Research Article

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American Journal of Ethnomedicine
ISSN 2348-9502

2020
Vol.7 No.1: 16

DOI: 110.36648/2348-9502.7.1.16

Comparative Study of Chemical Properties and Composition of Algerian and Tunisian *Mentha pulegium L*

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Citation: Barhoumi M (2020) Comparative Study of Chemical Properties and Composition of Algerian and Tunisian *Mentha pulegium L*. Am J Ethnomed Vol.7 No.1: 16.

Abstract

The aim of this study was to compare the chemical composition, antioxidant, antifungal and antileishmanial activities of the essential oils that were obtained from *Mentha pulegium L*. cultivated both in Algeria and in Tunisia. The kinetics of antioxidant activity was also studied.

The essential oils are obtained by steam distillation with a yield of 1.30 and analyzed by gas chromatography coupled to mass spectrometry (GC-MS) where 30 compounds were identified.

The Antioxidant activity was defined using the scavenging test of free radicals (DPPH), while ascorbic acid is used as positive control. The values of IC50 are between 95-107 $\mu g.mL^{-1}$.

The results obtained by the study of the antifungal activity, show that the essential oils of the Algerian and Tunisian *Mentha pulegium* have significant antifungal activity.

The antileishmanial activity was tested against *Leishmania Major and Donovani (LV9) axenic amastigotes*. The IC50 values evaluated in L. major promastigote and axenic amastigote forms were in the range of 100 $\mu g.mL^{-1}$.

Keywords: Essential oil; *Mentha pulegium*; Pulegone; Antifungal; Antoxidant and antileishmanial activities

Received: April 15, 2020, **Accepted:** April 22, 2020, **Published:** April 28, 2020

Introduction

Since antiquity, men have used essential oils for their cosmetic, nutritional and therapeutic needs [1-3]. Studies have also shown that vegetable extracts rich in phenolic compounds and with a marked antioxidant power could play an interesting role in the prevention of cancer because they are stabilizers of free radicals [4].

Several works have highlighted the different biological activities of aromatic and medicinal plants, especially their antifungal [5-7] antibacterial [8] antioxidant [9]. By these properties, the essential oils could therefore serve as a preservative of food. In this context, many studies have shown that extracts of certain aromatic plants have an inhibitory action on the growth and toxinogenesis of several bacteria and fungi responsible for food infections [10-14].

Mentha pulegium L. is one of the aromatic and medicinal plants widely used in traditional medicine; its antimicrobial potency has been demonstrated according to several studies [15].

Therefore, biological control through the use of natural antioxidant and antifungal substances can be an alternative to chemicals. Among these natural substances are the essential oils extracted from aromatic plants [16].

The use of natural products to fight against microbial pathogenesis and stress-related diseases is a very promising strategy to compact theses diseases. Indeed, medicinal plant secondary metabolites and their derivatives have shown antibacterial antileishmanial, antioxidant, and cytotoxic activities [17-18].

Fresh flower fumigations by *Mentha pulegium* were an efficient remedy to rid animals of chips. It is mostly grown as a condiment plant and its leaves are rich in aromatic menthol. In cosmetology, it is considered to be labeled herbaceous plant used especially in men's fragrance for its fresh and tonic character. Currently, in medicine, it is still used as an antiseptic and also as a stomachic and an analgesic [19].

Material and Methods

Plant material

The plants of *Mentha pulegium L* were collected in March 2018

from Bouira (Algeria) and Bizerte (Tunisia). The recovery of essential oils was achieved by using steam distillation and the recovery time was optimized to two hours. The obtained EO was dried over anhydrous sodium sulphate and, after filtration, stored at 4◦C until tested.

Methods

Gas chromatography coupled to mass spectrometry (GC-MS): Gas chromatography-mass spectrometry (GC-MS) is the most popular and strongest method for the determination of essential oil composition. Components existing in the essential oil can be identified by comparison of their relative retention indices and their mass spectra (MS). Identification of individual components of essential oils, however, is not always possible using MS data alone. Often different spectra are reported in a library for a single compound, with different common names, or systematic name, corresponding to an individual component sometimes apparent [20].

