



**HAL**  
open science

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

Zeineb Maaroufi, Sandrine Cojean, Philippe Loiseau, Marwa Yahyaoui, Florence Agnely, Manef Abderraba, Ghazlene Mekhloufi

### ► To cite this version:

Zeineb Maaroufi, Sandrine Cojean, Philippe Loiseau, Marwa Yahyaoui, Florence Agnely, et al.. In vitro antileishmanial potentialities of essential oils from Citrus limon and Pistacia lentiscus harvested in Tunisia. Parasitology Research, 2021, 120 (4), pp.1455-1469. 10.1007/s00436-020-06952-5 . hal-04305475

**HAL Id: hal-04305475**

**<https://universite-paris-saclay.hal.science/hal-04305475>**

Submitted on 28 May 2024

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

Zeineb Maaroufi<sup>a,b</sup>, Sandrine Cojean<sup>c</sup>, Philippe M. Loiseau<sup>c</sup>, Marwa Yahyaoui<sup>b</sup>, Florence Agnely<sup>a</sup>, Manef Abderraba<sup>b</sup>, Ghozlene Mekhloufi<sup>a\*</sup>

<sup>a</sup>Université Paris-Saclay, CNRS, Institut Galien Paris-Saclay, 92296, Châtenay-Malabry, France.

<sup>b</sup>Laboratoire Matériaux Molécules et applications, Institut préparatoire des études scientifiques et techniques (IPEST), Univ. De Carthage, La Marsa, Tunisie

<sup>c</sup> Université Paris-Saclay, CNRS, BioCIS, 92290, Châtenay-Malabry, France.

\* corresponding author

E-mail: [ghozlene.mekhloufi@universite-paris-saclay.fr](mailto:ghozlene.mekhloufi@universite-paris-saclay.fr)

## **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 IC<sub>50</sub> 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/m<sup>3</sup>) 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  $\alpha$ -pinene as major compounds. An interesting activity against *L. infantum* of the oil extracted from *Pistacia lentiscus* fruit (rich with limonene and  $\alpha$ -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<sup>•</sup>) 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<sup>•+</sup> radical was generated by adding a 7 mM of ABTS solution at pH = 7.4 (5 mM NaH<sub>2</sub>PO<sub>4</sub>; 5 mM Na<sub>2</sub>HPO<sub>4</sub>, and 154 mM NaCl) to a potassium persulfate solution K<sub>2</sub>S<sub>2</sub>O<sub>8</sub> 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<sub>50-AA</sub> (mg/L) is the concentration of the EssOil able to scavenge 50 % of DPPH<sup>•</sup> or ABTS<sup>•+</sup> 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  $\mu$ 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  $\mu$ m film thickness) interfaced with an Agilent mass selective detector 5975 C inert MSD. The carrier gas was N<sub>2</sub> 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  $\mu$ 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 (H<sub>3</sub>PW<sub>12</sub>O<sub>40</sub>) and phosphomolybdic acid (H<sub>3</sub>PMo<sub>12</sub>O<sub>40</sub>) 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  $\mu$ L of EssOil solutions were transferred into tube then mixed thoroughly with

500  $\mu$ 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  $\mu$ 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  $\mu$ M adenosine, 0.5 mg/L hemin, 10 % fetal bovine serum, and 50  $\mu$ g/mL gentamycin at 26 °C in a dark environment, under an atmosphere of 5 % CO<sub>2</sub>. One day before the treatment, differentiation of promastigotes into axenic amastigotes was achieved by dilution of  $1 \times 10^6$  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 CaCl<sub>2</sub>, and 2 mM MgCl<sub>2</sub>). Axenic amastigotes were grown under 37 °C and 5 % CO<sub>2</sub>.

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

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

priorly diluted in ethanol, were added at concentrations ranging from 100 to  $10^{-4}$   $\mu\text{g}/\text{mL}$  obtained by serial dilution. Miltefosine (HePC) was used as a reference compound with final concentrations ranging from 100  $\mu\text{M}$  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 %  $\text{CO}_2$  atmosphere. Then, plates were subjected to 3 freeze/thaw cycles. Amastigotes viability was assessed using the SYBR<sup>®</sup> green I incorporation method. This method consists in evaluating the parasite growth using SYBR<sup>®</sup> 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<sup>®</sup> 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 ( $\text{IC}_{50}$ ) calculated using ICEstimator<sup>®</sup> (<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  $\mu\text{L}$  of DMEM. After incubation in a 5 %  $\text{CO}_2$  incubator at 37 °C for 2 h, the culture medium was replaced by 100  $\mu\text{L}$  of fresh DMEM, supplemented with 2 mM  $\text{CaCl}_2$  and 2 mM  $\text{MgCl}_2$ , containing a suspension of  $3.2 \times 10^6$  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 % CO<sub>2</sub>. 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 (IC<sub>50</sub> and CC<sub>50</sub>, 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 <sup>a</sup>
	Area 2	0.25 ± 0.06 <sup>b</sup>
<i>Pistacia lentiscus</i>	Area 1	0.06 ± 0.01 <sup>c</sup>
	Area 2	0.06 ± 0.05 <sup>c</sup>

