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Screening of Antioxidant, Antibacterial and Antileishmanial Activities of *Salvia officinalis* L. Extracts from Morocco

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Authors' contributions

This work was carried out in collaboration between all authors. Study design was suggested by authors YB and AS followed by plant collection and extraction by authors AET and AB. Authors AET and AB managed the literature searches. Authors AET, HF, MM, ND, EHA and YB analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The aim of this study was to evaluate in vitro antioxidant and antimicrobial activities of organic extracts *from Salvia officinalis* L. (Lamiaceae) collected in the province of Ouezzane.
Study Design: Evaluation of *in vitro* antimicrobial and antioxidant activities of extracts and determination phenolic contents.
Place and Duration of Study: After plant collection from the Province of Ouezzane, further work was carried out in Parasitology Laboratory of the National Institute of health and Laboratory of

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Biochemistry-Immunology, Faculty of Science, Mohammed V University of Rabat, Morocco from Novembre 2015 to Mai 2016.

Methodology: The antioxidant activity was evaluated using DPPH scavanging assay. The antibacterial activity was tested against three reference strains (*Staphylococcus aureus*, *Escherichia coli* and *Listeria monocytogenes serovar*) by the diffusion method and the minimum inhibitory concentration (MIC) by microtitration assay. The antiparasitic activity was tested against *Leishmania major* using MTT (3- (4.5-dimethylthiazol- 2yl) -2.5-diphenyltetrazolium bromide) assay. The levels of polyphenols and flavonoids extracts were estimated by colorimetric assay.

Results: The methanol extract has shown a significant ability to trap the radical DPPH (IC_{50}=65.655 µg/ml) compared to *n*-hexane and ethanol extracts. This value is higher than that of ascorbic acid (13.198 µg/ml) and Trolox (22.484 µg/ml) used as standards. The three extracts tests revealed the inhibitory power of three bacterial strains with a significant difference in the diameters of inhibition. The largest area was registered by the hexane and methanol extracts against *S. aureus* (22±8 mm), while the weakest area was 11±0.22 mm expressed by the ethanol extract against *E. coli* and the methanol extract against *L. monocytogenes*. The antileishmanial activity was moderate with a value of cytotoxicity (IC_{50}) above 1 mg / ml. The extracts showed high concentrations of polyphenols and flavonoids, while biological activities were not very high when correlated with these levels.

Conclusion: These results will be completed by the determination of the active component and the extracts will be tested on other biological systems namely antifungal and antitumor activities.

Keywords: Salvia officinalis; antioxidant activity; antimicrobial activity; total phenols.

1. INTRODUCTION

The genus *Salvia* (sage) is one of the largest genera aromatic and medicinal and most representative of the Lamiaceae family and includes about 900 species, spread around the world [1]. Several species of the genus *Salvia* are used in folk medicine worldwide to treat microbial infections, the cancer, Malaria, inflammation and disinfect houses after the disease [2]. *Salvia* is a latin name which means " cure " while "*officinalis* " means medicinal, it is clear that the sage has a historical reputation of health promotion, and the treatment of diseases [3]. It is a species of great economic and industrial value. Worldwide, *Salvia* is cultivated for use in traditional medicines and for culinary purposes [4]. In fact, the essential oils and extracts of sage are used in the treatment of several diseases and have been shown to have an antioxidant, antimicrobial, virucidal, cytotoxic, antimutagenic, anti-inflammatory and antifungal effects [5,6]. In addition many phenolic compounds of this genus exerted variable anti-cancer activity or anti-carcinogenic / anti-mutagenic activities [7]. On the other hand, *S. officinalis* has been reported with excellent properties in the inhibition of lipid peroxidation, mainly attributed to polyphenols [8]. Many mediterranean countries get substantial economic wealth with the production and export of *S. officinalis*. The species is an aromatic plant, and thus was widely regarded for its essential oil content [9]. It was the subject of intensive investigation for its phenolic antioxidant components [10,11,12,13]. In North West Morocco (Ouezzane province), the vegetation is rich in aromatic and medicinal plants. However their use restricted to traditional level and few studies have been conducted to biological enhancement [13]. In this context, we launched an evaluation study *S. officinalis* from Ouezzane through biological activities tests.