The composition of essential oils was investigated by GC and GC-MS. GC analysis was performed in a gas chromatograph (HP 5890) using two fused silica capillary columns, HP5 (non-polar) and Innovax (polar) (30 m x 0.25 mm, film thickness 0.25 μm) and a flame ionization detector (FID). Injector and detector temperatures were set at 240°C and 280°C, respectively. The oven temperature programmed as 50°C for 3 min, then 50-280°C at 9°C.min^{-1} and finally 280°C for 3 min. Nitrogen was the carrier gas at a flow rate of 1 mL. min^{-1}. The samples were injected as 0.1 μL of 1% solution diluted in hexane in the split mode. The percentage of the constituents was calculated by electronic integration of FID peak areas and normalized without the use of response factor correction.

The Components identification was carried out by comparison of their MS spectra with the relative retention indices with those of standard compounds reported in the literature (Adams, 2007) [21].

IR measurements: A qualitative analysis with IR was made in order to characterize the different components of essential oils. The infrared spectrum was recorded using a ''Perkin-Elmer Spectrum 1000'' spectrophotometer. The scanning is performed in the 4000-400 cm^{-1} spectral domain**.**

The identification of functional groups was carried out by using the usual infrared tables and by comparing the frequencies of the peaks with those reported in literature [22].

Radical scavenging activity test: Scavenging test of free radical DPPH (2, 2-diphenyl-1-picrylhydrazyl) was carried out by using the method described by Braca and al [23]. A sample of 500 μL is placed in the presence of 500 μL of a solution of DPPH 0.4% in methanol. After stirring, the mixture is placed at 37°C for 15 minutes in the dark. For each concentration, the test is repeated three times and the absorbance measurement is made at 517 nm by a UV-visible spectrophotometer.

The control solution is composed of 500 μl of the DPPH methanol solution and 500 μl of methanol. Ascorbic acid was used as a positive control. The results are expressed as percentage inhibition (PI) according to the following formula:

$$PI = \frac{[Abs_{Blank} - Abs_{Sample}]}{Abs_{Blank}} \times 100$$

Where Abs_{Blank} is the absorbance of the control solution and Abs_{Sample} is the absorbance in the presence of the essential oil.

Sample concentration capable of scavenging 50% of the DPPH radicals (IC50) can be graphically determined by plotting the absorbance (the percentage of inhibition of DPPH radicals) against the log concentration of DPPH and determining the slope of the nonlinear regression.

The kinetic of the antioxidant activity was also studied. 50 μL of essential oil are added to 1.5 mL of a DPPH solution at 0.4% in methanol. The UV absorbance is read at a wavelength of 517 nm.

Antifungal activity: The study of antifungal activities was carried out at Dr. Robert McFeeters' Laboratory (University of Alabama in Huntsville USA). The minimum inhibitory concentrations (MIC) are determined according to the following protocol:

Microdilution assays were performed with *Aspergillus Niger* and *Candida albicans* to evaluate the antifungal potential of *Mentha Pulegium* essential oils. 100 μL of RPMI media buffered with MOPS was added to every well micro dilution plate. 100 μL of a 1% solution of the essential oils in DMSO, along with 100 μL of media (RPMI) and solvent (DMSO) controls, were added to the first well of each row and serial diluted down each respective column by 100 μL. 100 μL of *C. albicans* cells or C. neoformans at a concentration of 4.000 cells/mL or *A. niger* conidia at a concentration of 4.000 conidia/mL in PDB was added to all wells in the plate. *C. albicans* and C. neoformans plates were incubated at 37°C for 2 days and *A. Niger* plates at room temperature for 7 days [24-26]. All results were interpreted visually.

