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Chemical Constituents, *In Vitro* Antibacterial Properties and Antioxidant Activity of Essential Oils from *Marrubium vulgare* L. Leaves

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ABSTRACT

Aromatic and medicinal plants contain important bioactive compounds with many interesting biological activities, and Morocco is considered among the countries rich in this kind of plants. In this study, the chemical composition of essential oils of *M. vulgare L.* leaves growing in Morocco was investigated using gas chromatography/mass spectrometry (GC-MS) and gas chromatography coupled with flame-ionization detector (GC-FID). These essential oils were evaluated for their antibacterial activity against five pathogenic bacteria: *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. The antioxidant activity was evaluated via free radical scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH), and total antioxidant capacity (TAC). The results revealed the presence of thirty-three volatile compounds in the essential oils and the composition consists mainly of monoterpenes: α-Pinene (33.91%), 3-carene (8.68%), camphene (4.31%), β-myrcene (2.66%), longifolene (2.49%), and sesquiterpene like cadinol (5.86%). The essential oils exhibited an interesting antibacterial activity, where the inhibition zones for the bacterial strains, were in the range 8.00 ± 0.56 and 34.00 ± 0.05 mm, also, the essential oils revealed a minimum inhibitory concentration (MIC) ranging between 0.1 and 0.65 mg/mL. The evaluation of the antioxidant activity, showed that the essential oils gave IC_{50} = 108.75 ± 1.8 µg/mL, which was lower compared to BHT (IC_{50} = 7.71 ± 0.51 µg/mL), and ascorbic acid (IC_{50} = 1.16 ± 0.29 µg/mL) in DPPH method, and a total antioxidant capacity of 218.42 ± 8.67 mg EAA/g. The essential oils of *M. vulgare L.* can be used as sources of antioxidant and antibacterial activity.

Keywords: *M. Vulgare L.*, Essential oils, Chemical composition, Antibacterial, Antioxidant activity.

Introduction

Morocco is considered as one of the countries which contains a great wealth of aromatic and medicinal plants, in the northern inland of this country, the local population uses these plants daily in kitchen, cosmetics and traditional therapy, [1] also the traditional medicine hold an important place in this country, where many plants are used for the treatment of many diseases and for prevention. [2,3] Essential oils can be extracted from aromatics plants and used for their biological activities, as antioxidant, antimicrobial, and antiviral, in addition to that, essential oils are widely used by Arabs since the middle ages, in food, cosmetic and curative interest. [4,5] Isolation of essential oils and their component are the subject of many research above the years for medicinal and cosmetic purposes.[6] *Marrubium* genus contain approximatively 30 species, which considered as essential oil-poor species. [7,8] *M. vulgare* L. "horehound" is a perennial herb of the Lamiaceae family known in Morocco as "Merriwa", and its frequently used in traditional medicine to treat various illnesses, and reported in different studies, and proving many biological activities, such as analgesic, antihypertensive and anti-edematogenic activity. [9]This species has been known as a remedy for upper respiratory tract ailments. Nowadays, horehound is used in herbal medicine for treatment of liver diseases, biliary tract disorders, and for increasing the appetite and supporting the function of the stomach. *M. vulgare* L., is reported to possess antioxidant activity, [10] and also as anti-inflammatory, [11] hypoglycemic [12] and vasorelaxant. [13] The essential oils from *M. vulgare* L. was already studied and showed a good antioxidant effectiveness, [14,15] nociceptive,[16] antibacterial, [17] antifungal.[18] Nevertheless, there are few information on the antioxidant properties of horehound leaves essential oils. Moreover, there is no report on the chemical composition and antioxidant activity of the essential oils of horehound leaves from Morocco. Currently, the use of synthetic substances has been restricted in food because of its carcinogenic effect. Therefore, the search for new natural antioxidant sources has been greatly intensified. In the same context this study was performed to determine the chemical composition of the essential oils of *M. vulgare* L. leaves from Morocco and to evaluate their antibacterial properties and antioxidant activity.