2. MATERIALS AND METHODS

2.1 Collection of Plant Material and Preparation of Extracts

The selected plants were collected in different areas of Ouezzane province in July 2015 and were authenticated by Pr. ENNABILI Abdeslam (National Institute of Medicinal and Aromatic Plants, Taounate, Morocco). Samples were further transported to the laboratory. Samples were air-dried under the shade and milled into powder using an electric grinder, the investigated dried powdered plant materials were extracted by maceration. The powder (25 g) of leaves was placed in an Erlenmeyer flask containing 100 ml of solvent for 72 h. The plant extracts were filtered with a filter paper (Whatman. No. 1) and the combined filtrate was then dried under

vacuum using a rotary evaporator (Buchi Heating Bath B-490, Buchi Rotavapor R-200) at a temperature not exceeding 45°C. All extracts were stored in a dark bottle at 2 - 8°C until analysis.

2.2 Determination of Total Phenolic Content (TPC)

The concentration of the phenolic compounds in the plant's extracts was determined using the Folin Ciocalteu assay [14], with some modifications. In brief, the extract was diluted to the concentration of 1mg/ml, and aliquots of 100 µl or a standard solution of gallic acid (20, 40, 60, 80 and 100 mg/l) were mixed with 500 µl of Folin Ciocalteu reagent (previously diluted 10-fold with distilled water) and 400 µl of Na_2CO_3 (7%). After 40 min of incubation at room temperature (23±2°C), the absorbance was measured at 760 nm using a Spectro-photometer against a blank sample. The total phenolic content was calculated using a calibration curve for gallic acid (R^2= 0.998). The results were expressed as the gallic acid equivalent per gram of dry weight of extract (mg of GAE/g of extract). All samples were analyzed in triplicate.

2.3 Determination of Total Flavonoid Content (TFC)

The total flavonoid content of the extracts was determined using the aluminum chloride ($AlCl_3$) colorimetric method described by Brighente et al. [15] with minor modifications. Briefly, 1 ml of the extract (1 mg/ml in methanol) or a standard solution of quercetin (20, 40, 60, 80 and 100 mg/l) were mixed with 1 ml of 2% $AlCl_3$ in methanol. After 40 min of staying at room temperature (23 ± 2°C), the absorbance against blank was measured at 430 nm using a Spectrophotometer. The total flavonoid content was calculated using a calibration curve for quercetin (R^2= 0.985). The results were expressed as the quercetin equivalent per gram of dry weight of extract (mg of QE/g of extract). All samples were analyzed in triplicate.

2.4 Diphenyl-1-Picrylhydrazyl (DPPH) Radical Scavenging Capacity Assay

The ability of the plant extracts to scavenge DPPH free radicals was assessed using the standard method with some modifications [16]. In brief, aliquots (0.2 ml) of various concentrations (62.5–1000 µg/ml) of the plant extracts samples were added to 1.8 ml of a 0.004% methanolic solution of DPPH prepared daily.

The absorbance at 517 nm was measured at different time intervals (in seconds) until the reaction reached a plateau. The concentration of DPPH (mg/l) in the reaction medium was calculated starting from a calibration curve constructed with the following concentrations of DPPH [0.01, 0.015, 0.02, 0.025, 0.03, 0.035 mg/l] by a linear regression.

$$A_{517}\ nm = a \times [DPPH]_t + b$$

Where $[DPPH]_t$ was expressed as mg/l.

