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In vitro Screening of Antibacterial and Antioxidant Activities of Essential Oils from Four Moroccan Medicinal Plants

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Evaluation of antibacterial and antioxidant activities of essential oils extracted from *Salvia officinalis*, *Mentha viridis*, *Eucalyptus globulus* and *Myrtus communis* from Ouezzane province.
Study Design: *In vitro* evaluation of antibacterial and antioxidant activities of medicinal plants essential oils (EOs).
Place and Duration of Study: Department of Biology (Faculty of Sciences), July, 2015 to September, 2016 (15 Months).
Methodology: Essential oils were extracted by hydrodistillation method, while agar well diffusion,

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microdilution and spectrophotometry methods were used to evaluate the antibacterial and antioxidant activities respectively.

Results: The yields of EOs are 0.9, 1.2, 2.5, and 2.1% for *M. communis*, *E. globulus*, *M. viridis*, and *S. officinalis* respectively. EOs showed significant antibacterial activities against test bacterial strains: *Staphylococcus aureus* CECT 976, *Staphylococcus aureus* CECT 994, *Listeria monocytogenes* serovar 4b CECT 4032, *Proteus mirabilis*, *Staphylococcus aureus* MBLA, *Escherichia coli* K12, *Pseudomonas aeruginosa* and *Bacillus subtilis* 6633. *Salvia officinalis* EO was more active than the rest EOs on the test bacteria and exhibited the highest zone of inhibition (23 mm) against *B. subtilis* bacterial, while *P. aeruginosa* was the most resistant bacterial strain. *S. officinalis* and *M. communis* EO showed minimum inhibitory concentration at MIC=0.5 % (v/v) against *L. monocytogenes* and *P. mirabilis.* The antioxidant results indicated that *M. communis* and *S. officinalis* posess the ability to scavenge DPPH radicals. Their IC_{50} Values of 0.24 and 0.46 mg/mL respectively, suggest their anatioxidant capacity compared to reference drugs IC_{50} value (IC_{50}=0.027 mg/mL for ascorbic acid and IC_{50}=0.043 mg/mL for Trolox).

Conclusion: Our study showed that apart from the local uses of the plants extracts, the EOs of *S. officinalis*, *M. viridis*, *E. globulus* and *M. communis* plants poses strong antibacterial and antioxidant properties and may be useful as food preservatives.

Keywords: Essential oil; antibacterial activity; antioxidant activity.

1. INTRODUCTION

The resistant to antibacterial agents increases dramatically and become a major problem of health and economy, eradicating the discovery of antibiotics and their use in clinical medicine. Today, the bacterial resistance to antibiotics emerges in an accelerated way since their introduction into clinical use [1]. Indeed, bacterial resistance correlates positively with the use of antibacterial drugs in clinical practice [1,2,3].

The use of antibiotics may increase the selective pressure of a bacterial population and therefore the survival of resistant bacteria. Practically, we can arrive to a bacterial population that may resist all available antibiotics. Multi-drug resistance has been found in some pathogens bacterial strains such as *P. aeruginosa*, *E. coli*, *S. aureus* VRSA and *Mycobacterium tuberculosis* [4,5].

Oxidative stress is a phenomenon of chemical imbalance between the oxidized molecules and those reduced [6]. The result is the regeneration of free radicals including superoxide radical and hydrogen peroxide that are that are often shown as initiators involved in the pathogenesis of several diseases such as cancer, atherosclerosis, diabetes, etc. Synthetic antioxidants have often shown adverse effects on human health beyond their desired beneficial [7], hence the search for natural alternatives to bacterial resistant and some effects of synthetic antioxidants is necessary. Medicinal and aromatic plants are rich in secondary metabolites that can be used as antibacterial as well as antioxidants [8]. EOs are volatile substances of very diverse chemical structures [9]. They are now regarded as potential source of molecules with multiple biological activities. In recent times, some essential oil scientists have showed the antibacterial and antioxidant properties of some plants EOs [8,9,10]. The antibacterial and antioxidant activities of essential oils and their chemical compositions are related to the botanical origin of the plant and the method of extraction used [11].