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Materials and Methods

Plant material

M. vulgare L. (Lamiaceae) used in this study have been collected between May and June 2019 in Fez-Morocco 34°03'50.1"N 5°02'13.3"W, and identified by Pr. Amina BARI botanist at the Sidi Mohamed Ben Abdellah University, Fez, Morocco. A voucher (RM001617) sample was deposited at the herbarium of of the Laboratory of Natural Substances, Pharmacology, Environment, Modeling, Health & Quality of Life, Faculty of Sciences Fez, and for

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the part used, the leaves have been cut, and dried in the laboratory ambient air sheltered from light and moisture.

Essential oils extraction
The dry leaves of *M. vulgare* L. (200 g x 3) were extracted by hydrodistillation following the protocol described by Rezouki et *al.* [19] The extraction was done for 4h using a Clevenger-type apparatus, in a 2 liter flask containing 1 liter of distilled water, and surmounted by a column 60 cm long and 2 cm in diameter connected to a condenser, the distillation repeated three times to recover a considerable volumes for the biological study. The distilled essential oils were dried over anhydrous sodium sulfate, filtered and stored at -4°C. [20]

Identification of essential oils components
The analysis of essential oils of *M. vulgare* L. leaves was performed by gas chromatography coupled with mass spectrometry (GC/MS), and gas chromatography coupled with flame-ionization detector (GC-FID), at the city of innovation in Sidi Mohammed Ben Abdellah University, Fez, Morocco.

Gas chromatography (GC-FID) analysis
The quantitative analysis of the essential oils was analysed with GC/FID (Trace GC ULTRA S/N 210729, Thermo Fischer, France), Varian capillary column (5% poly diphenyl 95% dimethylsiloxane, TR5- CPSIL- 5CB; 50 m length, 0.32 mm of diameter and film thickness 1.25 µm). The column temperature was programmed from 40 to 280°C for 5°C/min and finally held at that temperature for 10 min. The temperature of the injector was fixed to 250°C and the one of the detectors (FID) to 260°C. The debit of gas vector (Azot) was fixed to 1 mL/min and split injection with split ratio 1:40. The volume injected was 1 µL of diluted oil in hexane solution (10%). The percentage of each constituent in the oil was determined by area peaks.

Gas chromatography - mass spectrometry (GC/MS)
The GC-MS analysis was performed with a gas chromatography (Trace GC ULTRA S/N 20062969 /Polaris Q Thermo Fischer, France) equipped with an ion trap mass spectrometry system. HP-5MS capillary column (60m x 0.32mm; coating thickness 0.25um) was used for the chromatographic separations. Temperatures of the transfer line and the ionic source were 300°C and 200°C, respectively; scan range, 40-650 amu; 3.9 scans/s. Oven temperature programmed from 50 to 260 °C ramp of 3°C /min; injector temperature was 250°C; carrier gas helium at 1 mL/min; injection of 1µL (10% cyclohexane solution); split ratio 1:30. Identification of the constituents was based on comparison of the retention times with those of authentic samples, comparing their linear retention indices relative to the series to C8 – C29 alkanes, and on computer matching against commercial (NIST-MS) and laboratory-developed library mass spectra built up from pure substances and components of known oils and MS literature data. [21]

Antibacterial activity assays
The evaluation of the antibacterial activity was performed by disc diffusion method, and the determining of the minimum inhibitory concentration against five strains : *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, according to published procedures with some modification. [22,23] The method of disc diffusion had for objective the determination of the zone of inhibition caused by the essential oil around the bacterial strain. For this, a disc of 0.6 cm in diameter from Whatman paper number 1 was used, then the discs immersed with 10 µL of essential oils, are placed in the surface of the petri dish already filled with agar (MH) culture medium and inoculated with bacterial strains ($1x10^8$ à $2x10^8$ CFU/mL). Antibacterial activity was evaluated after incubation (37 ° C) for 24 h by measuring the zone of inhibition around the discs in millimeters.
The determining of the minimum inhibitory concentration (MIC) was performed by dilution of essential oils in dimethyl sulfoxide (DMSO). For inoculation 50 µL of the culture medium (MH) was deposited in each well of the microplate. The microplate was incubated for 24 h at 37°C. MIC was defined as the lowest concentration that inhibited the visible bacterial growth. The growth of microorganisms is revealed by a white spot below the wells, and 10 uL of Resazurin (5 mg/mL) for the confirmation. The experiments were repeated twice and the antibacterial activity of *M. vulgare* L. leaves oils was examined using different bacterial species and tetracycline (5 mg/mL) was used as a positive control.