The percentage inhibition of DPPH by the extracts of phenolic compounds was calculated using the formula:

$$\%\ Inhibition = \frac{Abs\ (blank) - Abs(Sample)}{Abs\ (blank)} \times 100$$

Where Abs (blank) is the absorbance of the control with t=0.

t=0, and *Abs (Sample)$_t$* represents that in the presence of antioxidants at a time t, which varies according to the concentrations. Ascorbic acid and Trolox were used as positive control.

2.5 Antibacterial Activities

2.5.1 Bacteria strains

In order to evaluate the antibacterial activity of the various extract of *S. officinalis*, the flowing bacteria were used: *Escherichia coli* K12 (Laboratory of Food Microbiology, UCL, Belgium: MBLA), *Staphylococcus aureus* CECT 976, *and Listeria monocytogenes serovar* 4b CECT 4032 (Spanish Type Culture Collection: CECT).

2.5.2 Agar-well diffusion assay

A basal layer was prepared by Muller-Hinton agar. After the agar plates were solidified, sterile 8 mm diameter cylinders were deposited. Six ml of LB medium in superfusion containing 0.8% agar were inoculated by a fresh culture of indicator bacterial strain (a final concentration was 10^6 CFU/ml). After solidification, the wells were filled with 50 µl of diluted extracts at 2.5 mg/ml. After incubation at appropriate temperature for 24 h, all plates were examined for any zone of growth inhibition, and the diameter of these zones was measured in millimeters. All the tests were performed in triplicate.

2.5.3 Determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

MICs were determined using the broth micro-dilution assay [17]. Agar at 0.15% (w/v) was used as stabilizer of the extract–water mixture and resazurin as bacterial growth indicator. 50 µl of Bacteriological Agar (0.15% w/v) was distributed from the 2nd to the 8th well of a 96-well polypropylene microtitre plate. A dilution of the each extract was prepared in DMSO (10%), to reach a final concentration of 32 mg/ml; 100 µl of these suspensions was added to the first test well of each microtitre line, and then 50 µl of scalar dilution was transferred from the 2nd to the 7th well. The 8th well was considered as growth control, because no extract was added. Then, we added 50 µl of a bacterial suspension to each well at a final concentration of approximately 10^6 CFU/ml. The final concentration of the extract was between 16 and 0.25 mg/l. After incubation at 37°C for 18 h, 10 µl of resazurin was added to each well to assess bacterial growth. After further incubation at 37°C for 2 h, the MIC was determined as the lowest essential oil concentration that prevented a change in resazurin color. Bacterial growth was detected by reduction in blue dye resazurin to pink resorufin. A control was carried out to ensure that, at the concentrations tested, the extract did not cause a color change in the resazurin. The minimum bactericidal concentration (MBC) corresponded to the lowest concentration of the essential oil yielding negative subcultures after incubation at appropriate temperature for 24 h. It is determined in broth dilution tests by sub-culturing 10 µl from negative wells on PCA medium. All the tests were performed in triplicate.

2.6 Antileishmanial Activities

The in vitro antileishmanial effect of the extract obtained was evaluated on culture of *Leishmania major* (MHOM/MA/2009/LCER19-09). The promastigote forms were isolated and identified in National Reference Laboratory of Leishmaniasis, National Institute of Health, Rabat.