Morocco has a Mediterranean climate with his ecological and economic interest in aromatic and medicinal plants [12]. Some of these plants have showed several pharmacological properties in previous studies [13,14,15]. However, the province of Ouezzane (North-West of Morocco) despite the abundance of aromatic and medicinal plants, there is dearth of information of their bioactivity and economic value. Indeed, there are only few studies undertaken by our laboratory for pharmacological enhancement of extracts of some plants in this region [16,17,18, 19]. Therefore, we aimed in this study to evaluate the antibacterial and antioxidant activities of essential oils from above medicinal plants selected based on ethnobotanical study.

2. MATERIALS AND METHODS

2.1 Plant Material and Extraction Procedure

Medicinal plants were collected from Ouezzane province (Table 1). The identification was done by Pr. ENNABILI Abdesalam (National Institute

of Medicinal and Aromatic Plants, Taounate, Morocco). The collected materials were air dried at room temperature (≈23℃) in the shade and subjected to hydrodistillation, using a Clevenger-type apparatus for 3 h until total recovery of oil. The extraction essential oils was performed three times (3 × 150 g) and oils were dried with anhydrous sodium sulphate, weighed and stored at 4℃ until use. The EO yield is determined from the dry matter. Yields are expressed in mL /100 g of dry matter.

R = Pb/Pa *100

R: oil yield in %; Pb: weight of oil g; Pa: plant weight in g.

2.2 Antibacterial Activity

2.2.1 Bacteria strains

To evaluate the antibacterial activity of essential oils, we used the following bacteria: *E. coli* K12 and *S. aureus* MBLA (Laboratory of Food Microbiology, Université Catholique de Louvain (UCL), Belgium), *S. aureus* CECT 976, *S. aureus* CECT 994, *L. monocytogenes serovar* 4b CECT 4032 and *P. mirabilis* (Coleción Española de Cultuvos Tipo (CECT); Spanish Type Culture Collection), *P. aeruginosa* IH (Institute of hygiene, Rabat, Morocco: IH) and *B. subtilis* 6633 (Deutsche Sammlung von Mikroorganismen (DSM); German Collection of Microorganisms). Strains are maintained on an inclined agar medium at 4℃ (conserved in Lysogeny broth agar medium: LB agar). Before use, the bacteria were revived by two subcultures in an appropriate culture medium (Lysogeny broth liquid medium) at 37℃ for 18 to 24 h. For the test, final inoculum concentration was adjusted to 10^6 CFU/mL.

2.2.2 Agar-well diffusion assay

The principle of this technique is to estimate the bacteriostatic activity of the essential oils by measuring the growth inhibition zone of germs around wells. It is mostly used in a preliminary step to further study because it provides access to essentially qualitative results. We have adapted the method previously described by [15,16]. Briefly, a basal layer was prepared by Muller-Hinton agar. After the agar plates were solidified, sterile 8 mm diameter cylinders were deposited. Six mL of LB medium in superfusion containing 0.8% agar were inoculated by a fresh culture of indicator bacterial strain (a final concentration was 10^6 CFU/mL). After solidification, the wells were filled with 50 µl of essential oil. After incubation at appropriate temperature (37℃) for 24 h, all plates were examined for any zone of inhibition, and the diameter of these zones was measured in millimeters. The experiment was carried out thrice and each test was performed in triplicate.

2.2.3 Minimum inhibitory concentration (MIC)

MICs were determined using the broth microdilution assay, as previously described [15,16,17]. Briefly, agar was used at 0.15% (w ⁄ v) as stabilizer of the oil–water mixture and resazurin as bacterial growth indicator. First, 50 µl of Mueller Hinton Broth (Oxoid; UK) supplemented with bacteriological agar (0.15% w⁄ v) was distributed from the 2nd to the 12th well of a 96-well polypropylene microtitre plate (Costar; Corning Incorporated, Corning, NY, USA). A dilution of the essential oil was prepared in Mueller Hinton Broth supplemented with bacteriological agar (0.15% w⁄ v), to reach a final concentration of 16%; 100 µL of these suspensions was added to the first test well of each microtitre line, and then 50 µL of scalar dilution was transferred from the 2nd to the 11th well. The 12th well was considered as growth control, because no essential oil was added. We then added 50 µl of a bacterial suspension to each well at a final concentration of approximately 10^6 CFU/mL. The final concentration of the essential oil was between 8 and 0,125% (v⁄v). Plates were incubated at 37℃ for 18 h. After incubation, 10 µl of resazurin was added to each well to assess bacterial growth. After further incubation at 37℃ for 2 h, the MIC was determined as the lowest essential oil concentration that prevented a change in résazurine colour. Bacterial growth was detected by reduction in blue dye resazurin to pink resorufin. A control was carried out to ensure that, at the concentrations tested, the essential oil did not cause a colour change in the resazurin. Experiments were performed in triplicate, and modal values were selected.