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  $\beta$ -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
$\alpha$ -pinene	5.07	948	-	0.8
$\beta$ -thujene	5.61	969	-	2.6
$\beta$ -pinene	5.69	972	3.2	12.8

$\beta$ -myrcene	5.81	977	-	1.2
D-limonene	6.41	1000	12.9	<b>30.0</b>
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
$\alpha$ -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	<b>29.5</b>	16.7
geranyl acetate	14.54	1382	6.6	6.5
caryophyllene	15.52	1506	2.7	2.1
$\alpha$ -bergamotene	15.78	1512	0.5	-
$\beta$ -bisabolene	17.13	1544	0.8	-
(-)-spathulenol	18.34	1572	0.7	0.9
methyl arachidonate	18.45	1575	2.0	-
<b>Total (%)</b>			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  $\beta$ -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  $\beta$  and  $\alpha$ -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
$\alpha$ -phellandrene	4.96	945	3.2	-
$\alpha$ -pinene	5.07	948	<b>16.9</b>	13.6
camphene	5.29	957	2.0	2.3
$\beta$ -phellandrene	5.62	969	2.4	-
$\beta$ -pinene	5.69	972	3.2	6.2
$\beta$ -myrcene	5.81	977	11.3	<b>30.0</b>
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
$\beta$ -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
$\alpha$ -terpineol	9.22	1107	1.6	1.1
$\alpha$ -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	-
$\beta$ -elmene	14.86	1491	-	1.2
caryophyllene	15.52	1506	3.3	5.6
$\alpha$ -caryophyllene	16.21	1522	1.5	1.6
spatulanol	16.35	1526	0.9	-
$\gamma$ -cadinene	16.61	1532	3.3	2.6
germacrene D	16.73	1535	-	5.7
$\beta$ -cubebene	16.72	1534	1.1	-
$\alpha$ -elemene	16.96	1540	1.0	-
$\alpha$ -amorphene	17.29	1548	4.0	1.8
cadina-1(2),4-diene	17.43	1551	3.0	-
$\alpha$ -calacorene	17.77	1559	0.7	-
(-)-spathulenol	18.34	1572	0.6	1.6
caryophyllene oxide	18.44	1575	5.1	-
$\gamma$ -cadinene	19.24	1593	-	5.3
$\tau$ -muurolol	19.24	1595	4.3	-
$\alpha$ -cadinol	19.42	1597	4.4	2.2
$\alpha$ -bisabolol	19.76	1605	1.1	-
<b>Total (%)</b>			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  $\alpha$ -pinene (16.9 %), followed by  $\beta$ -myrcene (11.3 %), D-limonene (4.9 %), o-cymene (4.5 %), terpinen-4-ol (4.4 %),  $\beta$ -pinene (3.2 %), and  $\alpha$ -phellandrene (2.4 %). However, EssOil from area 2 exhibited a chemotype of  $\beta$ -myrcene (~ 30 %). Other components were also present in appreciable quantities such as  $\alpha$ -pinene (13.6 %),  $\beta$ -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  $\alpha$ -pinene, among other common constituents including  $\beta$ -myrcene,  $\beta$ -pinene, carvacrol, limonene,  $\beta$ -caryophyllene, verbenone,  $\alpha$ -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,  $\beta$ -caryophyllene, and  $\alpha$ -terpineol (Bachrouch et al. 2010). Aissi et al. (2016) found that  $\alpha$ -pinene was the most abundant compound of *Pistacia* EssOil from area 2 followed by limonene, terpinen-4-ol,  $\beta$ -caryophyllene, germacrene, and  $\gamma$ -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<sub>50-AA</sub>, (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 <sub>50-AA</sub> (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<sub>50-AA</sub>, 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  $\alpha$ -pinene,  $\beta$ -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 IC<sub>50</sub> value was lower than 10  $\mu$ g/mL, and moderately effective when IC<sub>50</sub> was between 10 and 20  $\mu$ g/mL. A sample with IC<sub>50</sub> value higher than 20  $\mu$ 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 IC<sub>50</sub> 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 <sub>50</sub>	IC <sub>50</sub>	CC <sub>50</sub> (µg/mL)	Selectivity index (SI)
		Axenic amastigotes (µg/mL)	Intramacrophage amastigotes (µg/mL)		
<i>Citrus limon</i>	Area 1	12.1 ± 1.8 <sup>a,A</sup>	4.2 ± 1.3 <sup>ab,A</sup>	49.6 ± 7.2 <sup>a,B</sup>	11.7
	Area 2	24.0 ± 3.6 <sup>b,A</sup>	13.8 ± 0.7 <sup>b,B</sup>	48.2 ± 4.2 <sup>a,C</sup>	3.5
<i>Pistacia lentiscus</i>	Area 1	0.5 ± 0.2 <sup>a,A</sup>	12.5 ± 1.4 <sup>b,B</sup>	68.5 ± 4.2 <sup>b,C</sup>	3
	Area 2	56.1 ± 20.5 <sup>c,A</sup>	35.6 ± 0.7 <sup>c,A</sup>	> 100 <sup>c,B</sup>	> 1
<i>HePC</i> <sup>*</sup>		2.7 ± 0.4 <sup>a,A</sup>	2.0 ± 0.6 <sup>a,A</sup>	62.1 ± 4.4 <sup>b,B</sup>	31.4