Antileishmanial activity: Leishmaniasis is a family of parasitic diseases that affect about 12 million people in tropical and subtropical areas in the form of three clinical expressions: visceral leishmaniasis, which is fatal in the absence of treatment, muco-cutaneous leishmaniasis, and cutaneous leishmaniasis, which is often self-curing. Classical drugs such as antimonials (Pentostam and Glucantime) are toxic, and drug resistance is increasing dangerously in the field. Toxicity and the appearance of drug resistance justify the search for new chemical series in order to find an orally safe and active drug [27, 28].

The study of antileishmanial activities was carried out at Pr. Philippe M Loiseau Laboratory (Faculty of Pharmaceutical Sciences. Chatenay-Malabry, France).

The IC50 values are determined according to the following protocols.

Protocol *in vitro*

Cell lines and cultures: The mouse monocyte/macrophage cell line RAW264.7 was maintained in culture in DMEM supplemented with 10% heat-inactivated fetal bovine serum.

L. Major and *L. Donovani* (LV9) were used for *in vitro* experiments. Promastigotes forms were grown in M-199 medium supplemented with 40 m M HEPES, 100 μM adenosine, 0.5 mg/mL haemin, 10% heat-inactivated fœtal bovine serum (FBS) and

50 µg/ml gentamycine at 26°C in a dark environment under an atmosphere of 5% CO_2. Differentiation of promastigotes into axenic amastigotes was achieved by dilution of 10^6 promastigotes in 5 mL of axenic amastigote medium (1 x M-199, supplemented with 40 mM HEPES, 100 µM adenosine, 0.5 mg/mL haemin, 10% FBS; 2 mM $MgCl_2$, 2 mM $CalCl_2$). The pH was adjusted to pH 5.5. Axenic amastigotes were grown at 37°C in 5% CO_2. All the experiments were performed with parasites in their logarithmic phase of growth.

***In vitro* antileishmanial evaluation of the compounds on Leishmania Major and Donovani (LV9) axenic amastigotes:** Two fold serial dilutions of the compounds from a maximal concentration of 100 µM were performed in 100 µl of complete medium in 96-well microplates.Triplicates were used for each concentration. A suspension of axenic amastigote forms was prepared to yield 10^7 cells/mL and amastigotes were then added to each well at a density of 10^6/mL in a 200 µL final volume. Cultures were incubated at 37°C for 72 h in the dark and under a 5% CO_2 atmosphere, then the viability of the amastigotes was assessed using the SYBR1 Green I (Invitrogen, France) incorporation method. Parasite growth was determined by using SYBR1 Green I, a dye with marked fluorescence enhancement upon contact with parasite DNA. After incubation, the plates were subjected to 3 freeze/thaw cycles to achieve complete hemolysis. The parasite lysis suspension was diluted 1:2 in lysis buffer (10 mM NaCl, 1 mM Tris HCl pH8, 2.5 mM EDTA pH 8, 0.05% SDS, 0.01 mg/mL proteinase K and 1X SYBR Green I). Incorporation of SYBR Green I in parasite was measured using the Master epRealplex cycler® (Eppendorf, France) according the following program to increase the SYBR green incorporation: 90°C for 1 min, decrease in temperature from 90°C to 10°C for 5 min with reading the fluorescence 10°C for 1 min and a new reading at 10°C for 2 min.

Axenic amastigotes viability could also be measured using a resazurin assay. After 72 h incubation time at 37°C with 5% CO_2, 10 µl of a resazurin solution at 450 µM was added to each well, and the plates were further incubated in the dark for 24 h at 37°C with 5% CO_2.Cell viability was then monitored by using the resazurin test. In living cells, resazurin is reduced in resorufin and this conversion is monitored by measuring OD570 nm (resorufin) and OD600 nm (resazurin; Lab systems Multiskan MS).

The IC_{50} was calculated by nonlinear regression. Fluorescence obtained was compared to those from the range obtained with different parasite densities. Miltefosine was used as reference compound. The antileishmanial activity was expressed as IC_{50} in µM (concentration of drug inhibiting the parasite growth by 50%, comparatively to the controls treated with the excipient only).