2.2.4 Minimum bactericidal concentration (MBC)

MBC corresponded to the lowest concentration of the essential oil yielding negative subcultures after incubation at appropriate temperature for 24 h. It is determined in broth dilution tests by sub-culturing 10 µL from negative wells on plate count agar (PCA) medium. The experiment was carried out thrice and each test was performed in triplicate.

Table 1. Plants used, family, common names, place of collection, part collected and some ethnopharmacological properties of plants tested

Plants species (family)	Trivial name	Place of collection	Part plant collected	Traditional use	Phytochemistry	Pharmacological properties
Salvia officinalis L. (Lamiaceae)	Salmia	Zoumi	Flowering tops	Cardiac disease, hypertension and diabetes [20] Asthma and Inflammation [21] Chill, rheumatism and cough [22] Diabetes, cardiovascular diseases and pathologies of the digestive system [23]	Polyphenols, flavonoids [19] α-Thujone, camphor, α-pinene, β-thujone, α-thujone, 1,8- cineole, camphor and viridiflorol [24]	Antioxidant, antibacterial and anti-leishmanial activities [18,23,24]
Mentha viridis L. (Lamiaceae)	Na'naa	Ain Beida	Flowering tops	Cold, system digestive [22] Pathologies of the urinary system, pathologies of the respiratory system and dermocosmotology [23]	Carvone, 1,8-cineole, limonene [25]	Antibacterial activity of essential oil [13]
Eucalyptus globulus L. (Myrtaceae)	Kellito	Bni kolla	Leaves	Diabetes [20], Asthma [21]	9-Epoxy-18-hydroxyoctadecanoic acid 9,10,18-Trihydroxyoctadecanoic acid 9(7–10),16-Dihydroxyhexadecanoic acid [26]	Antibacterial [27] and hypoglycemic activity [28]
Myrtus communis L. (Lamiaceae)	Rihan	Moukrisset	Leaves	Cardiac disease, hypertension and diabetes [20], Cardiac weakness, digestive system [22], Pathologies of the digestive system allergy and diabetes [23]	α-Pinene, limonene, myrcene, *p*-cymene, α-caryophyllene germacrene-D, gallic acid, cafeic acid, syringic acid, vanillic acid, verulic acid [29,30,31,32]	Antioxidant activity [33] Antibacterial activity [17] Anti-genotoxic effect [34]

2.3 Antioxidant Activity

2.3.1 DPPH radical scavenging capacity assay

The ability of the plant extracts to scavenge DPPH (2,2-Diphenyl-1-picrylhydrazyl) free radicals was assessed using the standard method [35]. In brief, Aliquots (3 mL) of various concentrations (0.1; 0.25; 0.5; 1; 2.5 mg/mL) of the essential oils samples prepared in methanol were added to 1 mL of a 0.004% methanolic solution of DPPH. After an incubation period of 30 min in dark at 23 ± 2℃, the absorbance was recorded against a blank at 517 nm with a spectrophotometer. Absorption of a blank sample containing the same amount of methanol and DPPH solution acted as the control. Samples were analyzed in triplicate.

$$\% \text{ Inihiition} = \frac{\text{Abs (blank)} - \text{Abs(sample)}}{\text{Abs (blank)}} \times 100$$

Where *A*bs (blank) is the absorbance of the control and *Abs (sample)* is the absorbance of the sample. Ascorbic acid was used as positive control and the concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage plotted against the essential oil concentration.

2.4 Data Analysis

The statistical analysis was performed by a one-way ANOVA analysis of variance followed by Duncan's test, and results were considered to be statistically significant with a 95% confidence level ($p < 0.05$).

