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***In vitro* antileishmanial potentialities of essential oils from *Citrus limon* and *Pistacia lentiscus* harvested in Tunisia**

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Abstract

Leishmaniasis is a tropical parasitic disease that affects up to 12 million people worldwide. Current chemotherapies have limitations such as toxicity, high cost, and parasite resistance. This work aims to select an essential oil (EssOil) isolated from the Tunisian flora as a new antileishmanial candidate. Two plants were chosen for their antileishmanial potential: *Citrus Limon* (*Citrus*) and *Pistacia lentiscus* (*Pistacia*). Each of these plants was harvested from two different sites (area 1 and area 2).

Extracted EssOils were characterized using GC-MS. Their antiparasitic activity against axenic and intracellular *Leishmania major* amastigotes and their cytotoxicity were assessed. *Citrus* EssOil from area 1 displayed an interesting activity against *L. major* intramacrophage amastigotes with IC₅₀ value at $4.2 \pm 1.3 \mu\text{g/mL}$. Interestingly, this activity was close to that of miltefosine. Moderate activities against intracellular amastigote were observed for *Pistacia* EssOil from area 1 and *Citrus* EssOil from area 2. However, low cytotoxicity with high selectivity index was proved only for *Citrus* EssOil from area 1, revealing its safety for macrophages. This study also demonstrated for the first time the antileishmanial activity of EssOil extracted from *Citrus limon* leaves. The EssOil interesting activity could be related to the lipophilic properties of terpenes that were shown in literature to contribute to the disruption of parasite intracellular metabolic pathways.

Keywords

Essential oil; Antileishmanial activity; Cytotoxicity; *Citrus limon*; *Pistacia lentiscus*;
Leishmania major

1. Introduction

Leishmaniasis is a tropical and sub-tropical disease caused by the protozoan *Leishmania* parasites and transmitted by the bite of infected female phlebotomine sandflies (Traoré et al. 2016). Its most common form, cutaneous leishmaniasis (CL), is endemic in 89 countries and about 0.6 to 1 million new cases occur worldwide annually (WHO, 2018). Among *Leishmania* species, *L. major* and *L. tropica* represent species that cause most CL cases in the Old World (Karami et al. 2013). Lesions caused by *L. major* appear as severely inflamed, exudative, large, and may be complicated by superficial and secondary bacterial infections. Spontaneous healing is generally observed within 2 to 8 months leaving often indelible scars on the exposed parts of the body, which usually causes serious disability and social prejudice (Aoun and Bouratbine 2014; Traoré et al. 2016).

Currently, in the absence of a vaccine, the use of pentavalent antimonial drugs, given parenterally or intra-lesionally as the first line treatment of CL, presents several limitations related to their reported toxicity (pancreatitis, cardiotoxicity, hepatotoxicity, etc.), high cost, drug resistance, and risk of relapse (Chakravarty and Sundar 2019). In case of treatment failure, alternative drugs can be used such as amphotericin B, paromomycin, pentamidine, or miltefosine. Despite their good clinical results, adverse side effects, such as nausea, anemia, fever, and possibly drug resistance, are limiting factors in their use (Akbari et al. 2017). Therefore, to reduce these side effects, different innovative formulations of amphotericin B have been developed like liposomal forms (AmBisome®), lipidic complex forms, and colloidal dispersion forms. However, their high cost is the main limiting factor of their use and is a barrier to effective disease control, especially in underdeveloped countries (Pham et al. 2013).

Consequently, the development of new drugs, less or nontoxic, more efficient, and cheaper, which would be accessible especially for low-income people, is becoming a priority (Oryan 2015). Within this framework, the WHO recommends the use of traditional medicine where

appropriate health services are inaccessible (Rocha et al. 2005). In this context, the molecule screening of medicinal plants could be a way to identify alternative natural, cheaper, and safe sources of antileishmanial molecules. Medicinal plants are source of a wide range of bioactivity due to the presence of secondary metabolites, acting through diverse modes of action. Within the group of natural products, essential oils (EssOils) are oily odorant products extracted by several extraction methods, from different parts of the plant raw material. Owing to their complex composition in terpenes, EssOils are known to have a broad spectrum of interesting biological activities including antileishmanial properties (El Asbahani et al. 2015). Moreover, their physicochemical properties, such as low density (around 0.94 g/m³) and lipophilicity, that allows a rapid diffusion through the cell membrane, can enhance the targeting of EssOils active components to intracellular parasites (Anthony et al. 2005).

The present work deals with the investigation of antileishmanial potentialities of EssOils extracted from medicinal and aromatic plants from Tunisian flora. Indeed, this latter is a rich source for bioactive molecules with interesting antiparasitic activity against different *leishmania* strains such as *L. major*, *L. infantum* or *L. amazonensis*, as many times reported in literature (Attia et al. 2019; Essid et al. 2015; Hajaji et al. 2018). Inspired by data on folk medicine and literature, two plants were chosen: *Pistacia lentiscus* (*Pistacia*) and *Citrus limon* (*Citrus*).

Pistacia lentiscus is a plant belonging to the family of *Anacardiaceae*. It is commonly used as a traditional cure for hypertension, gastrointestinal disorders (stomach aches and diarrhea), diseases of the urinary tract and the respiratory tract (asthma and chronic bronchitis), and for dental disorders (Rauf et al. 2017). Studies related to the antileishmanial potential of *Pistacia lentiscus* are very scarce. Recently, Bouyahya et al. (2019) showed the high potential against *L. major* parasites of EssOil extracted from leaves, with myrcene and α -pinene as major compounds. An interesting activity against *L. infantum* of the oil extracted from *Pistacia lentiscus* fruit (rich with limonene and α -pinene) was also described (Bouyahya et al. 2019;

Essid et al. 2015). It should be noted that minor compounds of *Pistacia l.* EssOil, especially terpinene (Pereira et al. 2017) and caryophyllene (Barros et al. 2016), were reported to have significant anti-proliferative effect by causing mitochondrial damage to the *Leishmania* genus (Machado et al. 2014). In the present work, this choice was inspired by previous work of Bachrouch et al. (2012) that showed an interesting activity of EssOil extracted from *Pistacia lentiscus* leaves against *L. donovani* parasite. This finding led to a national patent (N°TN2013/0129). Additionally, leaves of *Pistacia l.* are known in folk medicine for their wound healing activity (Aissi et al. 2016; Bozorgi et al. 2013).

In another hand, EssOil of *Citrus limon* was also selected because of its antiparasitic potential. This plant belongs to the *Rustaceae* family that is characterized by its high therapeutic potential. In folk medicine, leaves have been largely used to treat obesity, diabetes, cardiovascular diseases, and certain types of cancer (Bouzenna et al. 2016; Bruno et al. 2014). *Citrus* EssOil showed antioxidant, antibacterial, and anti-inflammatory properties that were interesting for the treatment of skin diseases (Sidana et al. 2013). Traditionally, this EssOil was frequently used to reduce skin itching and to nourish skin. Moreover, the lemon pulp could treat pimples and wrinkles or to soften facial skin (Bertuzzi et al. 2013; Otang and Afolayan 2016). Although the antileishmanial activity of *Citrus limon* was not investigated in previous studies, limonene, one of its major bioactive molecules exhibited an interesting antileishmanial activity *in vitro* against *L. major* and *L. amazonensis* parasites *in vitro*. When applied topically, limonene was effective against experimental CL due to *L. amazonensis* (Arruda et al. 2009).