Evaluation of compounds cytotoxicity using the rezasurin method: Cytotoxicity was evaluated on RAW 264.7 macrophages. RAW 264.7 cells were seeded into a 96-well microtiter plate at a density of 2 x 10^4 cells/well in 100 µl of DMEM. After incubation in a 5% CO_2 incubator at 37°C for 24 h, the culture medium was replaced with 100 µl of fresh DMEM containing two fold serial dilutions of the test compounds. The starting final concentration was 100 µM. After 48 h incubation time at 37°C with 5% CO2, 10 µl of a resazurin solution at 450 µM was added to each well, and the plates were further incubated in the dark for 4 h at 37°C with 5% CO_2.Cell viability was then monitored by using the resazurin test. In living cells, resazurin is reduced in resorufin and this conversion is monitored by measuring OD570 nm (resorufin) and OD600 nm (resazurin; Lab systems Multiskan MS). The cytotoxicity of the compounds was expressed as CC_{50} (Cytotoxic Concentration 50% concentration inhibiting the macrophages growth by 50%). Miltefosine was used as the reference drug.

***In vitro* antileishmanial evaluation on intramacrophage amastigotes:** The mouse monocyte/macrophage cell line RAW 264.7 was maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum. RAW 264.7 cells were seeded into a 96-wellmicrotiter plate at a density of 2 x 10^4 cells/well in 100 µL of DMEM. After incubation in a 5% CO_2 incubator at 37°C for 24 h, the culture medium was replaced with 100 µl of fresh DMEM containing a suspension of axenic amastigote forms to reach a ratio amastigotes/macrophage of 16:1.Each plate has 8 wells with axenic amastigotes (control of the parasite growth), 8 wells with only macrophages (control of the macrophage growth) and finally 8 wells with infected macrophages (control of the growth of intramacrophage parasites). After incubation in a 5% CO_2 incubator at 37°C for 24 h, the culture medium was replaced with 100 µL of fresh DMEM containing the test compounds for a new incubation of 48 h. The viability of the amastigotes into macrophages was then assessed using the SYBR1Green I (Invitrogen, France) incorporation method. Thus, the medium was removed and the cells were lysed in 100 µL lysis buffer.After the plates were subjected to 3 freeze thaw cycles to achieve complete lysis. The parasite lysis suspension was diluted 1:2 in lysis buffer with SYBR Green I like previously. Incorporation of SYBR Green I in parasite DNA amplification was measured using the Master epRealplex cycler® (Eppendorf, France) according the following program to increase the SYBR green incorporation: 90°C for 1 min, decrease in temperature from 90°C to 10°C for 5 min with reading the fluorescence 10°C for 1 min and a new reading at 10°C for 2 min. Fluorescence obtained was compared to those from the range obtained with parasite, infected cell and non-infected cell densities. The activity of the compounds was expressed as IC_{50} (concentration of drug inhibiting the parasite growth by 50%, comparatively to the controls treated with the excipient only). Miltefosine was used as the reference drug.

Results and Discussion

Gas chromatography coupled to mass spectrometry (GC-MS)

The identification of the constituents of *Mentha pulegium* leaves essential oils using GC-MS has enabled us to identify 31 compounds, with a contribution of 94.22% for Algerian sample and 30 compounds, with a contribution of 92.91% for the Tunisian. It was noted that pulegone is the major compound with 67.95% for Algerian *Mentha Pulegium* and 48.78% for the Tunisian.

The percentage content of the individual components, the retention indices and the chemical of the oil compounds are summarized in **Tables 1 and 2**.

The chemical composition of *Mentha pulegium* essential oil has been determined by previously studies in other counties [18] [29-

Table 1 Chemical composition of Mentha pulegium essential oils by GC–MS. RI: Kovats retention indices and Pc: percentage of each compound.

