i$, inflammations $(Mac)_i$, stomach pain $(Dec)_i$, contusions $(Dc)_i$

[a] Plant part used in the present study: Ap, aerial parts; Le, leaves; R, roots; S, seeds; St, steam bark; T, tow; Wp, Whole plant.

[b] Preparation: NR, not reported, Dc, direct contact to the skin/tissue; Dec, decoction; Inf, infusion; Ing, ingestion; Mac, maceration; $_i$: Internal administration, $_e$: External administration

Annex No. 2

Minimum inhibitory concentration from extracts of Salvadoran flora against *A. baumanni*

Scientific name	Plant parts used	Extract solvent	MIC (µg/mL)
Cocus nucifera	1CNA		>200
	1CND		200
	1CNH		>200
	1CNM		>200
Mangifera indica	2MID		100
	2NIM		>200
Spondias radlkoferi	3SRA-C		>200
	3SRD-C		>200
	3SRH-C		>200
	3SRM-C		>200
	3SRA-H		>200
	3SRD-H		>200
	3SRH-H		>200
	3SRM-H		>200
Anacardium occidentale	4ACD-C		>200
	4ACH-C		>200
	4ACM-C		>200
	4ACA-H		>200
	4ACD-H		>200
	4ACH-H		>200
	4ACM-H		>200
Pluchea odorata	5POA		200
	5POD		200
	5POH		200
	5POM		>200
Tridax procumtens	6TPA		>200
	6TPD		200
	6TPH		>200
	6TPM		>200
Tabebuia rosea	7TRA-C		>200
	7TRA-H		<50
	7TRD-C		>200
	7TRD-H		>200
	7TRH-C		>200
	7TRH-H		>200
	7TRM-C		>200
	7TRM-H		>200

Annex No. 3

Minimum inhibitory concentration from extracts of Salvadoran flora against *M. tuberculosis*

Specie	Plant parts used	Extract solvent	*M. tuberculosis* MIC µg/mL
Cocus nucifera	Tow	Dichloromethane	200
Magnifera indica	Cortex	Dichloromethane	100
Pluchea odorata	Leaves	Ethyl acetate Dichloromethane Hexane	200 200 200
Tridax procumbems L.	Whole plant	Dichloromethane	200
Tabebuia rosea	Leaves	Ethyl acetate	<50
Bixa Orellana	Leaves	Ethyl acetate Dichloromethane	200
Bursera simaruba	Cortex	Ethyl acetate Dichloromethane Hexane	100 100 <50
Buddleja americana	Leaves	Ethyl acetate Dichloromethane	100 <50
Nasturtium officinales	Whole plant	Dichloromethane Hexane	200 200
Jatropha curcas	Leaves Cortex	Methanol Ethyl acetate Dichloromethane	200 200 200
Erythrina berteroana	Cortex	Dichloromethane Hexane	200 100
Hymenaea courbaril	Cortex	Methanol Hexane	200 200
Cassia grandis	Cortex	Hexane	200
Gliricidia sepium	Cortex Leaves	Hexane Dichloromethane	200 200
Menta citrata	Leaves	Ethyl acetate Dichloromethane	200 100
Aloe vera	Leaves	Dichloromethane	200
Eucalyptus globulus	Leaves	Hexane	100
Punica granatum	Cortex	Ethyl acetate	200
Petiveria alliacea	Root	Ethyl acetate Dichloromethane Hexane	<50 <50 200
Cymbopogon citratus	Leaves	Ethyl acetate Dichloromethane Hexane	200 200 200
Ruta graveolens	Heart	Ethyl acetate Dichloromethane Hexane	200 100 100
Coutarea hexandra	Cortex	Dichloromethane Hexane	200 100

Hamelia patens	Leaves	Dichloromethane Ethyl acetate Hexane	200 200 200
Guazuma ulmifolia	Cortex	Hexane	100
Cecropia obtusifolia	Cortex	Ethyl acetate Dichloromethane	200 200
Lippia graveolens	Leaves	Ethyl acetate Dichloromethane Methanol	200 100 100