In the present work, after extraction and characterization of EssOils from the selected plants, the antileishmanial activity was assessed *in vitro* against two forms of *Leishmania major* parasite, *i.e.* the axenic amastigote and the intramacrophage forms. Additionally, the cytotoxic potential of EssOils was evaluated on macrophage cells. This study allowed assessing new potential antiparasitic molecules. To our knowledge, this is the first investigation of *Citrus*

limon and *Pistacia lentiscus* EssOils from different sampling sites in Tunisia for their antileishmanial potential *in vitro*.

2. Materials and methods

2.1. Chemicals and Reagents

All chemicals and reagents used for this study were of analytical grade (98.0 – 99.9 %). Reagents were purchased from Sigma Aldrich (Saint-Quentin, France). Miltefosine (HePC) was kindly supplied by Zentaris (Frankfurt, Germany). All solutions were prepared with ultra-purified water from a Millipore Synergy 185 apparatus coupled to a RiOs5™ with resistivity of 18.2 MΩ.cm. Distilled water was used for the EssOils extraction by hydrodistillation.

2.2. Selection and collection of plant materials

Pistacia lentiscus, and *Citrus limon* were collected in two different plant areas for each (see Tunisian map in Appendix A: supplementary material, Figure 1). The harvesting period extended from November 2015 to February 2016. The various data (plant species, harvested part, sampling site, harvesting period, and voucher specimen) are gathered in Appendix A (Tables 8 and 9). The freshly cut plants were sorted out and dried at ambient temperature in a drying room with active ventilation. Then, they were packed in paper bags and stored at ambient temperature for 3 – 4 weeks before use. Plant samples were transferred to the Laboratory of Biotechnology Applied to Agriculture (INRAT, Tunisia), where voucher specimens were delivered for further references.

2.3. Essential oils extraction by hydrodistillation

Hydrodistillation is the most recommended technique for extracting volatile compounds from a plant (El Haib 2011). For the extraction, 300 g of dried and crushed plant were brought into contact with 3 L of distilled water and brought to a boil during 2 to 3 h, according to the extraction process kinetic, using a modified Clevenger-type apparatus. Each EssOil obtained was dried over anhydrous sodium sulfate and stored in amber vials at 4 °C until further analysis.

The extraction yields (EY) were calculated according to Equation 1:

$$EY (\%) = \frac{\text{weight of essential oil obtained}}{\text{weight of dried plant material}} \times 100 \quad \text{Eq. 1}$$

2.4. Antioxidant activity

The ability to generate free radicals could play a very important role in antileishmanial activity since free radicals are involved in the process of defense against parasites (Krauth-Siegel and Comini 2008; Staerck et al. 2017; Stafford et al. 2002). *Leishmania* is one of the rare protozoa capable of surviving in hostile environment created by the host (Stafford et al. 2002) because it has an antioxidant defense system, which determines its pathogenicity, and serves as protection against macrophage attack (Staerck et al. 2017). Then, a good antioxidant could be able to strengthen the radical response of macrophages by Reactive Oxygen Species (ROS) generation, and thus fight the proliferation of parasites (Krauth-Siegel and Comini 2008).

Antioxidant activity of EssOils was thus assessed using two different tests: 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonate (ABTS) assays. Both tests provide radical scavenging ability of the studied sample. However, the two reagents (DPPH and ABTS) react with different kinetics and thus give different information. In fact, the antioxidant activity of a product is always assessed using several methods because of the complexity of the involved mechanisms (Schaich et al. 2015).

2.4.1. DPPH assay

In this assay, the antioxidant activity was measured in terms of hydrogen donating or radical scavenging ability, using the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH[•]) according to the method described by Ghazouani et al. (2016). Briefly, 100 μL of each sample (essential oil or standard) were introduced into a glass tube containing 900 μL of freshly prepared methanolic DPPH solution (0.2 mM). Samples were tested at different concentrations in order to establish a calibration curve. Concentration range depended on the

EssOil antioxidant capacity. For all EssOils, preliminary tests were firstly performed at 100 mg/L. The mixture was shaken using a vortex during nearly 30 s, until obtaining a solution with a homogenous color. After an incubation time of 25 min in dark at ambient temperature, the absorbance at 524 nm was measured using a UV/visible spectrophotometer (Helios, Unicam, Cambridge, UK).

2.4.2. ABTS assay

The radical scavenging capacity of antioxidant for the ABTS reagent (2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonate) was determined following the procedure described by Yahyaoui et al. (2017). The ABTS^{•+} radical was generated by adding a 7 mM of ABTS solution at pH = 7.4 (5 mM NaH₂PO₄; 5 mM Na₂HPO₄, and 154 mM NaCl) to a potassium persulfate solution K₂S₂O₈ solution (2.45 mM), the pro-oxidant prepared in ultra-purified water. The mixture was incubated overnight at 4 °C and then diluted until reaching an absorbance at 734 nm ranging from 0.70 to 0.90. 900 μL of ABTS solution were added to 100 μL of 100 mg/L of EssOil. Solutions were mixed using a vortex and incubated for 6 minutes in the dark at ambient temperature. Measurements of absorbance were performed at 734 nm.

For DPPH and ABTS tests, the percentage of inhibition (PI) of a given sample was calculated according to Equation 2:

$$PI (\%) = \frac{(Abs_{Control} - Abs_{Sample})}{Abs_{Control}} \times 100 \quad \text{Eq. 2}$$

where $Abs_{Control}$ is the absorbance of a blank experiment carried out applying the same procedure without the EssOil. Abs_{Sample} is the absorbance of the sample. IC_{50-AA} (mg/L) is the concentration of the EssOil able to scavenge 50 % of DPPH[•] or ABTS^{•+} radicals. It is determined from PI curve as a function of concentration. Ascorbic acid was used as a standard. Measurements for each sample were realized in triplicate.

2.5. Characterization of essential oils

2.5.1. Gas Chromatography coupled with Mass Spectrometry

The EssOil (10 μ L) was dissolved in 1 mL of ethyl acetate (HPLC grade). The sample was injected into the GC (7890A) -MS (5975C inert MSD with Triple-Axis Detector) Agilent unit under the following conditions: capillary column HP-5MS ((5% phenyl) – methylpolysiloxane (60 m \times 0.25 mm, 0.25 μ m film thickness) interfaced with an Agilent mass selective detector 5975 C inert MSD. The carrier gas was N₂ at 1 mL/min. The injector temperature was maintained at 200 °C. Then, oven temperature was varied from 40 °C to 280 °C at 10 °C/min, with temperature stages at 120 °C, 280 °C, and 300 °C. The spectra were recorded in electronic impact mode (EI) with an ionization energy of 70 eV, injection volume of samples was 1 μ L. The Kovats retention index (RI) of each eluent was calculated using a series of n-alkanes (C7-C30) that were analyzed in the same conditions. The identification of compounds was achieved by comparing their mass spectra with standard spectra existing library from National Institute of Standards and Technology 08 (NIST 08), Kovats indices, and mass spectra reported in the literature. Note that compositions lower than 0.2 wt % were not considered for the molecules identification.

2.5.2. Total amount of phenolic compounds

Phenolic compounds were quantified in order to determine if there is or not a correlation between polyphenols presence and antioxidant and/or antiparasitic activities. The total amount of phenolic compounds was determined with colorimetry using Folin-Ciocalteu method described by Yahyaoui et al. (2018). This reagent consists on a mixture of phosphotungstic acid (H₃PW₁₂O₄₀) and phosphomolybdic acid (H₃PMo₁₂O₄₀) that is reduced in presence of phenols. This reaction leads to the formation of a mixture of oxides of tungsten and molybdenum colored in blue that strongly absorbs at 765 nm.