Mentha pulegium Algeria				*Mentha pulegium* Tunisia		
N°	Compounds	RI	Pc (%)	Compounds	RI	Pc (%)
1	α-pinene	940	0.40	α-pinene	939	0.71
2	Camphene	946	0.05	Camphene	953	0.50
3	Sabinene	974	0.11	Sabinene	976	0.10
4	β-pinene	976	0.45	β-pinene	980	0.20
5	Oct-1-en-3-ol	977	0.52	Myrcene	991	0.82
6	Octan-3-one	985	0.18	α-phellandrene	1005	1,27
7	Myrcene	989	0.75	α-tepinene	1018	0.79
8	α-phellandrene	1003	0.20	limonene	1022	1.11
9	α-tepinene	1010	1.82	β-ocymene	1028	0.09
10	Para-cymene	1018	5.98	β-phellandrene	1034	0,22
11	limonene	1022	1.11	1,8-cineole	1044	4.33
12	β-phellandrene	1024	0,22	γ-terpinene	1066	0,22
13	1,8-cineole	1028	0.14	α-terpinolene	1095	0.18
14	Eucalyptol	1032	0.05	Piperitone oxide	1125	1.55
15	γ-terpinene	1052	4.65	Terpin-4-ol	1149	0.66
16	Terpinolene	1080	0.09	Menthone	1165	9.26
17	Octan-3-ol acetate	1125	0.13	α-tepineol	1169	0.27
18	Menthone	1160	2.65	Isomenthone	1173	4.35
19	Borneol	1163	0.08	Carvone	1178	6.40
20	Menthol	1166	1.55	Borneol	1184	0.08
21	Terpinen-4-ol	1172	0.14	Menthol	1190	7.58
22	pulegone	1251	**67.95**	pulegone	1293	**48.78**
23	piperitone	1255	0.35	Linalool	1304	0.06
24	Menthyl acetate	1288	0.51	piperitenone	1313	0.29
25	piperitenone	1339	1.22	α-copaene	1388	0.02
26	α-copaene	1370	0.02	β-caryophyllene	1452	0.42
27	β-bourbonene	1382	0.27	α-humulene	1458	0.08
28	β-caryophyllene	1411	0.42	Germacrene-D	1489	1.66
29	α-humulene	1449	0.70	δ-cadinene	1533	0.81
30	Germacrene-D	1479	0.17	Caryophyllene oxide	1999	0.98
31	δ-cadinene	1520	0.03			
Total		**92,91**		**Total**	**94.22**	

Table 2 Comparative results of the percentages of pulegone in the essential oil of *Mentha pulegium* in some different countries.

Country	Algeria	Tunisia	Egypt [18]	Morroco [29]	Brasil [30]	Iran [31]	Bulgaria [32]	Uruguay [33]	Portugal [34]	Turkey [35]
% pulegone	67.95	48.78	43.50	84	31.05	40.5	45.4	73.4	23.20	28.90

35]. A comparison between these studies showed the variability of volatile compounds.

Table 2 shows the percentages of the pulegone identified in some essential oils from *Mentha Pulegium* in some different countries.

The large variability in this species and the chemical differences can be most probably explained by the existence of different chemo types. So the geographical distribution of this plant influenced significantly the chemical composition of its essential oils.

Infrared spectroscopy

The results of IR spectroscopic analysis are shown in **Table 3**. We notice the presence of broad and intense bands located at 3332-3516 cm^{-1} corresponding to the alcohol functions (OH), confirmed by the presence of a characteristic band of the CO bond aliphatic around 1060-1140 cm^{-1} and aromatic or α, β unsaturated around 1200 cm^{-1}.

The unsaturation and aromaticity is confirmed by the presence of vibration bands of deformation outside the plane around 700 cm^{-1}.

Radical scavenging activity test: Antioxidants can scavenge the radical by hydrogen donation, which causes a decrease of DPPH (2.2-diphenyl-1-picrylhydrazyl) absorbance at 517 nm [36].

