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Essential Oil Chemical Composition of Myrtle Growing in Northeastern Algeria and Estimation of its Antibacterial Effectiveness

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Abstract: The aim of this study is to determine the antibacterial effectiveness of leaves essential oil extracted from myrtle growing in Algeria. A cluster analysis of soil and water was effectuated for performing chemical and granulometric analyses. The essential oil was isolated from leaves by hydro distillation and analyzed by Gas Chromatography-Mass Spectrometry (GC/MS). The leaves yield reached a peak at the flowering stage (1.14%) and a minimum in the fruiting time (0.6%). Several compounds in the leaf essential oil were characterized including 49 components. The major compounds of the oil are α-pinene (55%), 1,8-cineole (33,42%) and limonene (33,42%). The effect of essential oil and specific antibiotics was investigated *in vitro* against twenty Gram-negative bacteria. The essential oil effect was colicidal with best inhibition zone (35 mm). This study showed that the myrtle essential oil in relation with the chemical composition of soil give a promising perspective for the production of essential oils with constant composition and specific activities.

Keywords: *Myrtus communis*, Essential Oil, Chemical Composition, α-Pinene, 1,8-Cineole, Soil Analysis, Antibacterial Activity

Introduction

The problem of microbial resistance has grown significantly in the last several years due to the misuse of antibiotics and the increase of immunodeficiency (Grayer and Harborne, 1994). Nevertheless, plant extracts and natural products have been intensively investigated for their antimicrobial properties. The conventional antimicrobials from chemical synthesis, such as acetic, benzoic, lactic, propionic and sorbic acids, as well as nitrite and sulfites, have been used for many years to control the growth of microorganisms in food (Sofos *et al*., 1998). The absolute interdiction of chemical additives in aliments by the United States Food and Drug Administration (FDA) is due to the side effects of these chemicals on humans.

Plants have great advantages over microorganisms, making their exploitation in food or medicine more appropriate for replacing synthetic antioxidants and additives, which are being restricted due to their passivity. In addition, from a legislative point of view, it would be economically more feasible to use a whole spice or herb or a whole essential oil as an ingredient than to use essential oil component solely (Smid and Gorris, 1999). The exploitation of essential oils is expected to increase in the future because of the rise of 'green consumerism' motivating the use and development of products derived from plants for reliable applications.

Microbial contamination and food deterioration by microorganisms are an unresolved problem. It is represented, especially by Gram-negative bacteria. For instance, a large outbreak of diarrhea in Germany was caused by the most virulent strain of new *Escherichia coli* known to date and presented in steak. More broadly, these discoveries highlight the way in which the plasticity of bacterial genomes facilitates the emergence of new pathogens and therefore requires incessant monitoring (Rasko *et al*., 2011).

In Algeria, health problems are very challenging and still pose a crossroads in the scientific research field. The main objective is to discover an alternative source of safe, effective and acceptable natural preservatives. Actually, some Algerians commonly suffer from gastrointestinal disorders, such as ulcers, gastritis and infantile diarrhea. These crises are mostly caused by alimentary products that are frequently consumed in

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Algeria, particularly milk, dairy products, cheese, ice cream and eggs. The water used to wash vegetables may also contain pathogens that lead to the same problems.

Myrtle has played a key role as among the herbs used in alternative medicine. *Myrtus communis* is an evergreen, perennial and typical shrub belonging to the Myrtaceae family and widely spread in several regions globally, such as the Mediterranean ecosystem, the Middle East, North America and Australia (O¨zek *et al*., 2000). In addition, this specie is a very aromatic plant that has been used traditionally because of the high essential oil content in its leaves, flowers, and fruits (De Laurentis *et al*., 2005). Many essential oils and extracts from various plants have been investigated for their antimicrobial properties against a series of bacteria and yeasts (Cox *et al*., 2001).

The quantitative composition and the relative proportions of the myrtle oil components are widely influenced by the genotype, the ontogenic development and the environmental and growing conditions. Many phytochemical studies have simultaneously investigated the essential oil composition of leaves and fruits as well as the other parts of *M.communis* (Jerkovic *et al*., 2002; Tuberoso *et al*., 2006). Until now, many studies on myrtle has widely focused on the composition of the volatile compounds in leaves belonging to different regions and harvested in different periods (O¨zek *et al*., 2000; Asllani, 2000; Tuberoso *et al*., 2006). Recent studies were encouraged by the lack of reliable data on the antibacterial activity of essential oils of myrtle collected from the mountainous regions in northeastern Algeria.

The aim of this work is to characterize myrtle leaves collected from northeastern Algeria through its essential oil composition to determine its antibacterial effects and to try to valorize this myrtle part as a source of bioactive compounds. This may lead to the discovery of an analogous myrtle essential oil with a similar composition or activity. In light of the facts mentioned above, it appears necessary to evaluate the correlation between the chemical composition and the antibacterial activities of the Algerian essential oil of *M.communis* collected from Annaba region.

Materials and Methods

Biochemical Section

Plant Material

Fresh leaves of *M.communis* were collected from remote areas in the suburbs of the Annaba region (northeastern Algeria) during the vegetative, flowering and fruiting seasons (2012/2013). The taxonomic identification was performed in the Biology Department, Badji Mokhtar University, Annaba, Algeria. The leaves were then isolated from the other specimens and were dried in the shade for a week at room temperature. The dried leaves were conserved for the extraction process.

Essential Oil Extraction

The *M. communis* essential oil was extracted from 50 g of dried leaves after submission for 2 h to hydrodistillation using a Clevenger-type apparatus. This process was adopted in this study to extract the essential oil according to the method recommended in the European Pharmacopeia (2002). The essential oil was dried over anhydrous sodium sulfate. It was filtered and stored in the refrigerator (4°C) until antibacterial tests against clinical pathogens were performed.