<sup>a-c</sup> Means within a column with different letters were significantly different ( $p < 0.05$ ).

<sup>A-C</sup> Means within a line with different letters were significantly different ( $p < 0.05$ ).

HePC, Hexadecylphosphocholine (miltefosine), the positive control.

<sup>\*</sup> IC<sub>50</sub> and CC<sub>50</sub> 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 IC<sub>50</sub> 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 IC<sub>50</sub> < 10 µg/mL. Moreover, this activity was close to the IC<sub>50</sub> value obtained with miltefosine ( $p > 0.05$ ). However, EssOils of *Citrus* area 2 and of *Pistacia* area 1 presented a moderate activity with IC<sub>50</sub> 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 IC<sub>50</sub> 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, IC<sub>50</sub> 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 CC<sub>50</sub> 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 IC<sub>50</sub> 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 IC<sub>50</sub> values of  $17.60 \pm 0.88 \mu\text{M}$  (Essid et al. 2015) and  $19.80 \pm 0.23 \mu\text{M}$  (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  $\beta$ -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 <sub>50</sub> (µg/mL or µM) *	Cell line/animal	CC <sub>50</sub> /LD <sub>50</sub>	
<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)	

\*: IC<sub>50</sub> 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.

## Acknowledgements

The authors would like to thank Université Paris-Sud (now Université Paris-Saclay) (France), University of Carthage (Tunisia) and the laboratory of Materials Molecules and Applications (IPEST, Tunisia) for the funding granted to carry out this research work

## Appendix A: Supplementary materials

## References

Aissi O, Boussaid M, Messaoud C (2016) Essential oil composition in natural populations of *Pistacia lentiscus* L. from Tunisia: Effect of ecological factors and incidence on antioxidant and antiacetylcholinesterase activities. *Ind Crops Prod* 91:56–65. doi: 10.1016/j.indcrop.2016.06.025

Akbari M, Oryan A, Hatam G (2017) Application of nanotechnology in treatment of leishmaniasis: A Review. *Acta Trop* 172:86–90. doi: 10.1016/j.actatropica.2017.04.029

Amhamdi H, Aouinti F, Wathelet JP, Elbachiri A (2009) Chemical composition of the essential oil of *Pistacia lentiscus* L. from Eastern Morocco. *Rec Nat Prod* 3:90–95

Anthony JP, Fyfe L, Smith H (2005) Plant active components - A resource for antiparasitic agents? *Trends Parasitol* 21:462–468. doi: 10.1016/j.pt.2005.08.004

Aoun K, Bouratbine A (2014) Cutaneous Leishmaniasis in North Africa: a review. *Parasite* 21:14. doi: 10.1051/parasite/2014014

Arruda DC, Miguel DC, Yokoyama-Yasunaka JKU, Katzin AM, Uliana SRB (2009) Inhibitory activity of limonene against *Leishmania* parasites *in vitro* and *in vivo*. *Biomed Pharmacother* 63:643–649. doi: 10.1016/J.BIOPHA.2009.02.004

Arteche Garcia A, Vanaclocha B, Salazar JIG, Cobo RM (1998). *Fitoterapia, Vademecum de Prescripcion*. 3a ed. Masson, Barcelona.