Ethanol solutions of EssOils were prepared at concentrations ranging from 0 to 26 mg/L. Then, 100 μ L of EssOil solutions were transferred into tube then mixed thoroughly with

500 μ L of Folin reagent (Sigma Aldrich, France) previously diluted in ultra-purified water at 1:10 ratio. The mixture was shaken for 30 s using a vortex then incubated for 6 min in the dark. A total of 400 μ L of sodium carbonate (7.5 w/v %) was then added and mixed thoroughly for 30 s. The mixture was incubated in the dark for 15 min. The absorbance was then measured using UV/visible spectrophotometer (Helios, Unicam, Cambridge, U.K.) at 765 nm against a blank of distilled water (Yahyaoui et al. 2018).

For each sample, a control experiment was carried out applying the same procedure to a solution without the EssOil. In this assay, gallic acid, which is a phenolic acid characterized by a carboxylic function and phenolic hydroxyls, was used as a standard. A calibration curve was then obtained for gallic acid aqueous solutions at concentrations ranging from 0 to 30 mg/L. Results were expressed in terms of milligrams of gallic acid equivalent per gram of dry mass sample (mg GAE/g). All measurements were realized in triplicate.

2.6. Antileishmanial activity

2.6.1. Parasite cultures

Leishmania major (MHOM/PT/92/CRE26) promastigote forms were maintained in culture M-199 medium (Sigma, Saint Quentin Fallavier, France) supplemented with 40 mM HEPES, 100 μ M adenosine, 0.5 mg/L hemin, 10 % fetal bovine serum, and 50 μ g/mL gentamycin at 26 °C in a dark environment, under an atmosphere of 5 % CO₂. One day before the treatment, differentiation of promastigotes into axenic amastigotes was achieved by dilution of 1×10^6 promastigotes in stationary phase of growth in 5 mL of axenic amastigote medium at pH 5.5 (15 mM KCl, 8 mM glucose, 5 mM glutamine, $1 \times$ M199, 2.5 BBL™ trypticase™ peptone, 4 mM hemin, 10 % fetal bovine serum, 2 mM CaCl₂, and 2 mM MgCl₂). Axenic amastigotes were grown under 37 °C and 5 % CO₂.

2.6.2. *In vitro* antileishmanial activity on axenic amastigote forms

L. major axenic amastigote forms were suspended to yield 1×10^6 cells/mL and the assays were performed in 96-well microtiter plates under a volume of 100 μ L per well. EssOils,

priorly diluted in ethanol, were added at concentrations ranging from 100 to 10^{-4} $\mu\text{g/mL}$ obtained by serial dilution. Miltefosine (HePC) was used as a reference compound with final concentrations ranging from 100 μM to 97.6 nM obtained by serial dilution. Triplicates were performed for each concentration. Cultures were incubated at 37 °C in the dark under 5 % CO_2 atmosphere. Then, plates were subjected to 3 freeze/thaw cycles. Amastigotes viability was assessed using the SYBR[®] green I incorporation method. This method consists in evaluating the parasite growth using SYBR[®] Green I, which is a dye with marked fluorescence enhancement upon contact with parasite DNA. The parasite lysis suspension was diluted at 1:1 in lysis buffer (10 mM NaCl, 1 mM Tris HCl pH 8, 2.5 mM EDTA pH 8, 0.05 % SDS, 0.01 mg/mL proteinase K, and 10 X SYBR Green I) in qPCR plates. Fluorescence was measured with Mastercycler[®] ep realplex (Eppendorf, France) according to the following procedure to increase the SYBR Green incorporation: 90 °C for 1 min, decrease in temperature from 90 °C to 10 °C during 5 min with fluorescence reading. This experiment was processed thrice. Antileishmanial activity against amastigote axenic form was expressed as 50 % inhibitory concentration of growth (IC_{50}) calculated using ICEstimator[®] (<http://www.antimalarial-icestimator.net/MethodIntro.htm>).

2.6.3. *In vitro* antileishmanial activity on intramacrophage amastigote forms and cytotoxicity evaluation

The mouse monocyte/macrophage cell line Raw 264.7 was maintained in Dulbecco's Modified Eagles Medium (DMEM, Applied Biosystems) supplemented with 10 % heat-inactivated fetal bovine serum. Raw 264.7 cells were seeded into a 96-well microtiter plate at a density of 20,000 cells/well in 100 μL of DMEM. After incubation in a 5 % CO_2 incubator at 37 °C for 2 h, the culture medium was replaced by 100 μL of fresh DMEM, supplemented with 2 mM CaCl_2 and 2 mM MgCl_2 , containing a suspension of 3.2×10^6 promastigotes in stationary phase of growth, corresponding to 16 parasite-to-cell ratio. After 12 h incubation, the macrophage infection was checked using an inverted microscope (DMIL Led, Leica

Microsystems®). Culture medium was replaced by fresh DMEM, then, EssOils dilution in DMEM was processed in the same way as for the axenic test (see in the “In vitro antileishmanial activity on axenic amastigote forms” section). Miltefosine, the positive control, was also diluted to reach concentrations ranging from 100 µM to 97.6 nM. Samples were then transferred in plates containing infected macrophages. Both infected and non-infected macrophages without any drug were used as negative controls. Non-infected macrophages treated with EssOils or miltefosine allowed to determine their cytotoxicity. Plates were left 72 h at 37 °C in 5 % CO₂. After incubation, the cells were visualized using an inverted microscope to check their morphology or the presence of non-internalized free parasites. The medium was subsequently removed and replaced with the lysis buffer. Plates were subjected to 3 freeze/thaw cycles. Parasites growth or cells viability were determined using SYBR® Green I incorporation as previously described in the “In vitro antileishmanial activity on axenic amastigote forms” section. Macrophage alone or infected and non-treated macrophages were used as controls.

Experiments were carried out three times at least. Antileishmanial activity and cytotoxicity were expressed as 50 % inhibitory concentration (IC₅₀ and CC₅₀, respectively) and calculated using ICEstimator®, as described previously. Moreover, selectivity index (SI) was calculated according to Equation 3 in order to assess the selectivity of action of the treatment towards parasites.

$$SI = \frac{CC_{50}}{IC_{50}} \quad \text{Eq. 3}$$

2.7. Statistical analysis

Antioxidant activity, antileishmanial activity, cytotoxicity, and the total phenolic compounds amount were expressed as mean ± standard deviation (SD). All measurements were analyzed using one-way ANOVA (analysis of variance). Duncan test was then applied to the means to identify significant differences at a percent level of 0.05. These statistical analyzes were performed with the IBM SPSS Statistics 21.0 software®.

3. Results and discussion

3.1. Extraction yields

Yields of all extracted EssOils are shown in Table 1.

Pistacia l. exhibited the lowest extraction yield. It is worth noting that for *Pistacia* EssOils extracted from leaves, yields were not largely reported in literature. The study of Zaouali et al. (2018) showed that extraction yield in oil from Tunisian *Pistacia* leaves was around 0.15 %, which is 10-fold higher than our results.

Table 1 EssOils extraction yields.