Chemicals, Reagents and Solvents

All culture media, standard antibiotic discs and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. The water used was purified and distilled. All other chemicals were of analytical grade.

Chemical Analysis GC/MS

The samples of myrtle essential oil extracted from the leaves were analyzed by GC/MS using an HP 5890 series II gas chromatograph equipped with a flame-ionization detector and coupled to an HP 5972 mass spectrometer (Agilent Technologies) with electron-impact ionization (70 eV) and an HP-5MS capillary column (30×0.25 mm coated with 5% phenyl methyl silicone, 95% dimethylpolysiloxane; a 0.25-µm film thickness were used). The operating conditions were as follows: The column temperature was programmed to rise from 50 to 240°C at a rate of 5°C/min, and the transfer-line temperature was 250°C. The carrier gas was helium with a flow rate of 1.2 mL min^{-1} and a split ratio of 60: 1. The scan time and mass range were 1 s and 40-300 m/z, respectively. The mass-spectrometer conditions were the following: Injection of 2 µL aliquot of the sample and an HP-5MS capillary column (30×0.25 mm; coating thickness, 0.25 µm).

Compounds Identification

The components of the oil were identified by a comparison of the fragmentation patterns in the mass spectra with those stored in the GC/MS databases and other published mass spectra in relation to the retention time of a homologous series of alkanes (C7-C20/C30) (Adams, 2007). In addition, the percentages of the compounds were determined from their peak areas.

Ecological Section

Study Area and Raw Material

The area is located in the state of Annaba, a town situated in the northeastern of Algeria (latitude 36° 54' 15"N, longitude 7° 45′ 07″E) belonging to the

Mediterranean area. Single-season samplings were undertaken in December, 2013 (autumn season). The sampling strategy was a random zigzagged manner in which samples of the surface soil near the trees (0-20 cm depth) was collected manually from a single site at the same station using suitable, uncontaminated equipment, and then the samples were stored. The four samples were dried, ground and then homogenized. From this environment, four samples of water were chosen for measurement of the limnological variables. An aliquot of each sample was set aside for subsequent chemical and granulometric analysis.

Chemicals Parameters Measurements and Granulometric Analysis

For soil sampling, a depth of soil sample of approximately 0-20 cm was used for the identification and measurement of the pedogenetic horizons, aimed at obtaining a morphological description. The criterion adopted in the granulometric analysis followed the universal method of the Robinson pipette, which was proposed by the Association Française de l'Etude des Sols (AFES, 1995). In all collection samples, the pH, electrical conductivity, percentage of moisture, minerals and organic matter of the water and soil were measured. Principal component analysis was performed to identify which variables best explained the variability of the soil-analysis results, and a cluster analysis of the mean values of the chemical (soil and water) variables was also performed, calculating the average of the four samples. The presence of carbonates in the soil materials involved the reaction of HCl with soil carbonates and visual observation of the gaseous loss of CO_2 from the sample, as described by the U.S. Salinity Laboratory Staff. This method is not quantitative. Soils may be categorized as slightly, moderately or highly calcareous in accordance to the degree of effervescence.

Microbiological Section

Bacterial Strains and Culture Conditions

For the initial screening, eighteen pathogens and two reference strains were used to evaluate the myrtle essential oil activity *in vitro*. The clinical bacterial strains tested were obtained from the urine samples of patients in Annaba, Algeria. After culture enrichment, the bacterial samples were identified by standard biochemical parameters and morphological studies. Twenty Gram-negative bacteria were grown in aerobic or anaerobic cultures: *Escherichia coli* (EC), *Klebsiella pneumoniae* (KP), *Klebsiella oxytoca* (KO), *Shigella sonnei* (SS), *Serratia marcescens* (SM), *Serratia fonticola* (SF), *Acinetobacter baumannii* (AB), *Citrobacter koseri* (CK), *Citrobacter freundii* (CF), *Enterobacter aerogenes* (EA), *Enterobacter cloacae* (EL), *Enterobacter intermedius* (EI), *Enterobacter sakazakii* (ES), *Proteus mirabilis* (PM), *Proteus vulgaris* (PV), *Morganella morganii* (MM), *Salmonella typhimurium* (ST) and *Salmonella sp.* (S). In addition, two reference strains from the American-Type Culture Collection (ATCC), *Escherichia coli* EC® (ATCC 25922) and *Klebsiella pneumonaie* KP® (ATCC 700603), were also tested. All of the strains were cultured after identification in nutrient agar, and they were stored frozen at 4°C until the antibacterial tests were performed.

Preparation of Antibacterial Agents

Suspensions of the antibacterial agents to be tested were prepared immediately prior to use by the pure and the dilute essential oils of the leaves. The use of emulsifiers, such as ethanol or tween, was avoided because these agents may reduce the antibacterial effect of plant-oil components and possess membrane-disrupting activity, and especially to the crystallization of our essential oil in contact with these solvents. For this reason, the essential oil was dissolved in dimethylsulfoxide (DMSO) in equivalent concentrations (essential oil/DMSO: 50/50 v/v) and sterilized by filtration through a 0.45-µm membrane filter. The concentrations required for the experiments were prepared from this stock solution.