Asai T, Matsukawa T, Kajiyama S (2017) Metabolomic analysis of primary metabolites in *Citrus* leaf during defense responses. *J Biosci Bioeng* 123:376–381. doi: 10.1016/j.jbiosc.2016.09.013

Attia R, Messaoud C, Arraki K, Zedet A, Demougeot C, Boussaïed M, Girard C (2019) Phytochemical screening and arginase inhibitory activity of extracts from several Tunisian medicinal plants. *South African Journal of Botany* 120:313-318. Doi: 10.1016/j.sajb.2018.09.022

Bachrouch O, Abderraba M, Moussa F, Loiseau P (2013) Fractions bioguidées extraites à partir de l'huile essentielle foliaire du pistachier lentisque (*Pistacia lentiscus* L.) potentiellement actives contre les parasites de la leishmaniose (*Leishmania donovani*). Tunisian Patent TN 2013/0129.

Bachrouch O, Mediouni Ben Jemâa J, Wissem AW, Talou T, Marzouk B, Abderraba M (2010) Composition and insecticidal activity of essential oil from *Pistacia lentiscus* L. against *Ectomyelois ceratoniae* Zeller and *Ephestia kuehniella* Zeller (*Lepidoptera: Pyralidae*). *J Stored Prod Res* 46:242–247. doi: 10.1016/j.jspr.2010.07.001

Barros LM, Duarte AE, Morais-Braga MFB, Waczuk EP, Vega C, Leiyé NF, De Menezes IRA, Coutinho HDM, Rocha JBT, Kamdem JP (2016) Chemical characterization and Trypanocidal, Leishmanicidal and Cytotoxicity potential of *Lantana camara* L. (*Verbenaceae*) Essential oil. *Molecules Basel. Online* (21):201-209

Bertuzzi G, Tirillini B, Angelini P, Venanzoni R (2013) Antioxidative Action of *Citrus limonum* Essential Oil on Skin. *European J Med Plants* 3:1–9 Bitu VCN, Costa JGM, Rodrigues FFG, Colares AV, Coutinho HDM, Botelho MA, Portela AC, Santana NM, Menezes IRA (2012) Effect of Collection time on composition of Essential Oil of Schauer (*Verbenaceae*) growing in Northeast Brazil. *Journal of Essential Oil-Bearing Plants* 18:647-653

Bouyahya A, Chadon Assemian IC, Mouzount H, et al (2019) Could volatile compounds from leaves and fruits of *Pistacia lentiscus* constitute a novel source of anticancer, antioxidant, antiparasitic and antibacterial drugs? *Ind Crops Prod* 128:62–69. doi: 10.1016/j.indcrop.2018.11.001

Bouzenna H, Dhibi S, Samout N, et al (2016) The protective effect of *Citrus limon* essential oil on hepatotoxicity and nephrotoxicity induced by aspirin in rats. *Biomed Pharmacother* 83:1327–1334. doi: 10.1016/j.biopha.2016.08.037

Bozorgi M, Memariani Z, Mobli M, et al (2013) Five *Pistacia* species (*P. vera*, *P. atlantica*, *P. terebinthus*, *P. khinjuk*, and *P. lentiscus*): a review of their traditional uses, phytochemistry, and pharmacology. *Scientific World Journal* 2013:219815. doi: 10.1155/2013/219815

Brenzan MA, Nakamura CV, Prado Dias Filho B, Ueda Nakamura T, Young MCM, Aparicio Garcia Cortez D (2007) Antileishmanial activity of crude extract and coumarin from *Calophyllum brasiliense* leaves against *Leishmania amazonensis*. *Parasitol Res* 101:715–722. doi: 10.1007/s00436-007-0542-7

Bruno L, Spadafora ND, Iaria D, et al (2014) Developmental stimuli and stress factors affect expression of CIGLP1, an emerging allergen-related gene in *Citrus limon*. *Plant Physiol Biochem* 79:31–40. doi: 10.1016/j.plaphy.2014.03.003

Chakravarty J, Sundar S (2019) Drug Resistance in Leishmaniasis. *J Glob Infect Dis.*, 2:167–176. doi: 10.4103/0974-777X.62887

De Muylder G, Ang KKH, Chen S, Arkin MR., Engel JC, McKerrow JH (2011) A screen against *Leishmania* intracellular amastigotes: Comparison to a promastigote screen and identification of a host cell-specific hit. *PLoS Negl Trop Dis* 5:e1253. doi: 10.1371/journal.pntd.0001253