Plant	Harvest area	Extraction yield (wt %)
<i>Citrus limon</i>	Area 1	0.48 ± 0.09 ^a
	Area 2	0.25 ± 0.06 ^b
<i>Pistacia lentiscus</i>	Area 1	0.06 ± 0.01 ^c
	Area 2	0.06 ± 0.05 ^c

One-way ANOVA with Duncan post-test. Means values followed by different letters (a to c) within column for a given plant are significantly different ($p < 0.05$).

This may be due to difference between harvest periods. Indeed, seasonal variation is usually associated with the variation of yield in oils because of the variation of a set of bioclimatic and environmental factors such as air temperature, wind exposure, air humidity, water precipitation, sunlight, and UV radiation intensity, etc. From one year to another, environmental changes submit plants to modifications in morphology, physiology, and productivity that aim to prevent damage and adapt to stress conditions (Aissi et al. 2016).

Despite the differences in geographic, climatic, and ecological parameters, no notable yields variation ($p > 0.05$) was showed for *Pistacia* species. However, for *Citrus l.*, yield was about 2-fold higher in the area 1 in comparison with area 2. To our knowledge, extraction yield of *Citrus l.* EssOil from leaves has never been reported in previous works. Extraction yield strongly depends on the selected part of the plant. Significantly lower yields were obtained for *Citrus l.* flower (0.05 – 0.5 %), whereas, *Citrus l.* peels showed 10-fold greater yields around

2.5 % (Arteche Garcia et al. 1998). Nevertheless, we selected leaves EssOil for a matter of originality.

3.2. Chemical composition

The knowledge of the chemical composition of plant extracts is sought, not only for discovering new therapeutic substances but also for understanding the value of folkloric remedies. Prior to biological activities assessment, EssOils were subjected to GC-MS analysis in order to determine their compositions.

Chemical composition of EssOils extracted from *Citrus l.* leaves is given in Table 2. Seventeen components were identified representing 98.8 % of the total composition of EssOils from area 1 and from area 2. EssOil prepared from *Citrus l.* of area 1 was characterized by a high content of neryl-acetate (29.5 %), followed by nerol (13.8 %), D-limonene (12.9 %), geranial (7.8 %), geranyl acetate (6.6 %), and neral (5.8 %). In contrast, EssOil extracted from *Citrus l.* of area 2 presented D-limonene chemotype with a content of 30.0 %. Moreover, other compounds were present in high percentage as neryl acetate (16.7 %) followed by β -pinene (12.8 %), nerol (6.6 %), and geranyl acetate (6.5 %). Composition difference between these EssOils could be more clearly illustrated by the classification of terpenes family. Indeed, for *Citrus* EssOil from area 1, composition was mainly dominated by oxygenated monoterpenes (~ 76 %). However, *Citrus* EssOil from area 2 composition demonstrated that it contains similar amount of non-oxygenated (47.5 %) and oxygenated monoterpenes (48.2 %). For both *Citrus* EssOils, sesquiterpenes (oxygenated or not) were present at low content (< 4 %).

Table 2 Composition of *Citrus limon* EssOils extracted from areas 1 and 2 determined by GC-MS.

Compounds	Rt (min)	RI	Relative composition (%)	
			Area 1	Area 2
α -pinene	5.07	948	-	0.8
β -thujene	5.61	969	-	2.6
β -pinene	5.69	972	3.2	12.8

β -myrcene	5.81	977	-	1.2
D-limonene	6.41	1000	12.9	30.0
linalool	7.42	1038	2.1	2.6
citronellal	8.36	1074	3.4	3.8
(-)-terpinen-4-ol	8.96	1097	1.7	1.1
α -terpineol	9.23	1108	1.8	1.1
nerol	10.05	1238	13.8	6.6
neral	10.41	1252	5.8	3.4
geraniol	10.73	1264	3.5	2.5
geranial	11.26	1284	7.8	4.1
neryl acetate	14.07	1352	29.5	16.7
geranyl acetate	14.54	1382	6.6	6.5
caryophyllene	15.52	1506	2.7	2.1
α -bergamotene	15.78	1512	0.5	-
β -bisabolene	17.13	1544	0.8	-
(-)-spathulenol	18.34	1572	0.7	0.9
methyl arachidonate	18.45	1575	2.0	-
Total (%)			98.8	98.8
<i>Non oxygenated monoterpenes</i>				
(%)			16.1	47.5
<i>Oxygenated monoterpenes (%)</i>			76.1	48.2
<i>Non oxygenated sesquiterpenes</i>				
(%)			4.0	2.1
<i>Oxygenated sesquiterpenes (%)</i>			0.7	0.9
<i>Oxygenated diterpenes (%)</i>			2.0	-

Rt: retention time; RI: retention index; (-): < 0.2%. In bold: major compound (chemotype)

In the literature, only a small number of studies dealt with the composition of *Citrus Limon* EssOil extracted from leaves. Results of *Citrus* EssOil composition from area 2 are in accordance with the composition reported by Lota et al. (2002), characterized by limonene as major component, followed by β -pinene, and other components such as geranial or neral at appreciable levels. However, according to Vekiari et al. (2002), citral compounds (geranial and neral) are the predominant constituents of this EssOil followed by limonene, neryl acetate, geranyl acetate, and β and α -pinene. Besides, Njoroge et al. (1994) reported that Japanese *Citrus* EssOil extracted from leaves was mainly constituted of geranial followed by

limonene and neral. Generally, neral, geranial, geranyl acetate, and neryl acetate are characteristic of *Citrus* EssOil extracted from leaves and are in significantly lower quantities in EssOil extracted from the fruit peels (Lota et al. 2002; Vekiari et al. 2002). These components contribute significantly to the flavor and aroma of lemon. Their concentration is by far the most important index to determine the commercial value of lemon oil (Lota et al. 2002; Vekiari et al. 2002). High content in these components in *Citrus* EssOil from area 1 indicates that this EssOil is of high quality and is linked to its powerful and characteristic lemon aroma.

The composition of EssOils extracted from *Pistacia lentiscus* leaves from area 1 and 2 are presented in Table 3. Thirty-one and twenty-two components were identified representing 97.7 % to 97.1 % of EssOils composition of area 1 and area 2, respectively.

Table 3 Composition of *Pistacia lentiscus* EssOils extracted from areas 1 and 2 determined by GC-MS.

Compounds	Rt (min)	RI	Relative composition (%)	
			Area 1	Area 2
sabinene	4.95	944	-	1.5
α -phellandrene	4.96	945	3.2	-
α -pinene	5.07	948	16.9	13.6
camphene	5.29	957	2.0	2.3
β -phellandrene	5.62	969	2.4	-
β -pinene	5.69	972	3.2	6.2
β -myrcene	5.81	977	11.3	30.0
O-cymene	6.35	997	4.5	3.1
D-limonene	6.42	1000	4.9	5.4
isopentyl alcohol	6.74	1012	1.1	0.9
β -nonanone	7.30	1034	0.8	-
nitrofurazone	7.32	1036	-	0.7
2-ethylbutyl alcohol	7.41	1038	0.6	-
isopentyl isovalerate	7.49	1041	1.6	1.5
terpinen-4-ol	8.96	1097	4.4	1.3
α -terpineol	9.22	1107	1.6	1.1
α -thujenal	9.42	1115	-	-