Scavenging ability on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical
The evaluation of the antioxidant activity of the essential oils, was carried out according to the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity method described by Mssillou et *al*., [20] where 100 µL of essential oils at different concentrations is added to 750 µL of methanolic solution of DPPH at 0.004%. After incubation for 30 min at darkness and room temperature the absorbance was measured at 517 nm in a Shimadzu 160-UV Spectrophotometer, with negative control which does not contain the sample. The percentage inhibition (PI%) of the free radical DPPH was measured according to the following equation:

$$PI\ (\%) = (A_0 - A/A_0) * 100$$

PI (%): The percentage of inhibition of free radical DPPH
A_0: The absorbance of the control (the sample to be tested must be replaced by methanol)
A: The absorbance of the sample to be tested

The tests were performed in triplicate and the 50% inhibition (IC_{50}) is determined by the previous equation, and the inhibition of DPPH was compared with antioxidant such as BHT and ascorbic acid.

Test of total antioxidant capacity
Essential oils (100 µL) was mixed with 1mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95 °C for 90 min. Then, the absorbance was measured by the spectrophotometer at 695 nm in a Shimadzu 160-UV Spectrophotometer with a blank containing 100 µL of methanol instead of the extract, after cooling to room temperature. [24] The total antioxidant capacity was expressed in milligrams of ascorbic acid equivalent per gram of extracts (mg EAA/ g of extracts) from a calibration curve of Ascorbic acid.

Statistical analysis
The tests carried out in this study were repeated twice for the antibacterial activity and triplicated for the antioxidant activities (IC_{50} of DPPH and TAC value), and the results are expressed by mean ± standard deviation, using Microsoft Excel

Results and Discussion

Essential oils compositions
The hydrodistillation of *M. vulgare* L. leaves results an essential oil with a yield of 0.03 v/w (dry weight). The essential oils had a yellow color with a distinct odor. Many studies were performed on this plant in different countries, in the purpose to defined the chemical composition of its essential oils. In this study the chemical composition of produced essential oils was analyzed by GC/MS and GC-FID, and the results of this analysis are presented in the Figure 1 and Table 1.
The analysis of the chemical composition, showed the presence of thirty-three volatiles compounds identified in essential oils of *M. Vulgare* L. leaves, with a total of 65.49%. The major's compounds in the essential oils were: α-Pinene (33.91%), 3-carene (8.68%), cadinol (5.86%), camphene (4.31%), β- myrcene (2.66%) and longifolene (2.49%). In this study, the yield of this essential oils was 0.03%. Other studies from various countries showed that the yield obtained in this study is almost similar, like 0.07% in Slovakia, [25] 0.02% in Tunisia ,[15] and so less compared to Liban (1%). [26], and from 0.1% to 0.41% in Algeria. [27] Monoterpenes are essentially the major components of the

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essential oils of this plant, also monoterpenes and sesquiterpenes are generally the constituents of essential oils in different species of the genus *Marrubium.*[8] Other studies carried out on this plant, showed different chemical compositions compared to our study, like in Iran [28] where the major compounds are *β*-bisabolene (25.4%), *β*-caryophyllene (11.6%) and germacrene D (9.7%), in Slovakia [25] with *β*-caryophyllene (45.8%) and germacrene D (14.4%), in Tunisia [29] *β*-bisabolene (28.3%) and *β*-caryophyllene (7.8%), and in Algeria [30] eugenol and β-bisabolene. These differences due to the different part studied and also to the geographical area, and the difference of the environmental factors, however, the only chemical composition which is the closest one to our study, is that of the essential oils extracted from the leaves of *M. Vulgare* L., in Turkey, [31] *α*-pinene (28.85%), *β*-pinene (18.31%), and *β*-phellandrene (17.40%).