De Rycker M, Hallyburton I, Thomas J, Campbell L, Wyllie S, Joshi D, Cameron S, Gilbert IH, Wyatt PG, Drerson JA, Fairlamb AH, Gray DH (2013) Comparison of a high-throughput high-content intracellular *Leishmania donovani* assay with an axenic amastigote assay. *Antimicrobi. Agents Chemother.* 57:2913–2922. doi: 10.1128/AAC.02398-12

Dhifi W, Bellili S, Jazi S, Bahloul N, Mnif W (2016) Essential oils chemical characterization and investigation of some biological activities: A Critical Review. *Medicines* 3:25. doi: 10.3390/medicines3040025

El Asbahani A, Miladi K, Badri W, Sala M, Aït Addi EH, Casabianca H, El Mousadik A, Hartmann D, Jilale A, Renaud FN, Elaissari A (2015) Essential oils: From extraction to encapsulation. *Int J Pharm* 483:220–243. doi: 10.1016/j.ijpharm.2014.12.069

El Haib A (2011) Valorisation de terpènes naturels issus de plantes marocaines par transformations catalytiques. PhD thesis, University of Toulouse III, France

Escobar P, Leal SM, Herrera LV, Martinez JR., Stashenko E (2010) Chemical composition and antiprotozoal activities of Colombian *Lippia spp* essential oils and their major components. *Mem Inst Oswaldo Cruz* 105:184–190. doi: 10.1590/S0074-02762010000200013

Essid R, Rahali FZ, Msaada K, Sghair I, Hammami M, Bouratbine A, Aoun, K, Limam, F (2015) Antileishmanial and cytotoxic potential of essential oils from medicinal plants in Northern Tunisia. *Ind Crops Prod* 77:795–802. doi: 10.1016/j.indcrop.2015.09.049

Farag RS, Badei AZMA, Hewedi FM, El-Baroty GSA (1989) Antioxidant activity of some spice essential oils on linoleic acid oxidation in aqueous media. *J Am Oil Chem Soc* 66:792–799. doi: 10.1007/BF02653670

Ghazouani N, Abderrabba M, Bouajila J (2016) *Teucrium ramosissimum* (*Lamiaceae*): Volatile composition, seasonal variation, and pharmaceutical activity. *Anal Lett* 49:1258–1271. doi: 10.1080/00032719.2015.1082134

Hajaji S, Sifaoui I, Lopez-Arencibia A, Reyes-Batlle M, Jimenez A. I, Bazzocchi I. L, Valladares B, Akkari H, Lorenzo-Morales J, Pinero J. E (2018) Leishmanicidal activity of  $\alpha$ -bisabolol from Tunisian chamomile essential oil. *Parasitology Research* 117:2855-2867. Doi: 10.1007/s00436-018-5975-7  
Jenner PM, Hagan EC, Taylor JM, Cook EL, Fitzhugh OG (1964) Food flavourings and compounds of related structure I. Acute oral toxicity. *Food Cosmet Toxicol* 2:327–343. doi: 10.1016/S0015-6264(64)80192-9

Jomaa S, Rahmo A, Alnori AS (2012) The Cytotoxic Effect of essential oil of syrian *Citrus limon* peel on human colorectal carcinoma cell line (Lim1863). *Middle East J Cancer* 3:15–21

Karami M, Douidi M, Setorki M (2013) Assessing epidemiology of cutaneous leishmaniasis in Isfahan, Iran. 30–37

Krauth-Siegel RL, Comini MA (2008) Redox control in trypanosomatids, parasitic protozoa with trypanothione-based thiol metabolism. *Biochim Biophys Acta - Gen Subj* 1780:1236–1248. doi: 10.1016/j.bbagen.2008.03.006

Le TB, Beaufay C, Nghiem DT, Mingeot Leclercq MP, Quetin Leclercq, J (2017) *In vitro* anti-leishmanial activity of essential oils extracted from Vietnamese plants. *Molecules* 22:1–12. doi: 10.3390/molecules22071071

Leal SM, Pino N, Stashenko EE, Martínez JR., Escobar P (2013) Antiprotozoal activity of essential oils derived from *Piper* spp. grown in Colombia. *J Essent Oil Res* 25:512–519. doi: 10.1080/10412905.2013.820669

Lota ML, De Rocca Serra D, Tomi F, Jacquemond C, Casanova J (2002) Volatile components of peel and leaf oils of lemon and lime species. *J Agric Food Chem* 50:796–805. doi: 10.1021/jf010924I

Macedo DG, Souza MMA, Morais-Braga MFB, Coutinho HDM, Dos Santos ATL, Cruz RP, da Costa JGM, Rodriguez FFG, Quintas-junior LJ, Almeida JRGS, Menezes IRA