3. RESULTS AND DISCUSSION

3.1 Essential Oil Yield

The percentage of water content in the fresh plant materials are 67.3, 37.29, 32.08 and 26.41% for *S. officinalis, M. viridis*, *M. communis* and *E. globule* respectively. To carry out the hydrodistillation, the dry material was placed in a water-distiller with a water / plant material. The Table 2 summarizes the average yields of extracted essential oil. Yields are significantly varied between the four plants. The highest yield was recorded in *M. viridis* 2.5%, while *M. communis* had the lowest yield.

3.2 Antibacterial Activity

The antibacterial activity of four essential oils was assessed against eight bacterial strains by using Agar well diffusion method. The results are expressed as zones of inhibition as shown in Table 3. The results suggest that the essential oils from the four plants may have significant antibacterial activity against bacterial strains tested (Table 3).

Table 2. Humidity of plants tested and their yield of essential oil

Species	% of humidity	Yield (%)
Salvia officinalis (L.)	67.32	2.1
Mentha viridis (L.)	37.29	2.5
Eucalyptus globulus (L.)	26.41	1.2
Myrtus communis (L.)	32.08	0.9

This activity is depending on the essential oil ($p < 0.05$) and the nature of strain tested ($p < 0.05$). The variation of inhibition could be due mainly to the difference in the chemical composition of EO and presence of major compounds (Table 1). The highest inhibition zone (43 mm) was obtained with the essential oil of *S. officinalis* against *B. subtilis*. On other hand, *P. mirabilis* appears the most active to the bacteria tested, whereas *P. aeruginosa* is the most resistant to essential oils tested. In general, Gram-negative bacterial strains were more resistant than the Gram-positive bacteria [36,37,38,39]. The resistance of Gram-negative bacteria may due be to the character of their hydrophylique membrane which blocks the permeation of hydrophobic molecules such as phenolic compounds [40]. For example, the cell wall of *E. coli* is rich in lipopolysaccharides (LPS) that prevent hydrophobic molecules to pass through the membrane [41].

The evaluation of minimum inhibitory concentration and the minimum bactericidal concentration of plants essential oils against the most sensitive bacterial; *S. aureus*, *L. monocytogenes* and *P. mirabilis*; was carried out using microdilution assay (Table 4). The low MICs values (MIC=0.5%) were showed for *S. officinalis essential oil against L. monocytogenes* and for *M. communis essential oil against P. mirabilis.* While, *E. globulis* EO was less active against bacteria tested. The antibacterial activity of these EOs has been reported in some studies. The myrtle oil showed a significant antibacterial effect against several pathogenic strains [27]. This effect may be attributed to the presence of compounds such as Myrtenyl acetate, 1,8-cineol, α-pinene and linalool [29]. Another work carried

out by Nabavizadeh et al. revealed the bacteriostatic and bactericidal ability of this essential oil [42]. The EO of *S. officinalis* was reported be effective against certain bacterial strains [43,44,45]. The EO of *E. globulis* and *M. viridis* has also been shown active against pathogenic bacteria by previous studies [29,46,47,48].

3.3 Antioxidant Activity

The results of the antioxidant activity tested by scavenging DPPH radical of essential oils are shown in Table 5. They are expressed in scavenging percentage of DPPH radical. At the highest concentration (2.5 mg / ml), EOs from four plants (*S. officinalis*, *M. communis*, *E. globulis* and *M. viridis*) have proved a capacity reduction of DPPH free radical with percentages of trapping 87.26 ± 1.32, 98.05 ± 2.12, 53.29 ± 1.27 and 44.22 ± 0.74 mg/mL respectively.