Antibacterial activity evaluation

Results obtained in the antibacterial tests of the essential oils are shown on table 2., where the inhibition zone diameter in (mm), and the minimum inhibitory concentration (MIC) expressed in mg/mL. From the results of the table, we can see that the values of MIC vary between 0.08 mg/mL to 0.65 mg/ml, and *Bacillus subtilis* is the bacteria that represents the lowest growth rate in the presence of our essential oils (MIC = 0.08 mg/ml). The data indicated that the bacteria (*B. Subtilis)* was the most sensitive strain tested to the essential oils of *M. vulgare. L,* with the highest inhibition zone 34 ± 0.05 mm, followed by *S. aureus, E. Coli* and *K. pneumoniae*, with an inhibition zone of 32 ± 0.16 mm, 16 ± 1.21 mm and 11 ± 0.75 mm respectively. Modest activities were observed against *P. Aeruginosa*, with inhibition zones of 8 ± 0.56 mm for oils. The results obtained show a clear convergence between our essential oils and the positive control (tetracycline), with a greater effectiveness for this latter against *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa.* During this study, essential oils leaves of *M. vulgare* L., present a good activity against the pathogenic bacteria tested.

Antibacterial activity of the essential oils can be attributed, to a considerable degree, to the presence of α-pinene, which appears to possess an antibacterial activity according to Tantaoui-Elaraki et *al.* [32] The major components of this essential oils, *α*-pinene, has been known to exhibit antimicrobial activity against the bacterial strains (*E. coli*, and *S. Aureus)* according to the study conducted by de Sousa Eduardo et *al.* [33] In other studies, the aqueous, hexane, acetone and methanol and essential oil extracts of *M. vulgare* L. against *Mycobacterium tuberculosis* had an inhibitory effect over 200 µg/mL.[34] Other species essential oils rich in α-pinene demonstrated a good potential antibacterial activity.[35] Monoterpenes hydrocarbons, terpinenes, have also shown antibacterial properties against bacterial strains.[36] The bridged bicyclic monoterpenes α-pinene and β-pinene showed considerable biological activity and a good therapeutic potential according to Salehi et *al*. [37] Our results seems to be similar to those of other *M. vulgare* L. essential oils analyzed by Petrović et *al.* [38] Intensive research has been conducted on the antibacterial activity of *M. Vulgare* L. [17,39] On the other hand, enantiomers of α-pinene, β-pinene, limonene and linalool have a strong antibacterial activity. [40] The antimicrobial activities have been mainly explained through C_{10} and C_{15} terpenes with aromatic rings and phenolic hydroxyl groups able to form hydrogen bonds with active sites of the target enzymes, although other active terpenes, as well as alcohols, aldehydes and esters can contribute to the overall antimicrobial effect of essential oils. [41]

Antioxidant potential activity

DPPH free radical scavenging activity and total antioxidant capacity

Antioxidant activity of essential oils from *M. vulgare* L. are well documented in the studies of Firuzi et *al*. And Lopes-Lutz et *al.* [27,42] In this paper, two experimental methods based on different reaction mechanisms were applied to achieve a comprehensive evaluation of antioxidant activity of *M. vulgare* L. essential oils. The evaluation of the antioxidant activity of the essential oils by DPPH radical scavenging activity method was compared with that of BHT and ascorbic acid and the results are presented in Table 3 and Figure 2.

The ability of the essential oils to reduce free radical DPPH was expressed by percentage of inhibition and 50% inhibitory concentration (IC_{50}). The results obtained for the essential oils (IC_{50} = 108.75 ± 1.8 µg/mL) indicate a good antioxidant capacity and this due to the richness of the essential oils in chemical compounds and bioactive molecules. The essential oils are compared with two antioxidants: BHT (IC_{50}= 7.71±0.51 µg/mL) and ascorbic acid (IC_{50}= 1.160.29 µg/mL), we notice the weak antioxidant power of our essential oils comparing to them.