Screening of Antibacterial Activity

Solid diffusion tests: The susceptibility of the bacteria to the essential oil was determined by an agar diffusion disc method (Prabuseenivasan *et al*., 2006). After the Muller Hinton Agar (MHA) had solidified, the inoculums (DO≈0.1/λ = 625 nm) were streaked into agar plates using a sterile swab and were then dried at 37°C for 15 min. A sterile filter disc with a 6-mm diameter (Whatman paper N°3) was placed on the surface of the MHA. Then, 10 µL of the essential oil was dropped onto each disc and left for 30 min at room temperature for antibacterial agent diffusion. The Petri dishes were incubated at 37°C for 18 to 24 h. For quality control and comparative analysis, DMSO was used as a negative control. The effectiveness of the essential oils was calculated by measuring the diameter of the zone of bacterial-growth inhibition above the disc and the diameter was recorded in mm. The inhibition zones produced from the essential oils were compared with the inhibition zones produced by commercial standard antibiotics. An essential oil-inducing inhibition zone ≥ 3 mm around the disc was considered as antibacterial. All tests were performed in triplicate.

Determination of the Minimum Concentrations MIC and MBC

The minimum Inhibitory Concentration (MIC) was defined as the lowest concentration of the total essential oil at which the microorganism does not demonstrate visible growth (CLSI, 2006). Referring to the results of

the MIC assay, the Minimum Bactericidal Concentration (MBC) was determined. Serial dilutions of myrtle essential oil (10, 5, 2.5, 1.25 and 0.625 mg mL^{-1}) were made with dimethylsulfoxide (DMSO). Immediately, 10uL of each dilution of the essential oil were added to 9 mL of the MH-broth tubes, which were then incubated at 37°C for 24 h in stove. After incubation, from tubes without microbial growth, 0.1 mL was spread onto the nutrient agar plates. The minimum bactericidal concentrations were determined as the highest dilution at which no growth occurred definitely on the plates.

Susceptibility of Antibiotics

The twenty-one antibiotics that were used for comparison of their antibacterial effects with those of myrtle essential oil were amoxycillin (AMX, 25 µg), ampicillin (AMP, 25 µg), amikacin (AK, 30 µg), tetracyclin (TE, 30 µg), doxycyclin (DO, 30 µg), ticarcillin (TI, 75 µg), gentamycin (GEN, 10 µg), erythromycin (E, 15 µg), chloramphenicol (C, 30 µg), cefixim (CFM, 5 µg), ceftazidim (CAZ, 30 µg), cefalexin (CN, 30 µg), cefotaxim (CTX, 30 µg), ciprofloxacin (CIP, 25 µg), cotrimoxazol (COT, 25 µg), colistin (CL, 25 µg), nalidixic acid (NA, 30 µg), pipemidic acid (PA, 20 µg), nitroxolin (NO, 30 µg), ofloxacin (OFX, 10 µg) and imipenem (IMP, 10 µg). The antibiotics selected for the study to elucidate their mechanism of action by the direct-contact method are listed in the results with the diameters recorded in mm. The susceptibility of each of the twenty-one standard and specific antibiotics to twenty Gram-negative bacteria was assessed by the diffusion-agar method. The level of the resistance (R %) was calculated according to the critical diameter of inhibition for each antibiotic where each strain was represented as sensible or resistant (Cavallo, 2007).

Statistical Analysis

The data obtained from the antibacterial assays were expressed as the mean values. Statistical analysis was performed by one way Analysis of Variance (ANOVA) followed by Dunnett's or Tukey's multiple-comparisons tests using the MINITAB 16 package. The level of significance was significant when *$P \leq 0.05$; highly significant when **$P \leq 0.01$ and very highly significant when ***$P \leq 0.001$. All data were reported at least in triplicate for the various assays.

Results and Discussion

Essential Oil Yields

Essential oil obtained by hydrodistillation of leaves of *Myrtus communis* growing wild in Algeria had a pungent odor. The maximum, intermediate and minimum yields recorded were obtained in the flowering (1.14%), vegetative (0.62%) and fruiting (0.6%) stages, respectively (Fig. 1). According to Jerkovic *et al*. (2002) and Jamoussi *et al.* (2005), their results showed a strong correlation between the extraction yield and the vegetative cycle of the plant, with a maximum biosynthesis of essential oils near the blossom stage. In contrast, some authors reported that the leaf essential oils yield of myrtle did not vary significantly with the seasons (Zrira *et al*., 2003; Gardeli *et al*., 2008).

Chemical Composition of the Essential Oil

Analysis of the myrtle essential oil by GC/MS showed 49 identified compounds, presenting high fluctuations in its chemical profile (Table 1). The major components of this essential oil were α-pinene (55%), 1,8-cineole (33.4%), limonene (33.4%), geranyl acetate (2.04%) and linalool (1.43%). Bouzabata *et al*. (2010) were analyzed 27 samples of essential oils isolated from myrtle leaves of three localities of northeastern Algeria. The chemical composition of these oils was largely dominated by monoterpene hydrocarbons, with α-pinene (40-64%), 1,8-cineole (10,9-29,1%) and limonene (6,7-8,2%) being the major compounds, however with little amounts of limonene smaller than 1,8-cineole concentration.

Therefore, the differences in two main components (α-pinene, 1,8-cineole) of our sample and the myrtle essential oils from various origins are summarized in Fig. 2. Our results are in agreement with the findings of Jamoussi *et al*. (2005) who analyzed the Tunisian myrtle essential oils under GC/MS conditions and reported that the major volatile components were α-pinene, 1,8-cineole and limonene, among 39 identified compounds. However, it can be observed that Italian myrtle oil indicated the same main content (Cannas *et al*., 2013) in according with French myrtle oil which contained 14 compounds and showed also α-pinene and 1,8-cineole together representing around 86% (Curini *et al*., 2003).