bornyl acetate	11.87	1209	2.0	2.1
2-undecanone	11.99	1220	1.6	-
copaene	14.51	1483	1.4	-
β -elmene	14.86	1491	-	1.2
caryophyllene	15.52	1506	3.3	5.6
α -caryophyllene	16.21	1522	1.5	1.6
spatulanol	16.35	1526	0.9	-
γ -cadinene	16.61	1532	3.3	2.6
germacrene D	16.73	1535	-	5.7
β -cubebene	16.72	1534	1.1	-
α -elemene	16.96	1540	1.0	-
α -amorphene	17.29	1548	4.0	1.8
cadina-1(2),4-diene	17.43	1551	3.0	-
α -calacorene	17.77	1559	0.7	-
(-)-spathulenol	18.34	1572	0.6	1.6
caryophyllene oxide	18.44	1575	5.1	-
γ -cardinene	19.24	1593	-	5.3
τ -muurolol	19.24	1595	4.3	-
α -cadinol	19.42	1597	4.4	2.2
α -bisabolol	19.76	1605	1.1	-
Total (%)			97.8	97.10
<i>Non oxygenated monoterpenes (%)</i>			48.4	62.0
<i>Oxygenated monoterpenes (%)</i>			8.0	4.4
<i>Non oxygenated sesquiterpenes (%)</i>			19.8	25.4
<i>Oxygenated sesquiterpenes (%)</i>			15.9	2.2

Rt: retention time; (-): < 0.2%. In bold: major compound (chemotype)

Pistacia EssOil from area 1 was characterized by a high content of α -pinene (16.9 %), followed by β -myrcene (11.3 %), D-limonene (4.9 %), o-cymene (4.5 %), terpinen-4-ol (4.4 %), β -pinene (3.2 %), and α -phellandrene (2.4 %). However, EssOil from area 2 exhibited a chemotype of β -myrcene (~ 30 %). Other components were also present in appreciable quantities such as α -pinene (13.6 %), β -pinene (6.2 %), and D-limonene (5.4 %). However, these two EssOils had more similar composition in terms of terpene classes. Indeed, for both *Pistacia* leaves EssOils, non-oxygenated monoterpenes were the most abundant with

a content between 48 and 62 %, followed by the non-oxygenated sesquiterpenes that did not exceed 25.5 %. Nevertheless, *Pistacia* EssOil from area 1 was slightly more abundant in oxygenated monoterpenes and sesquiterpenes. In the literature, there are insufficient data on EssOils composition of *Pistacia* genus. Only few species have so far been studied. EssOil extracted from leaves has been described in several studies to be rich in α -pinene, among other common constituents including β -myrcene, β -pinene, carvacrol, limonene, β -caryophyllene, verbenone, α -terpineol, and linalool (Rauf et al. 2017). Regarding *Pistacia l.* from Tunisian flora, some previous studies described the EssOil composition extracted from leaves harvested from the same collection sites as ours. Regarding *Pistacia* from area 1, authors reported an EssOil composition considerably different from our results, rich in terpinen-4-ol, β -caryophyllene, and α -terpineol (Bachrouh et al. 2010). Aissi et al. (2016) found that α -pinene was the most abundant compound of *Pistacia* EssOil from area 2 followed by limonene, terpinen-4-ol, β -caryophyllene, germacrene, and γ -cadinene. These results are not in accordance with ours. This difference could be due to many reasons such as differences in procedures applied: drying conditions of the plant material, extraction duration, EssOil storage conditions, etc. Furthermore, significant differences in composition could be noticed for EssOils harvested from different sampling areas. It seemed that plant genetic characteristics and environmental conditions such as geography, altitude, temperature, day length, and nutrients might play a key role in the synthesis of bioactive molecules and thus in extract composition. All these parameters have an impact on the biosynthesis pathways and then on the main characteristic molecules and their percentage (Aissi et al. 2016; Amhamdi et al. 2009; Asai et al. 2017). For example, EssOils composition can be affected by seasonal variations such as water restriction, luminosity rate or the presence of predators altering the secondary metabolism of the plant. These variations may alter the EssOil composition throughout the year (Bitu et al. 2015). On the other hand, studies highlighted the influence of seasonality on the *in vitro* biological efficiency, such as the antifungal activity of *Psidium*

myrtooides and *Psidium saluarte* EssOils (Macedo et al. 2018; Macedo et al. 2020). Therefore, understanding the environmental conditions of the plant and the seasonal events that contribute to alter the quality of active compounds are essential to support pharmacological studies aiming to investigate EssOil greatest therapeutic potential. Interestingly, the odor of the two samples of EssOils from area 1 and area 2 was different, which reflected a difference in composition of volatile molecules.

3.3. Phenolic compounds and antioxidant activity of essential oils

Total phenolic compounds determined for all extracted EssOils are listed in Table 4.

Results showed that phenolic compounds did not exceed 5 equivalents of gallic acid per gram of dried extract for all EssOils.

Table 4 Total phenolic compounds (TFC) of different EssOils.

	<i>Citrus limon</i>		<i>Pistacia lentiscus</i>	
	Area 1	Area 2	Area 1	Area 2
TFC (EGA/g)	3.90 ± 0.42	0.21 ± 0.2	0.48 ± 0.14	2.99 ± 0.28

TFC, total phenolic compounds, expressed in (EGA/g) equivalent of Gallic Acid per gram of dried extract.

Regarding the antioxidant activity, the inhibition concentration at 50 %, IC_{50-AA}, (expressed in mg/L) for all EssOils samples and assessed by DPPH and ABTS tests are shown in Table 5.

The reference antioxidant was ascorbic acid (vitamin C).

Table 5 Antioxidant activity of different EssOils determined by DPPH and ABTS tests.

IC _{50-AA} (mg/L)	Reference Vitamin C	<i>Citrus limon</i>		<i>Pistacia lentiscus</i>	
		Area 1	Area 2	Area 1	Area 2
DPPH	3.1 ± 0.2	> 100	> 100	> 100	> 100
ABTS	2.1 ± 0.2	> 100	> 100	> 100	> 100

IC_{50-AA}, inhibition concentration at 50 %

Compared to ascorbic acid, no significant activity was found for all EssOils of the two plant species. This may be due to the dominance of non-phenolic compounds in these EssOils (Tables 2 and 3). This was confirmed by Folin-Ciocalteu test that showed that these EssOils did not contain significant amount of polyphenols (Table 4).

Phenolic compounds and terpenes with conjugated double bonds are responsible of substantial antioxidant activity (Dhifi et al. 2016). As shown in the previous section, chemical compositions of extracted EssOils (Tables 2 and 3) are abundant in non-oxygenated monoterpenes such as α -pinene, β -myrcene or limonene. These monoterpenes hydrocarbons have no antioxidant effect (Frag et al. 1989).

The presence of hydroxyl group could play a role in radical scavenging activity of an EssOil (Frag et al. 1989) However, geranyl acetate, present in *Citrus* EssOil, did not exhibit any antioxidant activity (Quintans-Junior et al. 2013). Other compounds in *Citrus* EssOil could present an important antioxidant activity thanks to the phenolic structure such as for nerol and geraniol. These phenolic compounds have redox properties and could play an important role in neutralizing free radicals (Dhifi et al. 2016). However, it seems that they were not present in sufficient amount in our samples to exert a significant antioxidant activity.

3.4. Antileishmanial activity in axenic amastigotes and intramacrophage amastigotes, and cytotoxicity

All EssOils were tested for their antileishmanial activity against *L. major* parasite (axenic amastigotes form and intramacrophage amastigotes). A sample was considered highly effective against the parasite when IC₅₀ value was lower than 10 μ g/mL, and moderately effective when IC₅₀ was between 10 and 20 μ g/mL. A sample with IC₅₀ value higher than 20 μ g/mL was not considered as promising. The cytotoxicity of all EssOils was also evaluated by calculating the selectivity index (SI). An EssOil was considered as selective to parasite and safe to macrophage when its SI value was higher than 10 (Le et al. 2017).