In addition, the antioxidant activity of our essential oils was exanimated by the total antioxidant capacity following the phosphomolybdenum protocol. This method consists of the transformation Mo (VI) to Mo (V), in the presence of antioxidant substance. The results showed that the essential oils present a capacity of 218.42 ± 8.67 mgEAA/g. The essential oils showed significantly weaker ability to scavenge the DPPH radical when compared to gallic acid, even that the essential oils from the leaves of *M. vulgare* L. showed a good antioxidant power. *M. vulgare* L. (Lamiaceae) collected from region of Fez city (Morocco) between May and June 2019 was characterized by an IC_{50} (108.75 ± 1.8 µg/mL**)**, it is relatively low than other *M. vulgare* L., studied in Algeria which the IC_{50} was ranging from (IC_{50} = 178.74 ± 4.37 to 379.64 ± 5.73 µg/mL).[27] Contrary, the IC_{50} obtained from this work was relatively higher than other plants of *M.vulgare* L. (IC_{50} = 70 µg/mL), (15) (IC_{50} = 15.10 µg/mL).[43] The different antioxidant activities observed, may be due to the difference in chemical constituents and geographic localities.

On the other hand, the major compound of this essential oils such as α-Pinene (33.91%) have been tested individually to evaluate its antioxidant capacity, and showed a lower antioxidant activity (IC_{50}= 12.57 ± 0.18 mg/mL) in DPPH method, than the essential oils exanimated in this study. [44] So, we can conclude that the antioxidant power of the essential oils is due to the synergy between all their volatile compounds and not only to the most representative ones**.** Or these activities found are probably in relation with the structure of the hydroxylated compounds, but a possible synergistic effect among oxygen containing compounds can be suggested too. The result of the antioxidant activity of our essential oils is close to that reported in Tunisia, [45] and to those from Lithuania.[46]

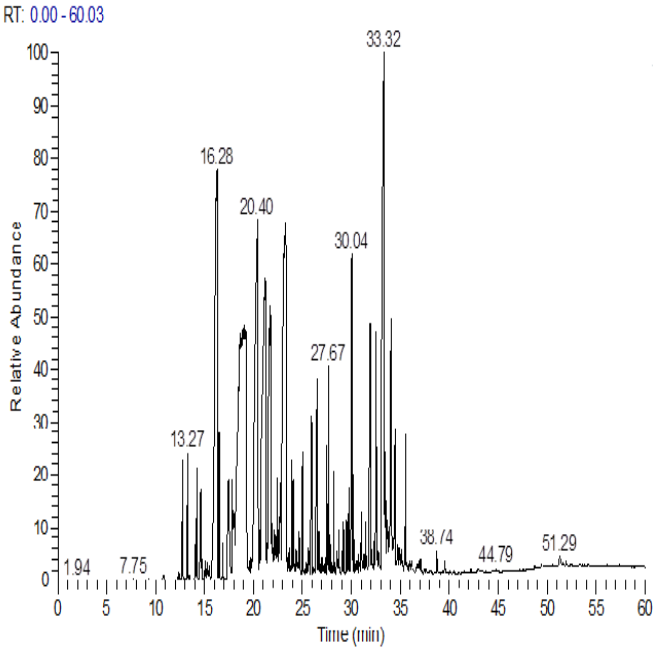

Figure 1: Chromatogram of *Marrubium vulgare* L. essential oils

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Table 1: Chemical composition (%), of *M. vulgare* L., essential oils from leaves