Parasites cultures of *Leishmania* promastigotes were washed with phosphate buffered saline (PBS) and centrifuged at 1500 rpm for 10 min. Cells were then re-suspended in RPMI 1640 (GIBCO) supplemented with 10% heat-inactivated fetal calf serum and 1% Penicillin-Spreptomycin mixture. Cultures were maintained at 23°C. The effect of the isolated extracts on cell viability was assessed using the 3-(4.5-dimethylthiazol-2-yl)-2.5 diphenyl tetrazolium bromid (MTT) assay, which measures the metabolic activity of mitochondria [18,19]. MTT assays are presently the preferred methods of cytotoxicity assessment in our laboratory [15,14]. The tests were conducted on 96 well microplates. Before treatment with extracts, 100 µL medium RPMI (GIBCO) containing $2.5x10^6$promastigotes/ml were placed in each well containing RPMI (GIBCO) and cultured at 23°C for 72 h. After incubation, samples were treated with crude extracts. Exactly from the stock solution (10 mg/ml), each extract sample was applied in a series of 6 dilutions (final concentrations ranging from 1 µg/ml to 500 µg/ml) in Dimethyl sulfoxid (DMSO 1%). Test solution (100 µl), was added in decreasing concentrations in duplicate. Microplates were then incubated for 72 h at 23°C. After, 10 µl MTT solution (5 mg/ml) (SIGMA) was added to the wells containing samples and were incubated for 3 h at 23°C. Tetrazolium salts are cleaved to formazan dye by cellular enzyme mitochondrial succinate dehydrogenase (only in the viable promatigotes) [20,19,18,21,22]. A solubilization solution (Isopropanol/hydrochloric acid) is added to dissolve the insoluble purple formazan product into coloration solution. The absorbance was measured at 570 nm, using microplate reader (Statfax 2100).The percentage of non-viable organisms which have failed to metabolize MTT and therefore does not produce the formazan product was determined by applying the following formula [23]:

$$\%\, Inibition = \left[1 - \frac{Abs(Test) - Abs(Blanc)}{Abs(Controle) - Abs(Blanc)}\right] \times 100$$

Data are presented as means ± SD of three different assays. Statistical analysis was performed by Origin 6.0 software.

3. RESULTS AND DISCUSSION

3.1 Phenols and Flavonoids Content

The total phenols and flavonoids content of *S. officinalis* extracts are shown in Table 1. The contents of total phenols of MeOH, *n*-hexane and EtOH extracts are respectively 115.23±0.82, 111.40±1.53 and 102.04±2.00 mg EAG / g of

extract. The contents of phenols and flavonoids vary significantly from one solvent to another.

This variability is due to differences in the drive force of each solvent. Other studies have shown the richness of *S. officinalis* in phenolic compounds [24]. Many studies have revealed the role of polyphenols and flavonoids in biological activities as antioxidant, antimicrobial, antitumor, etc. this activity is due in fact to the chemical groups, biologically functional, present in these chemical families. Bioguided research in medicinal plants has been oriented in most studies towards these types of molecules that have shown moderate side effects with safety.

Table 1. Total phenols content and flavonoids content of methanol (MeOH), ethanol (EtOH) and *n*-hexane of *S. officinalis* extracts

Extracts	Total phenol[a]	Flavonoids[b]
MeOH	115.23±0.82	31.05±0.62
EtOH	102.04±2.00	26.13±2.96
n-Hexane	111.40±1.53	33.64±1.02

[a]*gallic acid equivalent (EAG mg / g of extract).*
[b]*quercetin equivalent (EQ mg / g of extract).*

3.2 Antioxidant Activities

The antioxidant activity of *S. officinalis* extracts was evaluated by the DPPH method. Results obtained are shown in Fig. 1. The inhibition of DPPH is dose dependent for the three extracts (MeOH, EtOH and *n*-hexane extracts) and standards (Ascorbic Acid and Trolox). At concentrations below 500 µg/ml, it was observed that the methanol extract has the highest capacity reduction followed by *n*-hexane extract, while the ethanolic extract has shown the lowest.

The three extracts showed moderate activity compared to standard antioxidants. In fact, the antioxidant capacity (IC_{50}) of vitamin C and Trolox are 13.198 µg / ml and 22.484 µg/ml, respectively, whereas the IC_{50} of the methanol extract, *n*-hexane and ethanol are 65.655 µg/ml respectively, 133.423 µg / ml and 201.192 µg/ml (Table 2).

This difference is probably related to phenols and flavonoids concentrations in our extracts, which is correlated to the antiradical activity. Indeed, these compounds are influenced by some ecological extrinsic factors such as soil moisture, drought, and genetic factors, but also by plant phenological stage and storage time [25].