In the current study, the assessment of the antioxidant activity using the DPPH free radical method was evaluated. The DPPH scavenging index and the half maximal inhibitory concentration (IC50) value were determined to assess the antioxidant activity of essential oils.

IC50 value is negatively related to the antioxidant activity, the

lower the IC50 value, higher is the antioxidant activity of tested sample.

The results obtained show that the essential oil of Algerian *mentha pulegium* has antioxidant ability, with (IC50 = 95 µg.mL^{-1}) stronger than that of Tunisian *mentha pulegium* (IC50=107 µg.mL^{-1}).

The antioxidant activities of essential oils from aromatic plants are mainly attributed to the active compounds present in them. The most powerful scavenging compounds were reported to be the monoterpene, ketones, menthone and isomenthone [37].

The kinetic of the antioxidant activity of essential oils is presented by the curves of **Figure 1**. For the essential oils examined, the reaction is biphasic, with a rapid decline in absorbance in the first minutes, followed by a slower step, until equilibrium is reached, then there are two areas:

- Zone with strong kinetics of trapping of the radical observed after the first fifteen minutes for the Algerian essential oil and less fast of the order of 50 minutes for the Tunisian essential oil.
- Zone with low kinetics of trapping of the DPPH radical or zone of trend towards equilibrium for the two oils.

The results show that the reaction between DPPH and essential oils reaches equilibrium after a short time for the Algerian essential oil.

Antifungal activity: Regarding antifungal activity, we are interested in the three pathogens that are responsible for a majority of human fungal infections which are Aspergillus Niger, Candida albicans, and Cryptococcus neoformans. For each of the three pathogenic fungal species studied, MIC values essential oils have been determined.

The essential oils activity against C. neoformans is more

Table 3 Results and identification of the chemical constituents of essential oils by IR analysis.

Mentha pulegium Algeria			*Mentha pulegium* Tunisia		
Frequency (cm^{-1})	**Group**	**Identification**	**Frequency (cm^{-1})**	**Group**	**Identification**
879	CH	Terpinene-4-ol	775	CH arom	-pinene
937	CH arom	Myrcene	878	CH	Terpinene-4-ol
1028	C-O-C	1-8 cineole	970	CH arom	Myrcene
1131	C-O	Octan-3-ol	1028	C-O-C	1-8 cineole
1373	CH vinyl	Myrcene	1131	C-O	Octan-3-ol
1452	CH_3 ; CH_2 def	Terpinene-4-ol	1373	CH vinyl	Myrcene
1616	C=O α , β insat	β-pinene	1453	CH_3 ; CH_2 def	Terpinene-4-ol
1682	υ (C=O)	Pulegone	1616	C=O α , β insat	β-pinene
1707	υ (C=O)	Menthone	1682	υ (C=O)	Pulegone
2872	CH	Myrcene	1708	υ (C=O)	Menthone
2954	CH_3 ; CH_2	Limonene	2872	CH	Myrcene
3502	OH	Menthol	2955	CH_3 ; CH_2	Limonene
			3509	OH	Menthol