Peak	Compounds	[a]RT	[b]KI	Essential oils ([c]Air%)	Method of identification
1	*β*-pinene	12.74	847	0.12	KI, GC//MS
2	camphene	13.27	860	4.31	KI, GC//MS
3	3-Carene	14.50	848	8.68	KI, GC//MS
4	myrcene	16.28	683	2.66	KI, GC//MS
5	sabinene	18.64	833	0.65	KI, GC//MS
6	clofentezine	20.40	582	1.00	KI, GC//MS
7	D-verbenone	21.51	770	1.72	KI, GC//MS
8	isobornyl formate	22.44	794	0.13	KI, GC//MS
9	fenchyl acetate	22.76	738	0.04	KI, GC//MS
10	geranyl bromide	23.93	748	0.20	KI, GC//MS
11	bornyl acetate	24.06	829	0.16	KI, GC//MS
12	thymol	24.66	833	0.16	KI, GC//MS
13	isopulegyl acetate-	25.59	732	0.21	KI, GC//MS
14	à-bourbonene	26.68	814	0.19	KI, GC//MS
15	humulen-(v1)	27.57	847	0.08	KI, GC//MS
16	longifolene-(V4)	27.67	853	2.49	KI, GC//MS
17	copaene	27.82	795	0.19	KI, GC//MS
18	isocaryophillene	28.22	843	0.19	KI, GC//MS
19	isoledene	28.72	805	0.16	KI, GC//MS
20	epi-bicyclosesquiphellandrene	29.19	845	0.19	KI, GC//MS
21	ç-elemene	29.52	758	0.04	KI, GC//MS
22	aromadendrene oxide-(2)	30.04	809	0.64	KI, GC//MS
23	caryophyllene oxide	31.04	810	0.14	KI, GC//MS
24	gitoxigenin	31.93	744	0.20	KI, GC//MS
25	alpha-pinene	32.33	787	0.18	KI, GC//MS
26	guaiene	32.75	795	0.16	KI, GC//MS
27	*α*-Pinene	33.32	676	33.91	KI, GC//MS
28	ç-himachalene	33.46	742	0.18	KI, GC//MS
29	vatirenene	33.63	796	0.17	KI, GC//MS
30	farnesyl bromide	34.08	721	0.17	KI, GC//MS
31	carvacrol	34.47	758	0.14	KI, GC//MS
32	calarene epoxide	35.08	788	0.17	KI, GC//MS
33	cadinol	38.04	721	5.86	KI, GC//MS
Total Identified Constituents (%)				65.49	

[a]RT: Retention time obtained by chromatogram (Figure 1,2).
[b]KI: Kovats Index was determined by GC-FID on a HP-5MS column.
[c]Air% was determined by mass spectrometry (PlarisQ).

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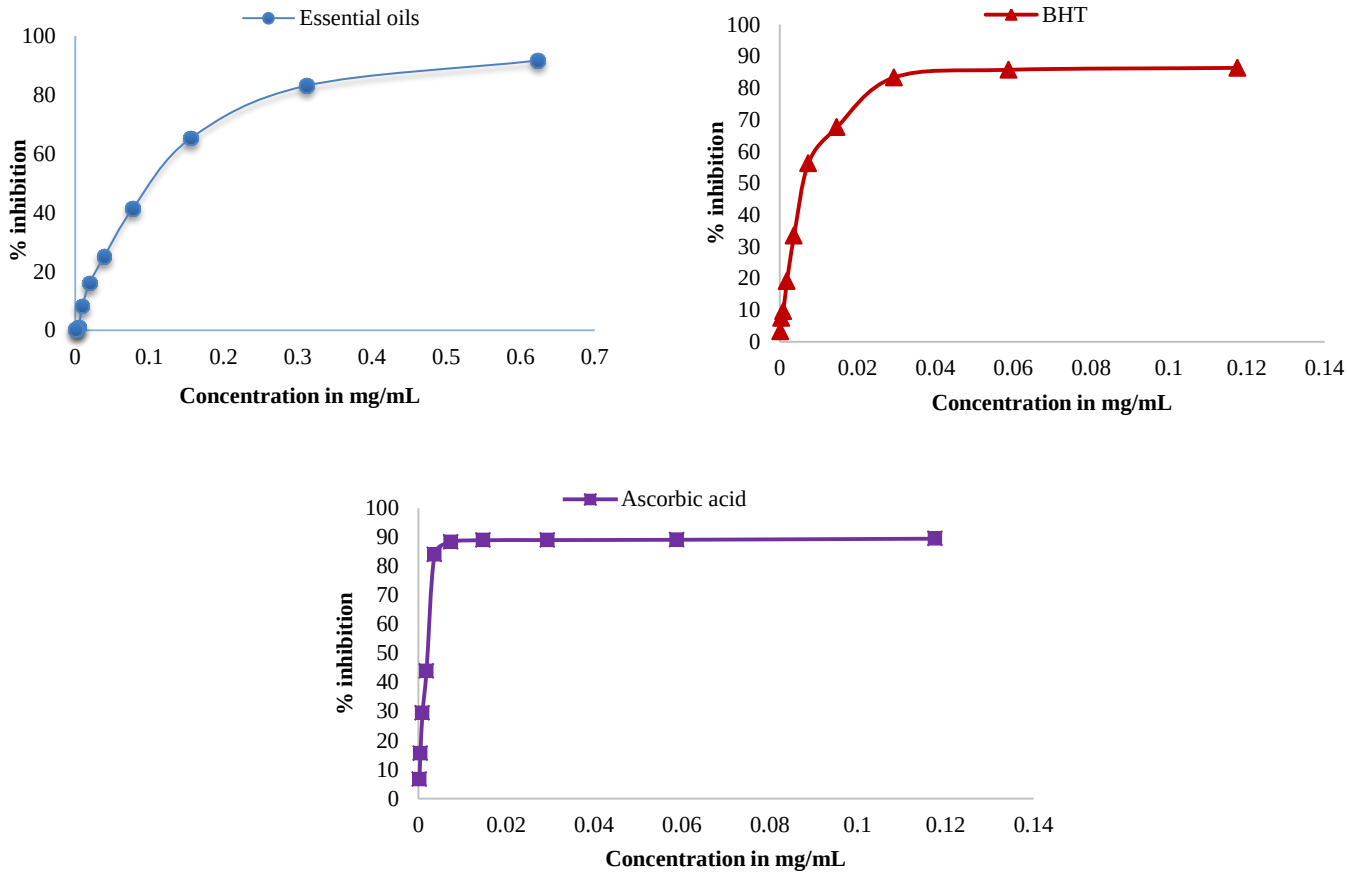