It is for this reason that we see an average scavenging activity of our extracts by granting their average rate of flavonoids. The antioxidant activity of phenols specifically flavonoids generally depends on their chemical structure and the distribution of hydroxyl groups [26]. Other studies have shown that the flavonoids are strong inhibitors of enzymes responsible of the production of free radicals as xanthine oxidase, which is an important biological source of superoxide radical [27,28]. The flavonoids are also known to modify the activity of enzymes such as protein kinase C, tyrosine protein kinase, aldose reductase, myeloperoxidase, NADPH oxidase, phospholipase, reverse transcriptase, ornithine decarboxylase, lipoxygenase. Antioxidant function and modification of enzymatic action of flavonoids explain many pharmacological activities [29,30]. The number of phenolic hydroxyl groups can play an important role in scavenging free radical activity [31,32]. The literature reports show that compounds hydroxylated in the aromatic ortho-position, such as quercetin, have antioxidant activity against ROS [33]. In the presence of H_2O_2, phenolic compounds, such as ortho-quinol, undergoes a reduction reaction to produce the ortho-quinones and water as products [34,33]. Phenolic compounds can also protect cellular components against oxidative damage and, therefore, to limit the risk of degenerative diseases associated to oxidative stress [35]. In plant extracts, except polyphenols, several secondary metabolites families may have antioxidant activity that, through their synergistic effect, can influence the activity of plant extracts [36].

Table 2. Antioxidant capacity (IC_{50})

Plant species	Type of extract	IC_{50} (µg/ml)
Salvia officinalis L.	Ethanol	201.192
	n-Hexane	133.423
	Methanol	65.655
Control	Trolox	22.484
	Acide ascorbique	13.198

Relationships between the levels of phenols and flavonoids content of *S. officinalis* extracts and antioxidant activities (IC_{50}) were established. The antioxidant capacity (IC_{50}) correlates with the total phenol content by a correlation coefficient R^2 = 0.944. While, this correlation coefficient between total flavonoids content and antioxidant

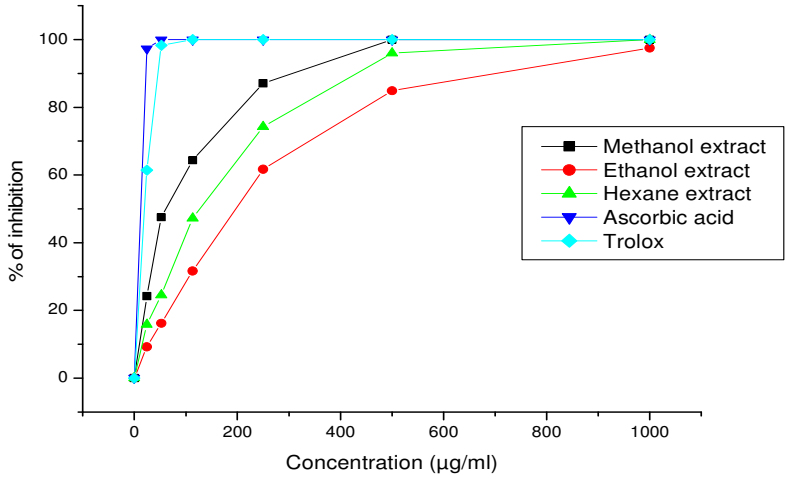

Fig. 1. Percent of inhibition of DPPH by methanol (MeOH), ethanol (EtOH) and *n*-hexane (C_6H_{14}) of *Salvia officinalis* extracts and ascorbic acid and Trolox (standards)

capacity (IC_{50}) was moderated (R^2 = 0.415). These results show the clear dependence of total phenol in the ability to scavenge DPPH and potentiate clearly the role of phenolic compounds in this activity. Except flavonoid compound, we have suggested that there are another compound of phenols has antioxidant activities.