(2018) Effect of seasonality on chemical profile and antifungal activity of essential oil isolated from leaves *Psidium saluarte* (Kunth) O. Berg. PeerJ 6:e5476Macedo DG, Almeida S, Morais-Braga MF, Coutinho DM, Dos Santos AT, Machado AJT, Rodriguez FFG, Costa JGMC, Menezes IRA (2020) Seasonality influence on the chemical composition and antifungal activity of *Psidium myrtoides* O. Berg. South African Journal of Botany 128:9-17

Machado M, Pires P, Dinis AM, Santos Rosa M, Alves V, Salgueiro L, Cavaleiro C, Sousa MC (2012) Monoterpenic aldehydes as potential anti-*Leishmania* agents: Activity of *Cymbopogon citratus* and citral on *L. infantum*, *L. tropica* and *L. major*. Exp Parasitol 130:223–231. doi: 10.1016/j.exppara.2011.12.012

Machado M, Dinis AM, Santos-Rosa M, Alves V, Salgueiro L, Cavaleiro C, Sousa M.C (2014) Activity of *Thymus capitellatus* volatile extract, 1, 8-cineole and borneol against *Leishmania* species. Vet. Parasitol. 200 (1):39–49.

Mikus J, Harkenthal M, Steverding DJ (2000) *In vitro* effect of essential oils and isolated mono-and sesquiterpenes on *Leishmania major* and *Trypanosoma brucei*. Planta Med 66:66–68

Moura IV, Wunderlich ML, Couto UA, Peres VJ, Katzin AM, Kimura EA (2001) Limonen arrests parasite development and inhibits Isoprenylation of proteins in *Plasmodium falciparum*. Antimicrobial agents and chemotherapy 9 (45):2553-2558

Njoroge SM, Ukeda H, Kusunose H, Sawamura M (1994) Volatile components of japanese yuzu and lemon oils. Flavour Fragr J 9:159–166. doi: 10.1002/ffj.2730090404

Oryan A (2015) Plant-derived compounds in treatment of leishmaniasis. Iran J Vet Res 16:1–19

Otang WM, Afolayan AJ (2016) Antimicrobial and antioxidant efficacy of *Citrus limon* L. peel extracts used for skin diseases by Xhosa tribe of Amathole District, Eastern Cape, South Africa. South African J Bot 102:46–49. doi: 10.1016/j.sajb.2015.08.005

Pereira PS, Maia AJ, Tintino SR, Oliveria-Tintino CDM, Raulino IRS, Vega MC, Rolom M, Corone C, Marivando-Barros L, Duarte AE, Menezes IRA, Countinho HDM, Da Silva TG (2017) Trypanoside, antileishmania and cytotoxic activities of the essential oils from *Rosmarinus officinalis* L. *in vitro*. *Industrial Crops and Products* 109:724-729.

Pham TT, Loiseau PM, Barratt G (2013) Strategies for the design of orally bioavailable antileishmanial treatments. *Int J Pharm* 454:539–552. doi: 10.1016/J.IJPHARM.2013.07.035

Piccolella S, Nocera P, Carillo P, Woodrow P, Greco V, Manti L, Fiorentino A, Pacifico S (2016) An apolar *Pistacia lentiscus* L. leaf extract: GC-MS metabolic profiling and evaluation of cytotoxicity and apoptosis inducing effects on SH-SY5Y and SK-N-BE(2)C cell lines. *Food Chem Toxicol* 95:64–74. doi: 10.1016/j.fct.2016.06.028

Quintans Júnior L, Moreira JCF, Pasquali MAB, Rabie SMS, Pires AS, Schröder R, Rabelo TK, Santos JPA, Lima PSS, Cavalcanti SCH, Araùjo AAS, Quinans JSS, Gelain DP (2013) Antinociceptive activity and redox profile of the monoterpenes (+)-camphene, p-cymene, and geranyl acetate in experimental models. *ISRN Toxicol* 2013:459530. doi: 10.1155/2013/459530

Rauf A, Patel S, Uddin G, Siddiqui BS, Ahmad B, Muhammad N, Mabkhot YN, Hassa TB (2017) Phytochemical, ethnomedicinal uses and pharmacological profile of genus *Pistacia*. *Biomed Pharmacother* 86:393–404. doi: 10.1016/j.biopha.2016.12.017

Rocha LG, Almeida JRGS, Macêdo RO, Barbosa-Filho JM (2005) A review of natural products with antileishmanial activity. *Phytomedicine* 12:514–535. doi: 10.1016/j.phymed.2003.10.006