Results presented in Table 6 showed that IC₅₀ obtained for both axenic and intramacrophage amastigote forms were significantly different ($p < 0.05$) depending on the harvesting region. This could be attributed to the difference of EssOils composition induced by the different environmental and climatic conditions that the plant underwent (previously described in the “Chemical composition” section).

Table 6 Antileishmanial activity and cytotoxicity of studied EssOils and miltefosine (HePC) as a reference compound.

Plant/harvest area		IC ₅₀	IC ₅₀	CC ₅₀ (µg/mL)	Selectivity index (SI)
		Axenic amastigotes (µg/mL)	Intramacrophage amastigotes (µg/mL)		
<i>Citrus limon</i>	Area 1	12.1 ± 1.8 ^{a,A}	4.2 ± 1.3 ^{ab,A}	49.6 ± 7.2 ^{a,B}	11.7
	Area 2	24.0 ± 3.6 ^{b,A}	13.8 ± 0.7 ^{b,B}	48.2 ± 4.2 ^{a,C}	3.5
<i>Pistacia lentiscus</i>	Area 1	0.5 ± 0.2 ^{a,A}	12.5 ± 1.4 ^{b,B}	68.5 ± 4.2 ^{b,C}	3
	Area 2	56.1 ± 20.5 ^{c,A}	35.6 ± 0.7 ^{c,A}	> 100 ^{c,B}	> 1
<i>HePC</i> [*]		2.7 ± 0.4 ^{a,A}	2.0 ± 0.6 ^{a,A}	62.1 ± 4.4 ^{b,B}	31.4

^{a-c} Means within a column with different letters were significantly different ($p < 0.05$).

^{A-C} Means within a line with different letters were significantly different ($p < 0.05$).

HePC, Hexadecylphosphocholine (miltefosine), the positive control.

*IC₅₀ and CC₅₀ of HePC are expressed in µM.

The best growth inhibitory effect on axenic amastigotes form of the parasite was observed for *Citrus* EssOil from area 1 and *Pistacia* EssOil from area 1. The antileishmanial activities of these EssOils were comparable ($p > 0.05$) to the activity of miltefosine, the reference drug. However, *Citrus* EssOil from area 2 and *Pistacia* EssOil from area 2 did not show any interesting inhibitory activity against axenic amastigote form of *L. major*, as their IC₅₀ values were higher than 20 µg/mL. Concerning the activity against intramacrophage amastigote form, *Citrus* EssOil from area 1 exhibited the best inhibitory effect with IC₅₀ < 10 µg/mL. Moreover, this activity was close to the IC₅₀ value obtained with miltefosine ($p > 0.05$). However, EssOils of *Citrus* area 2 and of *Pistacia* area 1 presented a moderate activity with IC₅₀ values ranging between 11 and 15 µg/mL. Moreover, as for the axenic amastigotes form, no significant activity ($p > 0.05$) was noticed for EssOil of *Pistacia* from area 2 against the intracellular amastigotes form.

According to these results, antileishmanial activity varied significantly from the axenic to intramacrophage amastigotes forms. Indeed, *Citrus* EssOil from area 2 was significantly more active ($p < 0.05$) against the intracellular form since the IC₅₀ value was 2-fold lower than that for the axenic form. Consequently, the antileishmanial potential may be dependent on the

proper cell functions of macrophages. In this context, De Muylder et al. (2011) tested the antileishmanial activity of naloxonazine. This compound exhibited no activity against axenic amastigote form but showed a good effect against intracellular amastigotes. Naloxonazine has an indirect action on pathogens *via* macrophage activation. Currently, this kind of compounds is of a particular interest for targeting host factors that are essential for parasite development. This emerging strategy is assumed to be less prone to induce drug resistant pathogens. It also offers the possibility to repurpose drugs by exploiting compounds currently used for diseases unrelated to microbial infections (De Muylder et al. 2011).

Conversely, IC₅₀ values could also increase from the free-living amastigote to intracellular amastigote. This case was observed for *Pistacia* EssOil from area 1 that exhibited a high inhibitory effect on the axenic form and a moderate inhibitory effect against intramacrophage form. This could be explained by the localization of the parasite inside the parasitophorous vacuole that constitutes a serious hurdle for small molecules, like terpenes in our case. Indeed, active molecules need to cross three biological barriers and resist to both neutral and acidic pH (De Rycker et al. 2013). It is important to point out that in literature, requirements of specific compounds to display activities against intracellular forms are higher (Leal et al. 2013). Nevertheless, compounds that exert an effect only against the free amastigote form may be of interest since these small molecules may be able to reach the vacuole and exhibit their antiparasitic effect after further chemical optimization. Other alternative delivery strategy, like encapsulation and targeting, could also be promising for such compounds (Leal et al. 2013).

It is relevant to consider treatment toxicity on macrophage cells. SI values showed that EssOil of *Citrus* from area 1 exhibited a selective antiparasitic effect with SI values higher than 10 (SI ~ 12). Thus, this EssOil is considered as safe for macrophages. However, despite the good antiparasitic effect of *Citrus* EssOil from area 2 and *Pistacia* EssOil from area 1, high cytotoxicity was observed with low selectivity values of 3.5 and 3, respectively. This study

demonstrated that EssOil of *Citrus* from area 1 may be a promising source for active molecules against *Leishmania major* parasite.

To have a general overlook at antileishmanial activity and cytotoxicity of the studied EssOils and to analyze the observed effects, we gathered in Table 7 literature data of EssOils extracted from these plants and their major compounds.

For the best of our knowledge, *Citrus Limon* EssOil was analyzed in our study for the first time for its antileishmanial potential. As seen above, the EssOil from *Citrus limon* leaves exhibited good growth inhibitory effect against especially intracellular *L. major* regardless the harvesting region. Unfortunately, the activity of EssOil of area 2 was cytotoxic and not selective to parasites as SI value was by far less than 10. In literature, it has been shown that *Citrus limon* EssOil were cytotoxic on LIM1863 colon carcinoma cell line with low CC₅₀ values ranging from 5.75 to 7.92 µg/mL (Jomaa et al. 2012). Besides, *Citrus* EssOil from area 1 was characterized by a remarkable intracellular activity and a good selectivity of action on *L. major*. The difference between *Citrus* EssOils from both areas is obviously due to the difference in composition of these EssOils obtained from different sites. *Citrus* EssOil from area 1 was characterized by higher content on neryl-acetate (29.5 %), nerol (13.8 %), and citral compounds (total of 14.4 %) than area 2. Among these compounds, only citral compounds, including geranial and neral, were widely studied for their antileishmanial activity. However, tests were basically performed on the promastigote stage of different *Leishmania* species. Citral did not reveal a significant growth inhibitory effect against *L. major*, *L. tropica*, *L. infantum* promastigotes, and *L. amazonensis* amastigotes (Machado et al. 2012; Santin et al. 2009). However, it exhibited good inhibitory effect on *L. amazonensis* and *L. donovani* promastigotes (Santin et al. 2009; Zheljzkov et al. 2008). Ultrastructure characterization performed with Transmission Electron Microscopy revealed that protozoa of *L. amazonensis* treated with citral compounds showed drastic alteration in their morphology, such as the presence of exocytic projections in the flagellar pocket, and swollen mitochondria,