3.3 Antibacterial Activities

The quantitative evaluation of antibacterial activity of *S. officinalis* extract was carried out against three bacterial strains (*E. coli, S. aureus* and *L. monocytogenes*) by the well diffusion method (Fig. 2). The results obtained are shown in Table 3. The antibacterial effect depends on both the type of extract and the nature of the strain. The *n*-hexane extract showed higher activity. *E. coli* was more resistant than three strains tested *vis-à-vis* the three extracts. Indeed, $Gram^-$ bacteria are more resistant to antibacterial molecules that $Gram^+$ bacteria [37,13]. This difference is tantamount to the difference of the chemical composition of their wall. $Gram^-$ bacteria have an outer membrane that prevents and/or sequester the antibacterial molecules inside cells [38]. The minimum inhibitory and bactericidal concentrations were determined by the microdilution method. The results shown in Table 3 show a significant difference in the activity of the extracts and confirm the general diffusion test wells. The lowest inhibitory value was found by the methanol extract against *S. aureus* (MIC = MBC = 2 mg/ml). In our results, the antibacterial activity obtained with extracts of *S. officinalis* does not correlate with the levels of polyphenols and flavonoids. Indeed, the biological effect of a plant extract does not only depend on their phenolic content, but here could be the result of all the compounds present in the extract. Several studies have been conducted on the antibacterial activity of *S. officinalis* and show the ability of extracts of this plant to inhibit the growth of pathogenic bacteria [39,23].

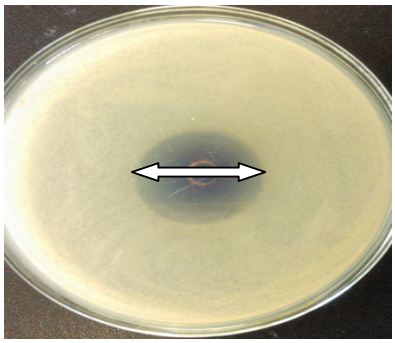

Fig. 2. Antibacterial effect of *S. officinalis* ethanol extract against *Staphylococcus aureus*

3.4 Antileishmanial Activities

The tests were performed against cultures *L. major* promastigotes strain, in the absence and presence of the three plant extracts (ethanol, methanol and *n*-hexane) and the N-methyl glucamine antimoniate (Glucantime®) at different concentrations. Results are summarized in the Fig. 3.

Table 3. Determining diameter of inhibition, MIC and MBC of different extracts (MeOH, EtOH and C_6H_{14}) for the three bacterial strains

Bacteria[a]	Extract	Diameter inhibition	MIC[b]	MBC[c]
E. coli K12	EtOH	11±0.89	>8	Nd
	MtOH	12±0.55	8	Nd
	n-Hexane	15±0.98	4	4
S. aureus	EtOH	19±0.24	4	8
	MtOH	22±0.81	2	2
	n-Hexane	22±82	8	>8
L. monocytogenes	EtOH	19±1.64	ND	ND
	MtOH	11±0.22	4	4
	n-Hexane	21±0.42	2	4

[a] bacterial density of approximately 10^6 CFU / ml, [b] Minimum Inhibitory Concentration (mg/ml), [c] Minimum Inhibitory Concentration (mg/ml)