In contrast, myrtenyl acetate was also the specific chemotype of the Croatian (Jerkovic *et al*., 2002), Greek (Gardeli *et al*., 2008), Spanish (Boelens and Jimenez, 1992) and Moroccan (Chalchat *et al*., 1998) myrtle essential oils, whereas in the present study, this compound was detected only in a low percentage in the leaf essential oil and did not exceed 0.03% in this variety. In various previous studies, Mahboubi's group studied the essential oils of Turkish myrtle, which presented a higher fraction of linalool and linalyl acetate, in disagreement with the compositions of our samples, and the Turkish findings illustrated a different chemical profile of myrtle growing in the Mediterranean area (Mahboubi and Ghazian Bidgoli, 2010). Otherwise, the essential oil of *M. communis* was established in Italian report to consist approximately of monoterpene hydrocarbons (58.3%), mainly a α-terpinene (51.8%); oxygenated monoterpenes (38.3%) represented primarily

by 1,8-cineole (35.6%); and small quantities of aldehydes (1.5%) and sesquiterpene hydrocarbons (1.2%) while α-pinene was not found in its chemical profile (Deriu *et al*., 2007).

Our results are influenced by several variables, such as the difference in myrtle essential oils yield or composition, which is due to the intervention of many important factors. Scora (1973) reported that environmental factors, such as the geographical location, temperature, day length and nutrients, were considered to play a key role in the chemical composition of myrtle oil. This leads to the existence of different chemotypes that differentiate the myrtle oil of different origins (Chalchat *et al*., 1998; Flamini *et al*., 2004) in according with the suggestion of Shu and Lawrence concerning the dependence of oil composition on the plant species, the chemotypes and the climatic conditions (Shu and Lawrence, 1997).

Several authors have even suggested that certain compounds, such as α-pinene, camphene, 1,8-cineole and geraniol, can be regarded as regional markers able to facilitate species-improvement research by studies on isoenzymatic polymorphism because there was a positive correlation between the variation of these compounds and the distribution of particular alleles due to the various enzymatic and chemical parameters, making it possible to select genotypes and enact elaborate conservation strategies (Messaoud *et al*., 2005). It can be deducted that the strong chemical variability in myrtle oils can be ascribed not only to the geographical origin of the sample and its environmental conditions but also to the variety type and genetic factors (Flamini *et al*., 2004).

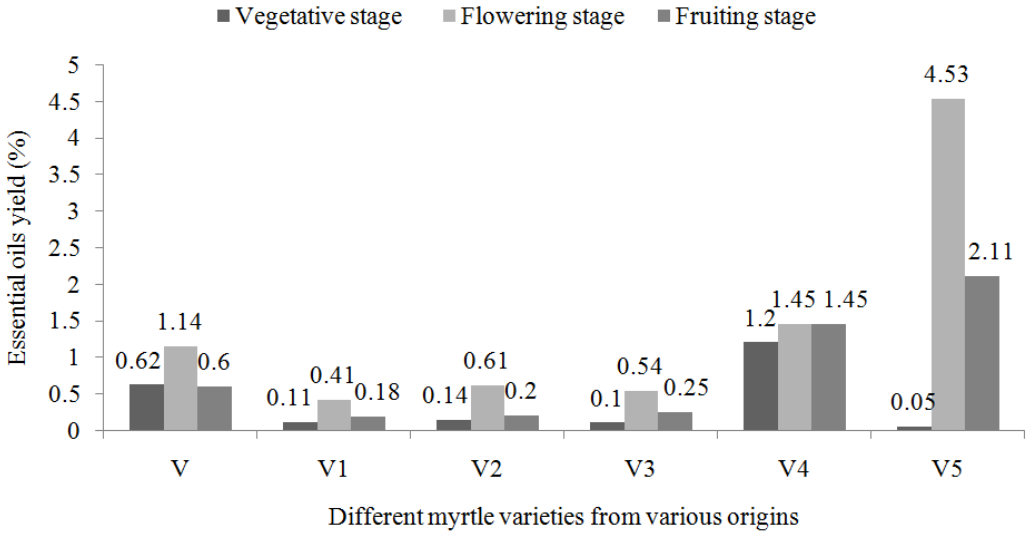

Fig. 1. Investigation of different myrtle leaves essential oils yields during successive stages of development (vegetative, flowering and fruiting periods). V: Sample studied (Algerian myrtle essential oil from leaves. V1, V2 and V3 (3 varieties of Tunisian myrtle leaves essential oils). V4 (Greek myrtle leaf essential oil). V5: Italian myrtle leaf essential oil. (Jamoussi *et al*., 2005; Aidi Wannes *et al*., 2008; Gardeli *et al*., 2008; Dell'Agli *et al*., 2012)

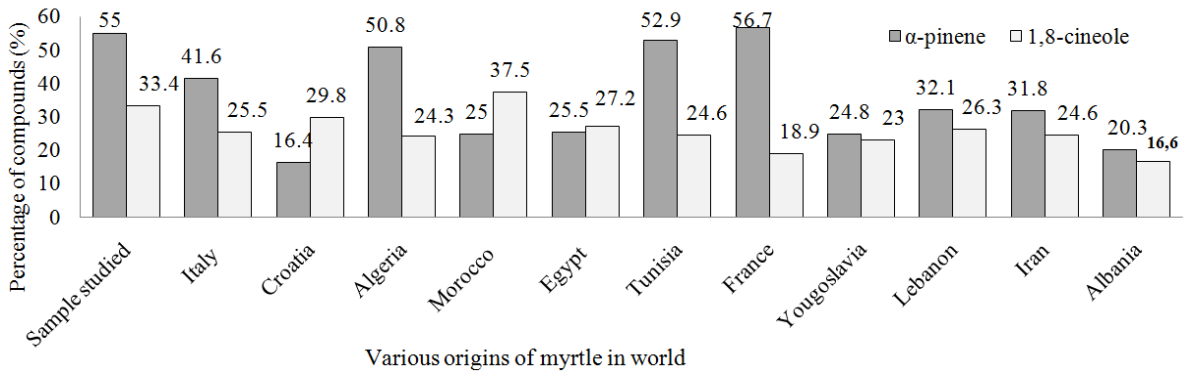

Fig. 2. Recaputilative of percentage composition of two main compounds in myrtle leaves oils from various origins, (Chalchat *et al*., 1998; Asllani., 2000; Jerkovic *et al*., 2002; Flamini *et al*., 2004; Ghasemi *et al*., 2010; Berka-Zougali *et al*., 2010; Nassar *et al*., 2010)

