CHEMICAL COMPOSITION AND ANTIMICROBIAL ACTIVITY OF ESSENTIAL OILS FROM ALGERIAN *Marrubium vulgare*

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Abstract: The development and use of traditional herbal medicine have a very long historical background that corresponds to the Stone Age. It has made a great contribution to the treatment of disease and also to the development of general health and well-being, in the prevention of many infectious diseases. The chemical composition of *Marrubium vulgare* oils and their antimicrobial properties were determined in this work.

The principal oil obtained by hydrodistillation was investigated using gas chromatography combined with mass spectrometry CG/MS to determine their chemical composition. Other parameters are also measured, such as refractive index, optical rotation, density, polarimetric deviation, freezing point and solubility in ethanol. The minimal inhibitory concentrations were investigated using disc diffusion and microdilution assays to characterize the antimicrobial activities of this essential oil. Antibacterial activity was evaluated against the yeast *Candida albicans* and against 5 bacteria including *Staphylococcus aureus*, *Salmonella typhimurium*, *Escherichia coli*, *Bacillus subtilis*, and *Pseudomonas aeruginosa*.

For chemical composition, 41 components have been determined. Results showed that major components in *M. vulgare* essential oil were β-Bisabolene (16.50%), followed by ß-Caryophylene (13.1%), α-Humulene (9.2%), E-β-Farnesene (6.4%), germacrene (5.95%).

For microbiological activity, *Marrubium vulgare* essential oil has a potent antimicrobial activity against a variety of pathogenic microorganisms including, *Staphylococcus aureus, Salmonella typhimurium, Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa* and *Candida albican*s showed the highest and broadest activity.

Finally, based on our results; *Marrubium vulgare* essential oil could be used as alternative and or therapeutic agent in treatments of such pathogens.

Key words: Antimicrobial activity; Essential oil composition; *Marrubium vulgare*; Mascara.

INTRODUCTION

Bacterial infectious diseases are a major cause of morbidity and mortality. In recent years, antimicrobial resistance has been a growing concern worldwide [2]. It has been defined as a global pandemic (EASAC, 2007), one of the major global public health threats, one of the major sanitary challenges of the twenty-first century [3]. Recently, there has been increasing research interest in the development of alternatives to address this problem. Natural products, mainly the plant-derived constituents, have long been considered as sources of drugs, and a great part (30-40%) of the pharmaceuticals available in modern medicine are directly or indirectly derived from natural sources [47]. According to the WHO [71, 72] about 70-80% of world population uses herbal medicines for their therapeutic effects [26-28].

Numerous studies have been published on the antimicrobial activity of medicinal and aromatic plant compounds [4]. Moreover, new pharmacologically active agents obtained from plants as clinically useful drugs play a promising role in treatment of human disease [6, 7, 49]. *Marrubium vulgare* is described as following: it is an aromatic plant including in the family of Lamiaceae, with a tough, woody, branched taproot or numerous fibrous lateral roots and numerous stems, which are quadrangular, erect, very downy, and from 20 to 100 cm high [70]. Leaves are roundish, ovate, usually toothed, petiolate, veined, and hoary on the surface, and they are arranged in opposite pairs on a long stem. Flowers in *M. vulgare* generally appear in the early spring, and they are regularly visited by nectar-gathering bees [75-76]. Seeds lie at the bottom of the calyx. The genus *Marrubium* comprises seven species, which are grown wild in many regions of Algeria. Among them, *Marrubium vulgare* L., a perennial herb, which is commonly known as ‘‘Horehound’’ in Europe, or ‘‘Merriouet’’ in Algeria [14]. It spreads along the Mediterranean area, and is geographically distributed to North Africa, Europe and temprate regions of Asia continent [48]. In Algeria, this plant is used in herbal medicine to treat a number of ailments, expectorant, bronchial asthma, non-productive cough, uterine, hepatic and diuretic effects [8,45]. *Marrubium vulgare* is increasingly recognized by their wide range of biological and pharmacological properties. Thus, including antimicrobial [9, 30], antioxidant [10, 11,13], anti-fungal [13], antiviral [13, 27], antiprotozoal [14-16, 39, 59], immunomodulatory [18], anti-diabetic [19], vasorelaxant [20, 25], antihypertensive [21, 26], cardioprotective [31], pain relievers [22-26], anti-inflammatory [26], antioedematogenic activity [23], anthelmintic [53] and many other biological activities has been reported [25-27, 56, 58, 68]. Using natural products of plant origin (botanical derivatives) is an alternative and recent approach for biological activities. *Marrubium vulgare* is used to prepare the well-known horehound candy, which, due to its pleasant taste is used to relieve cough, hoarseness, and bronchitis. It is generally recognized as safe in USA, and it is widely used as a flavoring agent [25-27, 70].