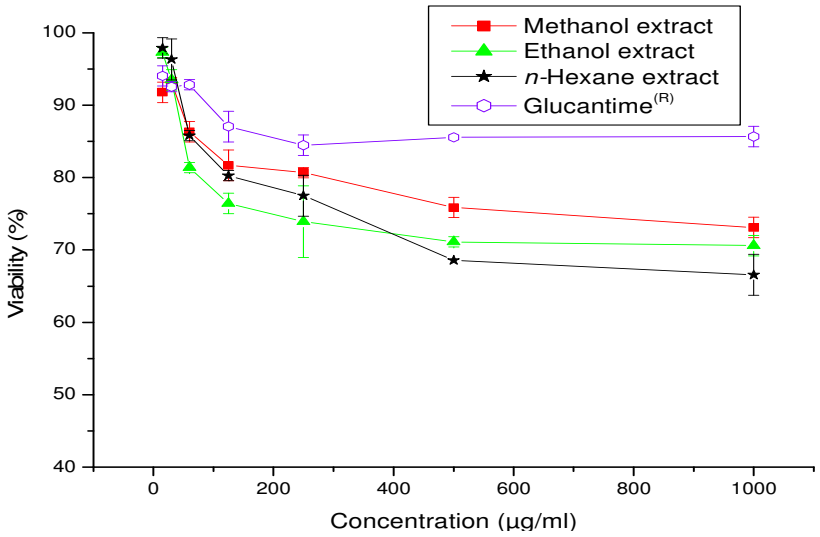

Fig. 3. Antileishmanial activity of *S. officinalis* extracts and witness against promastigotes of *Leishmania major*

The promastigotes were incubated with different concentrations of extracts of the plant rows of 15µg / ml to 1000µg / ml for 72 hours, cell viability was determined using MTT assay (n = 3). % Viability = (Absorbance of test / Absorbance of control) × 100

At the concentration 1 mg / ml, a major toxic activity is noted on the growth of promastigotes to different concentrations of the *n*-hexane, methanol, ethanol extracts and the control drug. The obtained viability percentages are all higher than 50%.

These results do not show in vitro an important antileishmanial activity of *S. officinalis* extracts against *L. major*, which is responsible for the cutaneous form raging in the North West of Morocco. To our knowledge, based on a literature search, no study has been conducted on effects of this plant extract on the *in vitro* growth of promastigotes. There is a general lack of effective chemotherapeutic agents and inexpensive for the treatment of leishmaniasis. Although antimony salts such as N-methyl glucamine antimonate are reference compounds for the treatment of this disease, with amphotericin B and pentamidine used as substitution drugs, all of which have several disadvantages, on the one hand the patient should be hospitalized for intravenous or intramuscular administration ranged between 20 to 28 days [40]. On the other hand, these drugs show several side effects of early anaphylactic treatment as muscle pain, rash, vomiting, hyperthermia, tachycardia and bleeding. The other side effects occur at the end of treatment and result in general signs, cardiac, hepatic, pancreatic, renal and haematological disorders

[41] and the resistance has also become a serious problem [42,43,44]. Thus, new drugs are urgentlyneeded. The substances of vegetable origin have always been a major source for the development of new substances with therapeutic properties to treat tropical diseases caused by protozoa [45]. The broad antimicrobial spectrum, high biodiversity, chemical and ubiquitous nature of plants of the genus *Salvia* has about 700 species, is one of the most common members of the Lamiaceae family. Many secondary metabolites are exceptionally helpful belonging to different chemical groups, such as essential oils, terpenoids and phenolics compounds were isolated from the genus, which figures prominently in the pharmacopoeia of many countries around the world [46,47,48,49] led us to evaluate the activity of extracts of the plant *Salvia* toward the promastigote forms of *L. major*. *Leishmania* tested show a similar sensitivity tested at or above concentrations 1000 µg/ml (Fig. 3). To our knowledge, Banafsheh et al. [50] show that *S. officinalis* has a significant effect on the species *L. major* promastigotes (strain MROH/IR/75/IR) with IC50 = 108.19 ± 8.6 µg/ml and this difference in results may be due to the chemical composition that differs by geographic region (climate, soil type, etc ...) [25].

4. CONCLUSION

Given the great concern of antibiotic resistance and pest control, it was found that these results support the fact that the use of *S. officinalis* is a valuable and it could be a source of bioactive compounds and an alternative for the treatment of infectious diseases caused by *E. coli*, *S. aureus*, *L. monocytogenes serovar* and parasitic diseases. However, other studies are needed to identify and quantify the constituents present in these extracts that are involved in biological effects.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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