with plasma membrane blebs seeming to detach from the parasite surface (Santin et al. 2009). These findings could indicate a specific activity of citral compounds related to the *Leishmania* live-stage and species. Hence, citral might contribute to the good inhibitory effect of *Citrus* EssOil from area 1 in our study. Note that citral compounds were also present in *Citrus* EssOil from area 2 but not in appreciable amount. On the other hand, citral did not exhibit a cytotoxic effect on the parasite host cell (Raw 264.7) (Machado et al. 2012) but revealed a toxic action on J774 G8 and kidney epithelial cells (Santin et al. 2009; Zheljzakov et al. 2008). Therefore, the cytotoxic effect of the extracted EssOil should further be investigated in different cell lines to confirm its safety. Moreover, Arruda et al. (2009) showed that limonene exhibited significant activity against *L. major* amastigote intramacrophage form and *L. amazonensis*-infected macrophages (with 78 % reduction in infection rates). *In vitro* antiparasitic activity evaluation of limonene showed that this non-toxic anticancer monoterpene efficiently inhibited the non-mevalonate pathway of isoprenoid synthesis in parasites (*Plasmodium falciparum*) (Moura et al. 2001). Antileishmanial efficacy was also assessed in cutaneous leishmaniasis experimental model in BALB/c infected mice. Treated topically or intrarectally with limonene, mice exhibited significant reduction in lesion area and significant decrease in parasite load. Moreover, limonene was able to enhance the percutaneous permeation of drugs (Arruda et al. 2009). These findings could explain the moderate activity against intracellular *L. major* observed for *Citrus* EssOil from area 2 whose major compound was limonene.

Regarding *Pistacia lentiscus* EssOils, a previous study confirmed the interesting activity of this EssOil from Tunisian flora with IC₅₀ ranged between 4 and 7.5 µg/mL (Bachrouh et al. 2012). More recently, a study proved that EssOil extracted from *Pistacia* leaves with myrcene and α-pinene as major compounds displayed an important activity against *Leishmania* species, especially *L. major* (Bouyahya et al. 2019). α-pinene, the major compound of the active *Pistacia* EssOil from area 1 (16.7 %), also exhibited a moderate antileishmanial

activity against two species of promastigote, *L. infantum* and *L. major*, with IC₅₀ values of 17.60 ± 0.88 µM (Essid et al. 2015) and 19.80 ± 0.23 µM (Mikus et al. 2000), respectively. However, activity was not noticed for other compounds present in appreciable amount in *Pistacia* EssOil from area 1, such as β-myrcene, o-cymene, terpinen-4-ol and phellandrene (Escobar et al. 2010; Essid et al. 2015; Machado et al. 2012; Mikus et al. 2000; Sibanda et al. 2004). Conversely, minor compounds in *Pistacia* EssOil such as caryophyllene were reported to inhibit efficiently *Leishmania* genus (Pereira et al. 2017). Caryophyllene and caryophyllene, present at low amount (< 6%) in the active EssOil (*Pistacia* area 1), caused a mitochondrial damage to both promastigotes and amastigotes of *Leishmania*. This mechanism of action was mainly attributed to the inhibition of isoprenoids biosynthesis (Santos et al. 2008). Interesting activity observed for *Pistacia* EssOil of area 1 could also be attributed to a synergistic effect between different terpenes. In addition, major compounds presented a considerable toxicity for several cell lines such as Vero and Raw 80 (Escobar et al. 2010; Essid et al. 2015). This could explain the cytotoxicity observed for *Pistacia* EssOils in our study.

The mechanism by which EssOils exert inhibition on parasite growth is still obscure because specific cellular targets have not been identified yet. However, previous studies have suggested that the antileishmanial activity is related to cell membrane disruption by virtue of essential oils composition in terpenes that contribute to their lipophilic characteristics. Indeed, these diverse compounds led to multiple mechanisms of action affecting either polysaccharides or fatty acids and phospholipids layers in plasma membranes of *Leishmania* parasites, which results in lyses and release of molecules (Pereira et al. 2017). Terpenes properties allow their diffusion through the parasite cell membrane affecting intracellular metabolic pathways and organelles (Brenzan et al. 2007; Ultee et al. 2002). Moreover, other studies indicated that EssOils activity could also be linked to the capacity of an EssOil to interact with mitochondrial membranes by generating free radicals that oxidize parasite

macromolecules leading ultimately to its death by apoptosis or necrosis (Tariku et al. 2010; Ultee et al. 2002). It is important to point out that the latter hypothesis did not correspond to the mechanism of action of EssOils studied here. Indeed, we proved in this work, through antioxidant activity assessment and polyphenols quantification, that none of the EssOils showed an ability to generate free radicals entities. Thus, the interesting antileishmanial activity of the studied EssOils could probably be attributed to the lipophilic properties of terpenes.

Table 7 Profile of *in vitro* antileishmanial activity and cytotoxicity of EssOils and their major compounds from *Citrus limon* and *Pistacia lentiscus* in literature.

Plant	Whole plant EssOil / Major compounds	Antileishmanial activity		Cytotoxicity		Ref.
		<i>L. species/form</i>	IC ₅₀ (µg/mL or µM) *	Cell line/animal	CC ₅₀ /LD ₅₀	
<i>Citrus limon</i>	Whole EssOil	nd	nd	LIM1863	5.75 - 7.92 µg/mL	(Jomaa et al. 2012)
	Limonene	<i>L. major/ama</i>	354 ± 33	LLCMK2	> 1012 µM	(Arruda et al. 2009)
		<i>L. amazonensis/ama</i>	< 300			
		<i>L. chagasi/prom</i>	185 ± 19	HEK-293		
		<i>L. braziliensis/prom</i>	201 ± 17			
	β-pinene	<i>L. major/prom</i>	200.1	HL-60	29.6 µg/mL	(Mikus et al. 2002)
		<i>L. donovani/prom</i>	19	Kidney epithelial cells	22.4 µg/mL	(Zheljazkov et al. 2008)
	Citral: Neral/Geraniol	<i>L. infantum/prom</i>	42	Raw 264.7	No toxicity	(Machado et al. 2012)
		<i>L. amazonensis/prom</i>	8.0 ± 0.1	J774G8	50.0 ± 0.1 µg/mL	(Santin et al. 2009)
		<i>L. amazonensis/ama</i>	25.0 ± 0.3			
		<i>L. major/prom</i>	36	Raw 264.7	No toxicity	(Machado et al. 2012)
<i>L. tropica/prom</i>		34				
Geranyl acetate	nd	nd	Rat	6330 mg/kg	(Jenner et al. 1964)	
<i>Pistacia lentiscus</i>	Whole EssOil	<i>L. donovani/prom</i>	4 -7.5	nd	nd	(Bachrouch et al. 2012)
		<i>L. major/prom</i>	17.52 ± 1.26	nd	nd	(Bouyahya et al. 2019)
		nd	nd	SH-SY5Y SK-N-BE (2) C	13.6 ± 0.5 µg/mL 26.3 ± 1.2 µg/mL	(Piccolella et al. 2016)
	α-pinene	<i>L. major/prom</i>	19.80 ± 0.23	HL-60	2.5 µg/mL	(Mikus et al. 2000)
		<i>L. infantum/prom</i>	17.60 ± 0.88	Raw 80	230.3±1.3 µg/mL	(Essid et al. 2015)
	β-myrcene	<i>L. infantum/prom</i>	164	Raw 264.7	No toxicity	(Machado et al. 2012)
		<i>L. major/prom</i>	219.17 ± 0.50	Raw 80	nd	(Essid et al. 2015)
	Cymene	<i>L. infantum/prom</i>	156.17 ± 0.45	nd	115.4 µg/mL	(Escobar et al. 2010)
		<i>L. chagasi/prom</i>	149.1 ± 1.0	Vero	157.3 µg/mL	
		<i>L. chagasi/ama</i>	> 30	THP-1	nd	
	Terpinen-4-ol	<i>L. major/prom</i>	335.9	Hs 578T	No toxicity	(Sibanda et al. 2004)
HL-60				20.5 µg/mL	(Mikus et al. 2000)	
Phellandrene	<i>L. major/prom</i>	32.8	HL-60	29.6 µg/mL	(Mikus et al. 2000)	