In Mascara western region of Algeria, few reports focused on the antimicrobial activity of *Marrubium vulgare* and the chemical composition of its essential oils. In this regard, the general aim of this study was to investigate chemical profile of essential oils extracted from *Marrubium vulgare* against variety of microorganisms including Gram positive and Gram negative bacteria.

MATERIALS AND METHODS

Plant material

During the flowering period, October 2007, the aerial part of *Marrubium vulgare* was collected in Mascara Province (North West part of Algeria). The plant was cleaned, dried and stored in the Institute of Biology, Faculty of Natural Sciences, University Mustapha Stambouli of Mascara, Algeria.

Oil isolation and analysis

Clean plant material was washed with distilled water, and was used for distillation. Essential oils were prepared by steam-distillation from *Marrubium vulgare.* Plant material (100 g) was performed in sufficient volume of boiling water for 3 hours. n-Hexane (3×50 mL) was added to the aqueous phase. The resulting essential oil was dried over anhydrous sodium sulphate Na_2SO_4 and stored at 4°C until examination [24, 25,51, 61].

The yield of the essential oil was measured by gravimetric method on a dry weight. The refractive index, density, optical activity, freezing point, solubility in ethanol at 90°, and acidity are measured in the essential oil. For gas chromatography and for mass spectrometry analysis, the oil was solubilized in n-hexane solvent.

Determination of the acid number: According to (NF ISO 1242: 1999 (T 75-103) [1]

This is the number of mg of KOH required for neutralization of free acids contained in 1g of EO. Free acids are neutralized with an EtOH solution titrated from KOH. The acid number gives an idea of the rate of free acids. An increase or rise in acid value indicates degradation of EO (ester hydrolysis) during storage. Conversely, an acid number less than 2 is proof of good conservation of essential oils (small quantity of free acids).

Chromatography conditions

In the chemical laboratory in Algeria the analysis of volatile constituents was performed by gas-chromatography. The volatile constituent analyzes were performed on a Hewlett-Packard CG/SM Gas Cromatograph (FID) detector system (CG: 5890 Series II, MSD5972) the fused silica HP-5MS capillary Colun (30 m X 0.25 mm) was directly connected to the MS. The carrier gas was helium, with a flow rate of 1.2 mL/min. The injector port 250°C and the oven temperature were designed as flows: isotherm at 50°C for 1 minute, then increased to 280°C at a rate of 5°C/minute and held isothermic for 20 minutes afterwards. Detector: 280°C, injected volume: 0.1 µL of 1% diluted solution and 1:50 split ratio. Ionization voltage: temperature 2080°C of 70eV ions, mass range: 40:300, time of scanning of mass units 1.5 seconds. Software adopted to handle mass spectra and chromatograms was a skin station. The percentage of the extract composition was determined from the peak area of the CG. For the determination of the retention index (RI), a hydrocarbon sequence was chromatographed on apolar columns along with essential oil, and their retention times were used to convert CG retention values to RI by linear interpolation with those of authentic compounds, literature data and also by computing them with the NIST (National Institute of Standards and Technology) [56]. Mass spectral library data as defined in Joulain *et al.* [38] and the data published in Adams [5].

Microorganisms

The strains selected for study were: Gram positive cocci *Staphylococcus aureus* (ATCC 25923) and Gram negative bacilli *Escherichia coli* (ATCC 25922), *Bacillus subtilis* (ATCC 6633), *Pseudomonas aeruginosa* (ATCC 10145), *Salmonella typhimurium* (ATCC 4296) supplied by the Algerian Pasteur Institute, and the yeast *Candida albicans* obtained from the microbiology laboratory of Yessad Khaled hospital, Mascara, Algeria.