Santin MR, Dos Santos AO, Nakamura CV, Dias Filho BP, Ferreira IC, Ueda Nakamura T (2009) *In vitro* activity of the essential oil of *Cymbopogon citratus* and its major

component (citral) on *Leishmania amazonensis*. Parasitol Res 105:1489–1496. doi: 10.1007/s00436-009-1578-7

Santos AO, Ueda-Nakamura T, Dias Filho BP, Junior VFV, Pinto AC, Nakamura CV (2008) Effect of Brazilian copaiba oils on *Leishmania amazonensis*. Journal of Ethnopharmacol. 120 (2): 204-208

Schaich KM, Tian X, Xie J (2015) Hurdles and pitfalls in measuring antioxidant efficacy: A critical evaluation of ABTS, DPPH, and ORAC assays. J Funct Foods 14:111–125. doi: 10.1016/J.JFF.2015.01.043

Sibanda S, Chigwada G, Poole M, Gwebu ET, Noletto JA, Schmidt JM, Rea AI, Setzer WN (2004) Composition and bioactivity of the leaf essential oil of *Heteropyxis dehniae* from Zimbabwe. J Ethnopharmacol 92:107–111. doi: 10.1016/j.jep.2004.02.010

Sidana J, Saini V, Dahiya S, Nain P, Bala S (2013) A review on *citrus* - “the boon of nature.” Int J Pharm Sci Rev Res 18:20–27

Staerck C, Gastebois A, Vandeputte P, Calenda A, Larcher G, Gillman L, Papon N, Bouchara JP, Fleury MJ (2017) Microbial antioxidant defense enzymes. Microb Pathog 110:56–65. doi: 10.1016/j.micpath.2017.06.015

Stafford JL, Neumann NF, Belosevic M (2002) Macrophage-mediated innate host defense against protozoan parasites. Crit Rev Microbiol 28:187–248

Tariku Y, Hymete A, Hailu A, Rohloff J (2010) Essential oil composition, antileishmanial, and aoxicity study of *Artemisia abyssinica* and *Satureja punctata* ssp. *punctata* from Ethiopia. Chem Biodivers 7:1009–1018. doi: 10.1002/cbdv.200900375

Traoré B, Oliveira F, Faye O, Dicko A, Coulibaly CA, Sissoko IM, Sibiry S, Soboga N, Sangare MB, Coulibaly YI, Traoré P, Traoré SP, Anderson JM, Keita S, Valenzuela JG, Kamhawi S, Doumbia S (2016) Prevalence of cutaneous Leishmaniasis in districts of high and low endemicity in Mali. PLoS Negl Trop Dis 10:1–12. doi: 10.1371/journal.pntd.0005141

Ultee A, Bennik MHJ, Moezelaar R (2002) The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. *Appl Environ Microbiol* 68:1561–8. doi: 10.1128/AEM.68.4.1561-1568.2002

Vekiari SA, Protopapadakis EE, Papadopoulou P, Papanicolaou, D, Panou, C, Vamvakias M (2002) Composition and seasonal variation of the essential oil from leaves and peel of a *cretan* lemon variety. *J Agric Food Chem* 50:147–153. doi: 10.1021/jf001369a

Yahyaoui M, Bouajila J, Cazaux S, Abderrabba M (2018) The impact of regional locality on chemical composition, antioxidant and biological activities of *Thymelaea hirsuta* L. extracts. *Phytomedicine* 41:13–23. doi: 10.1016/J.PHYMED.2018.01.010

Yahyaoui M, Ghazouani N, Sifaoui I, Abderrabba M (2017) Comparison of the effect of various extraction methods on the phytochemical composition and antioxidant activity of *Thymelaea hirsuta* L. aerial parts in Tunisia. *Biosci Biotechnol Res Asia* 14:997–1007. doi: 10.13005/bbra/2534

Zaouali, Y., Bel Hadj Yahya, I., Jaoudi, R., Messaoud, C., Boussaid, M., 2018. Sex-related differences in essential oil composition, phenol contents and antioxidant activity of aerial parts in *Pistacia lentiscus* L. during seasons. *Ind. Crop. Prod.* 121, 151–159. doi.org/10.1016/j.indcrop.2018.04.067

Zheljzakov V, Cantrell C, Tekwani B, Khan S (2008) Content, composition, and bioactivity of the essential oils of three basil genotypes as a function of harvesting. *Planta Med* 74:380–385. doi: 10.1055/s-2008-1075204

WHO | Leishmaniasis (2018) Leishmaniasis, <https://www.who.int/en/news-room/fact-sheets/detail/leishmaniasis>. Accessed 4 June 2020