Table 1. Chemical composition of leaves essential oil of myrtle

Peak	Compound	RT (min)	Area (%)	RI	MM	MF
1	Isobutyl isobutyrate	7.963	0.19	807	144.21	$C_8H_{16}O_2$
2	Alpha-pinene	8.676	55.00	925	136.23	$C_{10}H_{16}$
3	Camphene	8.935	0.76	968	136.23	$C_{10}H_{16}$
4	1-(Methylencyclopropyl)-1-cyclopentene	9.099	tr	995	120.19	C_9H_{12}
5	Beta-pinene	9.751	0.38	1103	136.23	$C_{10}H_{16}$
6	Ethylidene cyclopropane	10.265	tr	1188	68.11	C_5H_8
7	Isobutyl-2-methylbutyrate	10.597	0.22	1243	158.24	$C_9H_{18}O_2$
8	Delta-3-carene	10.784	0.25	1274	136.23	$C_{10}H_{16}$
9	Isoamyl isobutyrate	11.044	0.10	1317	158.24	$C_9H_{18}O_2$
10	Limonene	11.54	33.42	1399	136.23	$C_{10}H_{16}$
11	1,8-cineole	11.54	33.42	1399	154.24	$C_{10}H_{18}O$
12	Gamma-terpinene	12.301	0.16	1525	136.23	$C_{10}H_{16}$
13	Alpha-terpinolene	13.177	0.12	1670	136.23	$C_{10}H_{16}$
14	Linalool	13.648	1.43	1748	154.24	$C_{10}H_{18}O$
15	Ethylbutyl acetylene	14.041	tr	1813	110.19	C_8H_{14}
16	α-Campholenal	14.325	tr	1860	152.24	$C_{10}H_{16}O$
17	Pinocarveol	14.754	0.24	1931	152.24	$C_{10}H_{16}O$
18	Cis-verbenol	14.947	tr	1963	152.24	$C_{10}H_{16}O$
19	Cycloocta-1,4-dien-3-one	15.376	tr	2034	108.18	C_8H_{12}
20	4-Terpineol	15.908	0.15	2122	154.24	$C_{10}H_{18}O$
21	Linalyl propionate	16.367	1.20	2198	210.31	$C_{13}H_{22}O_2$
22	Linalyl acetate	18.017	0.44	2471	196.29	$C_{12}H_{20}O_2$
23	Geraniol	18.137	0.25	2491	154.24	$C_{10}H_{18}O$
24	Myrtenyl acetate	19.249	tr	2675	194.27	$C_{12}H_{18}O_2$
25	2,6-Dimethylene-7-octen-3-one	19.932	tr	2788	150.21	$C_{10}H_{14}O$
26	Exo-2-hydroxycineole acetate	20.367	tr	2860	212.28	$C_{12}H_{20}O_3$
27	Isohexane	20.596	0.64	2898	86.17	C_6H_{14}
28	Neryl acetate	20.965	0.16	2959	196.29	$C_{12}H_{20}O_2$
29	Geranyl acetate	21.491	2.04	3046	196.29	$C_{12}H_{20}O_2$
30	Methyleugenol	22.137	0.64	3153	178.22	$C_{11}H_{14}O_2$
31	Beta-caryophyllene	22.445	0.29	3204	204.35	$C_{15}H_{24}$
32	Alpha-humulene	23.315	tr	3348	204.35	$C_{15}H_{24}$
33	1-(2,6,6-Trimethyl-1-cyclohexen-1-yl)ethanol	23.424	tr	3366	84.14	C_4H_4S
34	Germacrene-D	24.004	0.11	3462	204.35	$C_{15}H_{24}$
35	3-Ethyl-3-methyl-2-pentanol	24.379	tr	3524	130.22	$C_8H_{18}O$
36	Cinerolone	24.910	0.13	3612	166.22	$C_{10}H_{14}O_2$
37	Artemisia acetate	25.472	0.12	3705	196.28	$C_{12}H_{20}O_2$
38	Germacrene B	25.841	0.10	3766	204.35	$C_{15}H_{24}$
39	Caryophyllene oxide	26.481	0.55	3872	220.35	$C_{15}H_{24}O$
40	2-Methyl-2-cyclopentenone	27.098	0.13	3974	96.12	C_6H_8O
41	P-Pentyloxynitrobenzene	27.224	tr	3995	209.24	$C_{11}H_{15}NO_3$
42	Cubebene	27.406	tr	4025	204.35	$C_{15}H_{24}$
43	Patchulane	27.690	tr	4072	206.36	$C_{15}H_{26}$
44	Adamantane	27.768	0.16	4085	136.23	$C_{10}H_{16}$
45	4,5-Diethyloctane	28.227	0.14	4161	170.33	$C_{12}H_{26}$
46	Cis-8-methylenebicyclo(5.1.0)octan	28.590	tr	4221	122.20	C_9H_{14}
47	(-)-Pinane-3-carboxylic acid	28.663	tr	4233	182.25	$C_{11}H_{18}O_2$
48	(N-propyl)-1,2,4-triazole	28.983	tr	4286	243.96	$C_6H_{12}Br_2$
49	2-Butanoylthiazole	29.490	tr	4370	155.21	C_7H_9NOS
	Identified compounds (%)		99.52			