However this activity remains below that of ascorbic acid used as a positive control ($p < 0.05$). For each EO, the ability to reduce the DPPH radical is concentration dependent. Therefore, the calculation of the effective concentration which reduces by 50% the initial concentration of DPPH (IC_{50}) is desirable to express the antiradical activity of EOs studied (Table 5). This capacity was determined from the graph plotted of inhibition capacity (AA in %) against extracts and standards concentrations, by using linear regression equations. The value of the antioxidant capacity 50 is inversely proportional to the antiradical effectiveness of the product tested. It is clear that the essential oil of *M. communis* showed great efficiency in reducing the DPPH radical (IC_{50}= 0.24 mg/mL). This is followed by essential oil of *S. officinalis* with an IC_{50} of 0.46 mg/mL. This high activity may be explained by the presence in these two essential oils for a large proportion of phenolic compounds with antioxidant activity. These different activities are indeed related to the nature and proportion of the active compounds present in the different oils.

The DPPH assay is a very common spectrophotometric method to determine the activity of any antioxidant. The advantage of this method is that the antioxidant activity is measured at ambient temperature, and thus, the risk of the thermal degradation of the molecule tested is eliminated [49]. Free-radical scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation [50]. Ours results are in consensus with others studies demonstrating the antioxidant activity of *M. viridis* *S. officinalis*, *M. communis*, and *E. globulis* EOs [43,46,48,51].

Table 3. Antibacterial activity of four essential oils against strains tested

Bacterial strains	Inhibition zones diameter* (mm)			
	M. viridis	***S. officinalis***	***E. globulis***	***M. communis***
S. aureus MBLA	18±1.34	14±0.81	13±0.46	11±0.34
S. aureus 976	09±1.9	07±0.6	8±2.33	08±1.3
L. monocytogenes	21±3.11	10±1.55	9±1.42	14±0.89
S. aureus 994	07±0.66	NA	07±0.98	NA
B. subtilis 6633	15±0.80	23±0,00	NA	NA
E. coli K12	09±0.65	NA	NA	NA
P. aeruginosa IH	NA	NA	NA	09±0.75
P. mirabilis	19±0.41	16±0.83	14±3.21	21±2.23

**Diameter of inhibition zone produced around the well. The values are the average of three replicates and the final cell density is about 10^6 CFU/mL*
NA: no active

Table 4. Determination of minimum inhibitory concentration (MIC % (v/v)) and minimum bactericidal concentration (MBC)

Microorganisms	***M. viridis***		***S. officinalis***		***E. globulis***		***M. communis***	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
S. aureus MBLA	4	>8	2	2	>8	>8	1	2
L. monocytogenes	1	4	0.5	2	4	4	2	4
P. mirabilis	4	>8	2	8	8	>8	0.5	1

MIC: Minimum Inhibitory Concentration
MBC: Minimum Bactericidal Concentration
Density is about 10^6 CFU/mL

Table 5. Antioxidant properties of ascorbic acid and essential oils from *S. officinalis, M. viridis, E. globulis* and *M. communis* as determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging

Essential oils	Antiradical activity (%)						IC_{50} (mg/mL)
S. officinalis	EO concentration (mg/mL)	0.1	0.25	0.5	1	2.5	0.46
	Scavenging effect on DPPH (%)	23,25	31,73	53,94	67,49	87,26	
M. viridis	EO concentration (mg/mL)	0.1	0.25	0.5	1	2.5	> 2.5
	Scavenging effect on DPPH (%)	3,05	9,37	23,25	31,75	44,22	
E. globulus	EO concentration (mg/mL)	0.1	0.25	0.5	1	2.5	2.37
	Scavenging effect on DPPH (%)	5,53	8,35	16,75	26,398	53,29	
M. communis	EO concentration (mg/mL)	0.1	0.25	0.5	1	2.5	0.24
	Scavenging effect on DPPH (%)	29,23	53,244	67,234	93	96,05	
Ascorbic acid	Ascorbic acid concentration (µg/mL)	30	60	125	250	500	0.027
	Scavenging effect on DPPH (%)	97,32	97.23	98.42	97.87	98.52	
Trolox	Ascorbic acid concentration (µg/mL)	30	60	125	250	500	0.043
	Scavenging effect on DPPH (%)	61.47	98.38	97.59	98.35	98.18	

4. CONCLUSION

Our study showed that apart from the local uses of the plants extracts, the EOs of *S. officinalis*, *M. viridis*, *E. globulus* and *M. communis* plants poses strong antibacterial and antioxidant properties and may be useful as food preservatives. Further study is however vital to identify and isolate the active compounds in these EOs.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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