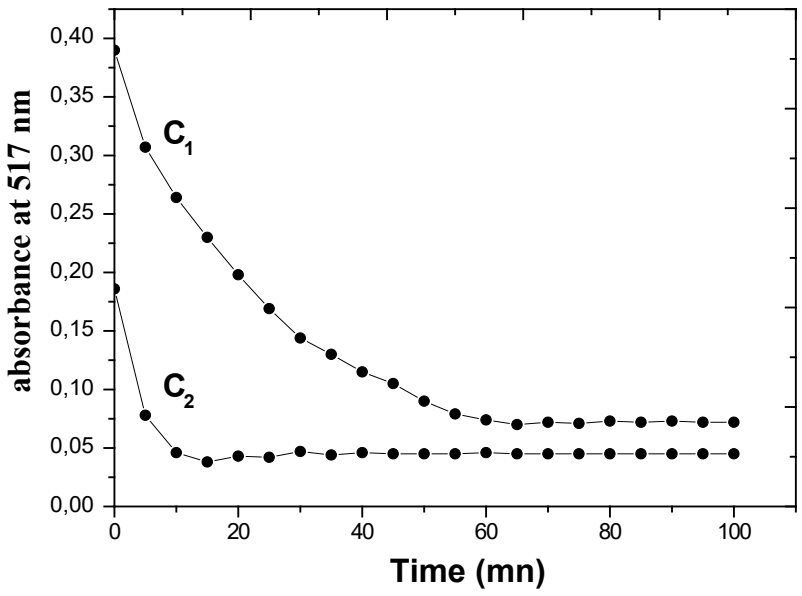

Figure 1 DPPH reduction kinetics obtained with the essential oils of *Mentha pulegium* L. C_1 corresponds to Tunisian M.P. Tunisian while C_2 is that of Algerian M.P.

Table 4 MIC of essential oils.

	Mentha pulegium Algeria	*Mentha pulegium* Tunisia
A. niger MIC (ppm)	313	625
C. albicans MIC (ppm)	625	625
C. neoformans MIC (ppm)	313	313

Table 5 Minimum and maximum **(**MIC) of some essential oils according to (Robert L. McFeeters).

A. niger MIC (ppm)	*Pogostemon cablin* (Indonesia): 160	*Myrtle communis* (Albania): 625
C. albicans MIC (ppm)	*Cedrus atlantica* (Morocco): 80	*Citrus junos* (Japan): 1250
C. neoformans MIC (ppm)	*Santalum spicatum* (Australia): 40	*Nymphaea caerulea* (India): 625

Table 6 *In vitro* antileishmanial activity results for essential oil.

Compound	Cytotoxicity CC50 ± SD (μg. mL^{-1})	*In vitro* antileishmanial activity on *L. dovani* axenic amastigotes IC50 aa ± SD (μg.mL^{-1})	Intramacrophages amastigotes IC50 ia ± SD (μg.mL^{-1})
Mentha pulegium Algeria	80	90	100
Mentha pulegium Tunisia	90	100	100

interesting than A. Niger and C. albicans. However the results obtained show that the essential oils of the Algerian and Tunisian *mentha pulegium* have significant antifungal activity.

Results of the minimum inhibitory concentrations (MIC) are summarized in **Table 4**.

In **Table 5** we propose some values of the minimum inhibition concentration for the three pathogens studied, we therefore present the minimum and maximum values (MIC) of certain essential oils according to (Robert L. McFeeters) [25].

The essential oils of both species have shown significant antifungal activity against the tested fungi. This bioactive power observed in the two oils is mainly attributed to their high contents in terpene phenols [38].

Antileishmanial activity: Antileishmanial properties of essential oils are described in **Table 6**. The IC50 values evaluated in L. major promastigote and axenic amastigote forms were around 80-100 μg.mL^{-1}.

In the present study, the extracts of *Mentha pulegium* had *in vitro* effects on *Leishmania Major and Donovani (LV9) Axenic amastigotes*.

Secondary metabolites such as alkaloids, flavonoids, saponins and terpenoids were known to possess antileishmanial activities. Thus the inhibitory effect of the extracts could be due to presence of wide range of secondary metabolites with different polarities [39-41].

This activity was explained by the mechanism of extract either by killing parasites or causing metabolic disorders to inhibit the reproduction of parasites [42].

Conclusion

The present study has allowed us to describe and to compare chemical composition of Algerian and Tunisian *Mentha Pulegium* essential oils. We have also studied antioxidant, antifungal and antileishmanial activities.

It can be concluded that the essential oils of Algerian and Tunisian *Mentha Pulegium* seem to be a natural means for the antifungal and antileishmanial treatment and also seem good antioxidants.

These essential oils can be used as an antibiotic or organic food preservative, therefore a natural replacement for harmful synthesized chemicals. However, the toxicological effects of these oils have to be investigated before human consumption before considering their use for food preservation or medicinal purposes.

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