RT (min): Retention time in minute, Area (%): Percentage of each compound, RI: Retention indices. MM: Molecular Mass (g/mol). MF: Molecular formula. tr: Traces <0,1%

In the end of this comparison between the chemical composition of our leaves essential oil and the various oils previously studied, it can be concluded that the most analogous oils are the Tunisian oils obtained from the leaves of two *M.communis* varieties (*baetica* and *italica*), which contained 49 compounds, with α-pinene as the predominant component (Aidi wannes *et al*., 2008). Subsequently, the Italian oil also has a chemical profile that is closely analogous to that of our oil,

combining the same major components (Zanetti *et al*., 2010). In a practical sense, this homology was related with the content (major and negligible minor components of all compared essential oils extracted from the myrtle leaves) and is most likely due to the close connection between the soil types, edaphic factors and climate effects, and it may also be connected to the infrastructural area of the Algeria, Tunisia and Italy triangle before the continental division of Africa and Europe.

Ecological Study

Many authors such as Flamini *et al*. (2004) reported that the type of soil played an important role in the essential oils composition variation. These facts encouraged our research to analyze the soil and surface water of one station where the myrtle leaves were sampled manually during a single season (autumn season). After designing and performing experiments on the soil and water from our sampling location, a description of the chemical parameters and the granulometric analysis was presented in Table 2. However, the results showed data obtained from the granulometric analysis of the sediment when the site had diversity in soil composition, with 57.35% sand and 42.64% mud, which was divided into silt and clay. The results showed that the water is most fresh when the environment was rich neither in minerals nor in organic matter. In addition, the soil is acidic with a high proportion of sand (57, 35%) indicating siliceous soil type.

In disagreement with our results, the Italian research detected linalool, linalyl acetate and trans-myrtanol acetate in greater amounts in leaves essential oil of myrtle growing in siliceous soils, which demonstrated an important variation in the chemical profile of the myrtle essential oils of the two localities in comparison with myrtle growing in calcareous soil (Flamini *et al*., 2004). In our case, α-pinene, 1,8 cineole and limonene (Table1) were detected in high levels in the siliceous soil. It would be interesting to follow a variety of myrtle grown on a particular soil with goal of producing the essential oils with a constant composition and, consequently, having a specific biologic activity. It is a long and difficult work that our laboratory hopes to achieve because we observed that the essential oils treated by the literature had very different compositions.

Antibacterial Activity Study

Extracts and essential oils of myrtle have been extensively tested against a broad spectrum of bacteria, fungi, yeasts, insects and parasites. They are mixtures of various lipophilic and volatile substances, such as monoterpenes, sesquiterpenes and/or phenylpropanoids. Therefore, one of the most striking features of this disparity of the myrtle essential oils effectiveness is its relationship with the active ingredients, including α-pinene and 1,8-cineole, which were reported as a major contributor to imparting a antimicrobial effect on *E. coli, S. aureus* and *C. albicans* (Cox *et al*., 2001). Several works claim that oxygenated terpenes, such as 1,8-cineole, linalool, and *α*-terpineol, exhibit powerful antibacterial activity (Randrianarivelo *et al*., 2009). However, limonene was also active against Gram positive and Gram negative bacteria (Pepeljnjak *et al*., 2005). These characteristics make it a perfect model of study which was carried to determine the antibacterial activity of Algerian myrtle essential oil extracted from leaves against clinical strains displaying primary resistance to some antibiotics. In Algeria, the majority of human infections are particularly provoked by the consumption of contaminated food, especially by *Salmonella sp.* and *Escherichia coli*, which represent the most food-borne Gram-negative bacteria that are frequently distributed in nature as well as in a large number of Algerian aliments.

The results for the antibiotics tested are summarized in (Table 3), which showed strong activity (40-mm zone) and weak inhibition (6-8-mm zone). Ampicillin and third-generation ceftazidim did not produce good zones for inhibition with the Gram-negative bacteria, which revealed the highest level of resistance at 90% (Fig. 3). As results, the antibacterial bioassays of the essential oil are summarized in (Table 4), where it is obvious that the myrtle essential oil of leaves showed moderate to strong antibacterial activity and the diameters of the inhibition zones ranged from 08-35 mm. According to the estimated diameters, the highest activity was observed against *E. coli* with inhibition zone (35 mm) recorded for the fresh essential oil isolated from the leaves. The MIC values ranged from 0.6 to 2.5 mg mL^{-1}, but the MBC tests indicated the entire bacteriostatic effect of our essential oils, which is demonstrated by the remarkable growth that occurred on the plates.

The inhibitory effect of different concentrations of oil on microbial growth varied with bacteria (Kivanc and Akgul, 1986). All the assayed essential oils significantly inhibited the growth of at least some of the bacterial strains tested. With comparison to both of diluted and undiluted oils; it is evident from the results that the inhibitory effect of diluted oil was moderately weak on all strains.

Based on these finding, the results summarized in the (Table 4) agreed with previous studies where myrtle essential oils demonstrated approximately weak inhibitory effects against *Proteus vulgaris*. It is also attributable to validate the slight activity *in vitro* of both the Turkish and the Italian oils towards the same strain while there was no activity of the Egyptian myrtle essential oil detected against *Proteus vulgaris* (Aboutabl *et al*., 2013; Senatore *et al*., 2013).