Figure 2: Antioxidant activity with DPPH method of essential oils compared to BHT and Ascorbic acid

Table 2: Antibacterial properties of leaves essential oils of *M. vulgare* L

	Essential oils		Tetracycline	
Microorganisms	**INZ**	**MIC**	**INZ**	**MIC**
Bacillus subtilis	34 ± 0.05	0.08	23 ± 2.5	0.062
Staphylococcus aureus	32 ± 0.16	0.10	18 ± 2	0.062
Escherichia coli	16 ± 1.21	0.20	20 ± 3	0.25
Klebsiella pneumoniae	11 ± 0.75	0.40	16 ± 3	0.003
Pseudomonas aeruginosa	8 ± 0.56	0.65	14.33 ± 1.1	0.25

INZ: Inhibition zone (mm), MIC: minimum inhibitory concentration mg/Ml, Disc diameter 6mm average of two consecutive trials

Table 3: Inhibitory concentration of essential oils in µg / mL compared to that of BHT and ascorbic acid

	Essential oils	**BHT**	**Ascorbic acid**
(IC_{50}) µg/mL	108.75 ± 1.8	7.71 ± 0.51	1.16 ± 0.29

Conclusion

In this study the chemical composition of the essential oils extracted from the leaves of *M. vulgare* L. was identified, and also their antioxidant activity by two methods. The results obtained have shown the richness of the essential oils in monoterpenes compounds, and α-pinene as the major component, and a good antioxidant potential wit an IC_{50} = 108.75 ±1.8 µg/mL, for essential oils in the DPPH method. Antibacterial properties of the essential oils were due to abundance of the α-pinene and also the special chemical constituents of this essential oils. Moreover, the results presented from previously studies indicated that the essential oils obtained from M. vulgare L., showed significant variability in their chemical composition, antibacterial properties and antioxidant activity. The antibacterial activity besides several biological properties can be employed in place of costly antibiotics for effective control of food borne pathogens. So, these essential oils can be used as a natural food preservative and enhance the human health as natural antioxidant. Other investigations of compounds are necessary to assess the effectiveness of this oils in food system, perfumes and pharmaceuticals fields. However, further scientific studies are needed to explore clinical efficacy, toxicity and to explore the therapeutic effect of major secondary metabolites of *M. Vulgare* L. leaves from Morocco.

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Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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