*: IC₅₀ expressed in µg/mL for essential oils and in µM for major compounds; **Prom**: promastigote; **ama**: amastigote; **nd**: not determined

4. Conclusion

This study showed the high potential of the EssOil extracted from leaves of *Citrus limon* from area 1 of Tunisia to inhibit the *Leishmania major* (axenic and intracellular amastigotes) growth, without having a cytotoxic effect on host cell. Selective activity proved the safety of this EssOil for macrophages. This study demonstrated for the first time the antileishmanial activity of EssOil extracted from *Citrus* leaves. This interesting result could be linked, either to the activity of major compounds previously reported such as citral compounds, or to synergistic effect between different EssOil compounds. On the other hand, *Pistacia* EssOil from area 1 displayed a moderate antileishmanial activity with important toxicity towards macrophages. Despite this observed cytotoxicity, the encapsulation of this EssOil could be a promising strategy, which could eventually reduce its toxicity and therefore improve its antiparasitic efficacy. The promising results of this study offer prospects for further investigations such as the antileishmanial evaluation of major compounds of active EssOils, and their mixture. A bioguided fractionation of EssOils could allow identifying molecules that are responsible of the observed effects. Moreover, an assessment of EssOils activity against other *Leishmania* species and the test of cytotoxicity on another cell line could be considered. In addition, *in vivo* evaluations of EssOils and their major isolated components could be performed on the *L. major*/BALB/c mice model. This research could contribute to the discovery of new effective drugs against leishmaniasis.

Declaration of Interest

The authors declare that they have no conflict of interest.

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Appendix A: Supplementary materials

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Appendix A: supplementary materials

Table 8 Geographical and climatic characteristics of the harvesting areas of the chosen plants

Harvesting area	Geographical coordinates	Mean pluviometry (mm/year)	Mean temperature *	Bioclimate	Soil quality
<i>Pistacia lentiscus</i>					
Area 1: <i>Seliana</i>	Long 9° 34'; Lat 36°05' Altitude 1266 m (Official website of the municipality of Seliana 2019)	440-560 (Agence de réhabilitation et de rénovation urbaine 2017)	25.9 °C, July (OpenStreetMap 2019)	Semi-arid medium to cool winter (Agence de réhabilitation et de rénovation urbaine 2017)	It may have developed on alluvia derived from red older soils (Fournet 1969)
Area 2: <i>Zaghouan</i>	Long 10°06'43"; Lat 36°24' Altitude 1295 m (Official website of the municipality of Zaghouan 2019)	483 (OpenStreetMap 2019)	26.8 °C, August (OpenStreetMap 2019)	Semi-arid climate superior to mild and temperate winter (CNEA 2007)	The soil is predominantly calcimagnetic with calcareous brown soil with medium to good fertility. The soil pH is 6.1 (CNEA 2007, Belkhodja et al. 1971)
<i>Citrus limon</i>					
Area 1: <i>Béja</i>	Long 9°10'55"; Lat 36°43'30" Altitude 222 m (Official website of the municipality of Beja 2019)	622 (OpenStreetMap 2019)	27.3 °C, August (OpenStreetMap 2019)	Sub-humid bioclimatic stage (CNEA 2007)	Slightly evolved soil, of non-climatic origin, alluvial supply, with a vertic nature (Ben Hassine et al. 2012)
Area 2: <i>Menzel Bouzelfa</i>	Long 10°35'; Lat 36°41' Altitude 48 m (Official website of the municipality of MenzelBouzelfa 2019)	397 (OpenStreetMap 2019)	26.3 °C, August (OpenStreetMap 2019)	Semi-arid upper bioclimatic stage with mild and temperate winters (CNEA 2007)	n.r

*: Mean temperature of the hottest month of the year, n.r: not reported in literature.

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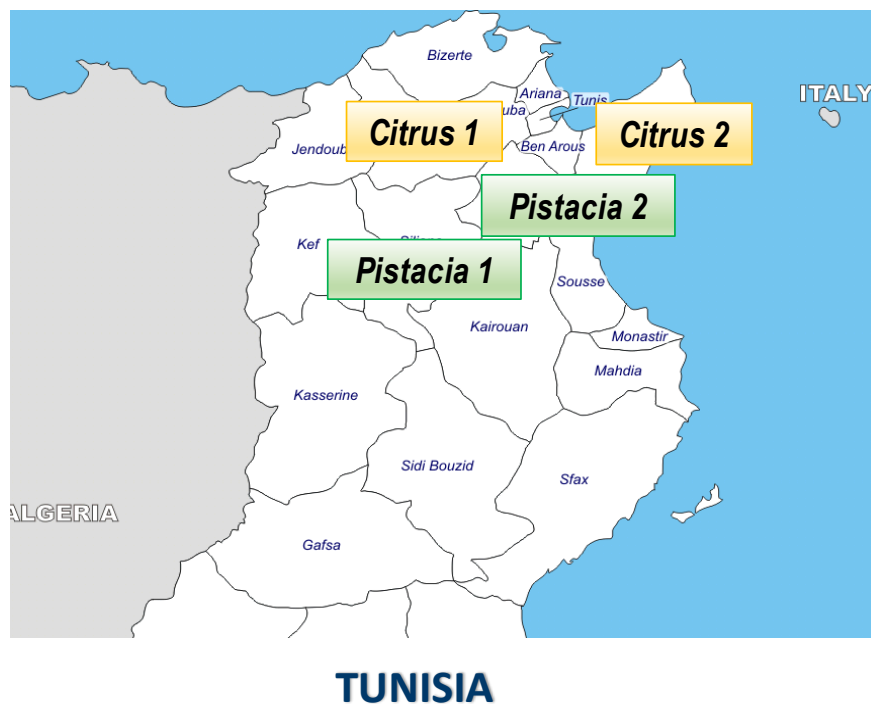
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Table 9 Harvesting conditions and voucher specimens of selected plants

Plant Species	Harvested part	Harvesting period	Voucher specimen
<i>Pistacia lentiscus</i>	Leaves	November to December 2016	P.1.08003
<i>Citrus limon</i>	Leaves	January to February 2017	C.L.001.2015.LMMA



Adapted image of www.d-map.com

Figure 1 Tunisian map presenting the different sampling areas of *Pistacia lentiscus* from Seliana (area 1: *Pistacia 1*) and Zaghouan (area 2: *Pistacia 2*), and *Citrus lemon* from Béja (area 1: *Citrus 1*) and Menzel Bouzelfa (area 2: *Citrus 2*).