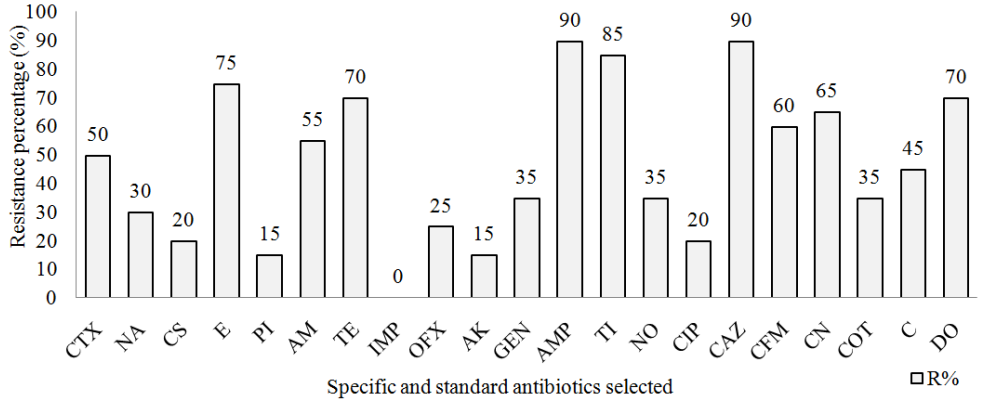

Fig. 3. Resistance percentage of selected antibiotics against Gram negative bacteria, AMX (Amoxycillin), AMP (Ampicillin), AK (Amikacin), TE (Tetracyclin), DO (Doxycyclin), TI (Ticarcillin), GEN (Gentamycin), E (Erythromycin), C (Chloramphenicol), CFM (Cefixim), CAZ (Ceftazidim), CN (Cefalexin), CTX (Cefotaxim), CIP (Ciprofloxacin), COT (Cotrimoxazol), CL (Colistin), NA (Nalidixic acid), PA (Pipemidic acid), NO (Nitroxolin), OFX (Ofloxacin), IMP (Imipenem)

Table 2. Chemical parameters measurements and granulometric analysis (water and soil of one sampling location)

	Parameters	**Values**
Water	Humidity (%)	99.91
	Organic matter (%)	0.015
	Minerals (%)	0.068
	pH	7
	Conductivity (µs)	476
Soil	Humidity (%)	2.8
	Organic matter (%)	7.8
	Minerals (%)	89.6
	pH	6.65
	Conductivity (µs)	103
	Clay (%)	38.38
	Silt (%)	4.26
	Sand (%)	57.35
	DSS: Depth of soil sample (cm)	[0-20]
	Soil + HCl 3N	No effervescence

Table 3. Selected antibiotics susceptibility to twenty Gram negative bacteria (Diameter of inhibition zones in mm)

	CTX	NA	CS	E	PI	AM	TE	IMP	OFX	AK	GEN	AMP	TI	NO	CIP	CAZ	CFM	CN	COT	C	DO
EC®	29	31	18	18	29	16	20	33	30	30	30	5	25	30	20	10	19	19	31	34	26
KP®	20	21	20	8	19	5	15	33	18	28	19	5	5	15	21	5	8	5	20	13	14
AB	5	5	15	17	5	5	8	33	5	23	11	5	15	8	5	5	5	5	5	14	13
CK	30	29	16	13	30	25	6	38	16	24	20	20	9	30	40	14	26	16	5	28	7
CF	36	31	6	13	27	6	7	32	30	25	22	6	30	25	35	16	34	20	30	27	6
EC	27	24	16	11	23	20	5	27	27	25	21	7	7	29	30	6	26	6	32	30	6
EA	5	5	17	5	5	16	5	30	8	23	21	6	7	28	36	15	27	10	24	25	17
EL	6	8	16	18	20	6	13	33	24	27	8	6	6	6	44	6	6	6	6	8	13
EI	25	6	17	6	5	20	5	30	8	30	19	14	19	26	40	6	6	6	6	11	22
ES	30	28	17	20	30	6	25	47	33	24	22	6	6	6	30	14	21	6	6	10	18
KP	28	25	20	5	30	24	5	34	32	26	16	6	6	30	34	6	6	6	34	32	10
KO	30	5	20	10	20	30	21	25	30	28	21	7	6	30	30	12	25	6	30	30	8
MM	14	5	20	10	10	6	40	40	36	6	6	7	6	25	27	6	6	24	35	28	8
PM	36	30	5	7	30	30	5	32	30	24	17	6	6	24	38	19	32	20	34	27	8
PV	20	23	25	25	28	6	8	32	34	7	7	6	6	6	40	10	20	6	6	13	8
S	7	7	20	7	5	10	5	32	11	23	15	6	6	26	16	6	6	6	15	27	9
ST	5	36	16	16	30	5	25	30	35	25	13	5	5	7	37	5	5	5	5	12	20
SS	33	30	20	5	28	8	30	32	35	22	20	7	7	27	30	15	23	6	16	16	20
SM	30	25	6	15	30	20	6	35	30	27	26	6	27	29	40	19	25	16	33	30	19
SF	14	23	8	13	30	6	8	30	32	7	7	6	6	6	40	10	19	6	6	10	6

Bacterial strains: *Escherichia coli* (EC), *Klebsiella pneumonia* (KP), *Klebsiella oxytoca* (KO), *Shigella sonnei* (SS), *Serratia marcescens* (SM), *Serratia fonticola* (SF), *Acinetobacter baumannii* (AB), *Citrobacter koseri* (CK), *Citrobacter freundii* (CF), *Enterobacter aerogenes* (EA), *Enterobacter cloacae* (EL), *Enterobacter intermedius* (EI), *Enterobacter sakazakii* (ES), *Proteus mirabilis* (PM), *Proteus vulgaris* (PV), *Morganella morganii* (MM), *Salmonella typhimurium* (ST) and *Salmonella sp.* (S). *Escherichia coli* (EC®), *Klebsiella pneumonaie* (KP®).
Antibiotics: AMX (Amoxycillin), AMP (Ampicillin), AK (Amikacin), TE (Tetracyclin), DO (Doxycyclin), TI (Ticarcillin), GEN (Gentamycin), E (Erythromycin), C (Chloramphenicol), CFM (Cefixim), CAZ (Ceftazidim), CN (Cefalexin), CTX (Cefotaxim), CIP (Ciprofloxacin), COT (Cotrimoxazol), CL (Colistin), NA (Nalidixic acid), PA (Pipemidic acid), NO (Nitroxolin), OFX (Ofloxacin), IMP (Imipenem).