## **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.



## Table 8 references'

Agence de réhabilitation et de rénovation urbaine (2017) Plan de gestion environnementale et sociale du projet de réhabilitation du quartier populaire El Ons dans la commune de Rouhia Gouvernorat de Siliana. <http://www.collectiviteslocales.gov.tn/wp-content/uploads/2017/06/PGES-REHABILITATION-QUARTIER-EL-ONS-COMMUNE-ROUHIA-SILIANA.pdf>. Accessed 5 March 2019

Belkhodja K, Bortoli L, Cointepas J, Dimanche P, Fournet A, Jacquinet J.C, Mori A (1971) Les sols de la Tunisie septentrionale. Documents from Tunisian Ministry of Agriculture.

Ben Hassine D, Abderrabba M, Yvon Y, Lebrihi A, Mathieu F, Couderc F, Bouajila, J (2012) Chemical composition and *in vitro* evaluation of the antioxidant and antimicrobial activities of *Eucalyptus gillii* essential oil and extracts. *Molecules* 17, 9540–9558.

CNEA (2007) Elaboration d'une étude sur l'état de désertification pour une gestion durable des RN/Avril 2007. Documents from Tunisian Ministry of Environment and Sustainable Development

Fournet A (1969) Etude pédagogique de la dorsale Tunisienne (Transversale Kébir-Bargou-Serj - Oussltic), PhD thesis, Faculty of Pharmacy, Paris France.

Official website of the municipality of Beja (2019) [www.commune-beja.gov.tn](http://www.commune-beja.gov.tn). Accessed 5 March 2019

Official website of the municipality of Menzel Bouzelfa (2019) [www.commune-menzelbouzelfa.gov.tn](http://www.commune-menzelbouzelfa.gov.tn). Accessed 5 March 2019

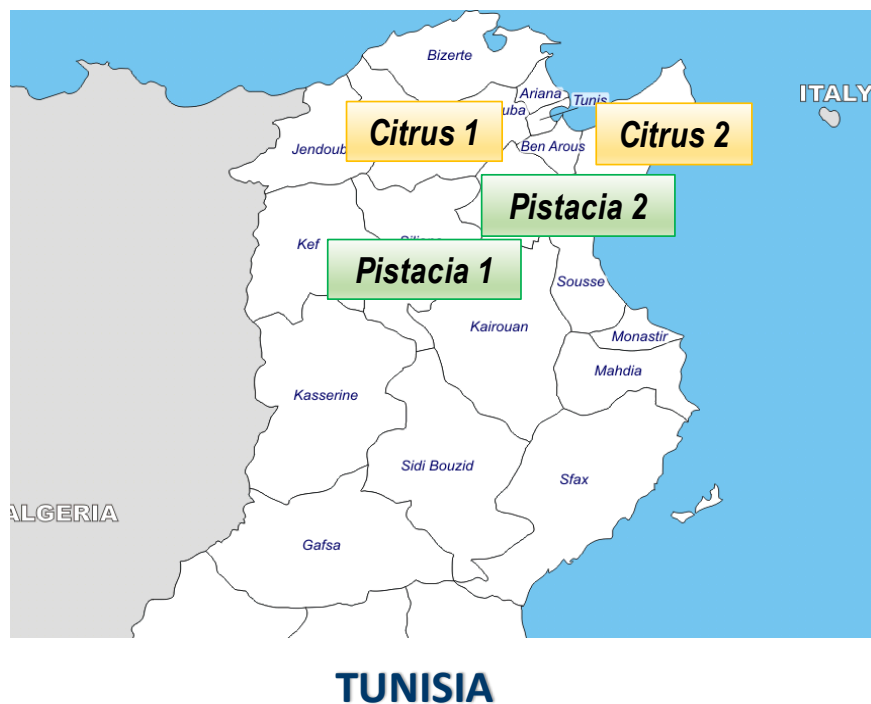
Official website of the municipality of Seliana (2019) [www.commune-siliana.gov.tn/](http://www.commune-siliana.gov.tn/). Accessed 5 March 2019

Official website of the municipality of Zaghouan (2019) [www.commune-zaghouan.gov.tn](http://www.commune-zaghouan.gov.tn). Accessed 5 March 2019

OpenStreetMap (2019) Les données climatiques pour les villes du monde entier. <https://fr.climate-data.org/>. Accessed 29 May 2019

**Table 9** Harvesting conditions and voucher specimens of selected plants

<b>Plant Species</b>	<b>Harvested part</b>	<b>Harvesting period</b>	<b>Voucher specimen</b>
<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](http://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*).