Table 4. Antibacterial activity of myrtle essential oil (leaves) against different strains bacteria (mm) with MIC results (mg/ml)

Strain	Symbol	Undiluted EO	Dilute EO (**)	MIC (mg/ml) (***)
Escherichia coli®	EC®	25	09	2.50
Klebsiella pneumonia®	KP®	09	07	2.50
Acinetobacter baumannii	AB	09	05	1.25
Citrobacter freundii	CF	09	05	2.50
Citrobacter koseri	CK	08	05	2.50
Escherichia coli	EC	35	10	1.25
Enterobacter aerogenes	EA	10	10	0.60
Enterobacter cloacae	EL	10	07	1.25
Enterobacter intermedius	EI	10	08	1.25
Enterobacter sakazakii	ES	10	07	1.25
Klebsiella pneumonaie	KP	09	05	1.25
Klebsiella oxytoca	KO	10	05	2.50
Morganella morganii	MM	08	05	1.25
Proteus mirabilis	PM	09	05	2.50
Proteus vulgaris	PV	11	07	0.60
Salmonella sp.	S	08	05	1.25
Salmonella typhimurium	ST	10	09	1.25
Shigella sonnei	SS	10	09	0.60
Serratia marcescens	SM	09	05	1.25
Serratia fonticola	SF	09	09	1.25
Negative control	DMSO	-	-	-

(05): No zone inhibition, (EO): Essential Oil, (MIC): Minimal Inhibition Concentration, ®: Reference strain, (DMSO): Dimethylsulfoxide, (**): $p \leq 0.01$, (***): $p \leq 0.001$

However, the results clearly indicated that the *Salmonella* sp. susceptibility to myrtle oils appears in an inhibition zone lower than those found in literature; this in agreement with previous reports by the several workers in North Cyprus (Akin *et al*., 2010). This ubiquitous environmental bacterium presented a slight activity that was achieved by Italian and Turkish myrtle essential oils (Senatore *et al*., 2013). In contrast, this Gram-negative bacterium was the most sensitive one to both the oils of Algerian and Tunisian populations, with high vulnerability to *M.communis* oil attack under static circumstances (Ben Ghnaya *et al*., 2013).

Many studies have proved the synergistic effect of essential oils or their fractions from different plants with synthetic drugs as antifungal and antibacterial agents particularly eucalyptol, geraniol were established to interact synergistically with norfloxacin against *B. subtilis, B. cereus, S. aureus,* and *E. coli* (Rosato *et al*., 2007). Interestingly, synergistic effects of the combinations of 1,8-cineole and aromadendrene against Methicillin-Resistant *Staphylococcus Aureus* (MRSA) and Vancomycin-Resistant Enterococci (VRE) and *Enterococcus faecalis* by using checkerboard and time-kill assays respectively were reported (Mulyaningsih *et al*., 2010). Other combinations including a monoterpene hydrocarbon (α-pinene) with limonene or linalool also showed additive and synergistic effects (Tserennadmid *et al*., 2011). In addition, *In vitro* synergistic efficacy of combination of amphotericin B with *Myrtus communis* essential oil against clinical isolates of *Candida albicans* was also performed to explore the opportunity of emerging a more powerful combination therapy (Mahboubi and Ghazian Bidgoli, 2010). The interaction between essential oils and microbes which ultimately induces the antimicrobial activity is not well understood. Hitherto, different target sites and mode of action are discussed. Based on these results, it was assumed that the essential oils may have antimicrobial activity by influencing bacterial and fungal targets involved in cytoplasmatic and cell wall metabolism. It is stated by several researchers that especially monoterpenes will increase cytoplasmic membrane fluidity and permeability, disturb the order of membrane embedded proteins, inhibit cell respiration, and alter ion transport processes (Reichling *et al*., 2009).

Besides, most of the studies on the incidence of a synergistic interaction between the essential oil and their phenolic constituents have concentrated on their effects on cell walls and cell membranes that are known to provoke damage to each structure, which disintegrates the external membrane of Gram-negative bacteria. They represented also by releasing lipopolysaccharides, increasing the permeability of the cytoplasmic membrane to ATP, causing leakage of cellular materials, and ultimately the bacteria death but also by influencing the membrane functions such as electron transport, enzyme activity or nutrient uptake (Amensour *et al*., 2010). Further studies concerning the antibacterial activity and the mode of action of myrtle essential oils are in progress.

Conclusions and Perspectives

The correlation between the chemical composition of the myrtle essential oil and its potent antibacterial

activity could be useful in the search for novel active compounds, and these findings may partially validate the alternative use of this plant, but further *in vivo* and clinical studies are required to justify the rationale of its traditional exploitation scientifically. Currently, the rapid growth of *Myrtus communis* makes it a promising candidate as a valuable natural resource for the commercial production of drugs, and its essential oil can also be play a key role in the preparation of specific microbiologic culture media for scientific research fields or for conservation procedures. Additionally, the production of essential oils with a constant chemical composition would be interesting.

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Author's Contributions

Barhouchi Badra: Conceived of the research, designed the study, drafted and revised the paper.

Aouadi Saoudi: Involved in study design, sample preparation, manuscript writing and scientific discussion.

Abdi Akila: Involved in antibacterial study and performed in the laboratory work.

Ethics

This article is original containing unpublished materials. All authors have read and approved the manuscript and no ethical issues involved.

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