Bacterial strains were grown and incubated at 37°C in nutrient broth (NB). The yeasts were grown and incubated at 25°C in Sabouraud broth (SB) [36, 40].

Antimicrobial activity

The antibacterial activity was assessed using two methods: the paper disc diffusion method as defined in NCCLS [53], Griffin *et al*. [36], and liquid Broth microdilution method as described by Lambert [44].

Paper disc diffusion method

The essential oils were dissolved for 2 minutes in vortexes of aqueous dimethyl sulfoxide (DMSO 10%) to the test concentration (20%, 10%, 5%, and 2.5%). Sterile Mueller Hinton agar was used to make Petri dishes with 100 µL of inoculum containing 10^6 CFU/mL or a McFarland Scale optical density of 0.5. A sample of 20µL of essential oil, were impregnated with sterile filter paper disks of 6 mm diameter as described by Griffin *et al.* [36]. Disc was aseptically transferred to the surface of the agar plates. The plates were 24 hours incubated at 37°C. Streptomycin (20µg) was used as positive controls and negative controls were performed on plates using aqueous DMSO (20µL) loaded paper disks. The plates were incubated aerobically over 24h at 37°C. The antimicrobial activity was evaluated by measuring the growth inhibition zone that surrounds the disks. After that, Vernier calipers were measuring the inhibition zone in millimetres. To minimize test error, all experiments were repeated three times. A 14 mm or greater inhibition zone (including disk diameter) was considered high antibacterial activity as described by *Koshy et al.* [43]. The aqueous DMSO control test alone showed no toxicity in the concentration used for those bacteria.

Broth microdilution assay for minimum inhibitory concentration (MIC)

The 10% DMSO was used to dissolve the *Marrubium vulgare* essential oil (EO) and then diluted to the highest concentration (200µL/mL). A serial doubling dilution of the oil was prepared in a 96 well microliter plate in inoculated Mueller Hinton broth. In brief, EO dilutions of 0.75, 1.55, 3.1, 6.2, 12.5, 25, 50, 100, and 200 µL/mL were prepared using a 96-well microtiter plate). Twenty µL EOs with specific concentration and 160 µL Mueller Hinton broth (were transferred to each well. Then, 20 µL inoculum was added to each well [2, 17, 19, 42].

The final concentration of bacteria in each microwell was 2×10^6 CFU/mL (estimated using the surface plate counting method). As a positive control, 20 µL inoculum was added to 180 µL Mueller Hinton broth lacking EO (0%). (Without antimicrobial substance). Negative regulation contained only 200µL of Mueller Hinton broth. Finally, 20 µL EO and 180 µL Mueller Hinton broth were also transferred to a well to evaluate the possible contamination of EO. All the extracts were checked in triplicates. The plates were incubated at 37˚C for 24h. Microbial growth was determined by turbidity being observed under daylight and the growth in each well was compared with that of the positive control. The lowest concentration exhibiting no turbidity is the MIC [47]. Turbidity suggested the development of the microorganisms [30, 33, 38, 43, 50, 64, 73].

Statistical analysis

Statistics were presented as mean ± standard deviation (S.D.) of duplicate solvent extractions and triplicate of assays, and analyzed with Student's test by Statview 5.0, Abacus Concepts, Berkeley, to assess significant differences between treatment levels.

RESULTS

Oil yield and chemical constituents

Physicochemical analysis showed a yellow color, with good aroma. The essential oil yield obtained from plant hydro-distillation was 0.54%. The determination density was obtained by double weighting d = 0.813, optical activity = +14.5 by polarimetry, and refractive index n = 1.4344 by interferometric method (Table 1). GC-MS analyzed the chemical composition of the obtained essential oils which enabled identification. Table 2 shows the findings of the chemical review of the examined essential oils. Forty-one components were detected in the oil of *Marrubium vulgare*, representing 97.78% of the total oil. The key constituents of the essential oil are β-Bisabolène (16.50%), its relative abundance was followed by ß-Caryophylene (13.1%), α-Humulene (9.2%), E-β-Farnesene (6.4%), germacrene (5.95%), germacrene D-4-ol (5.31%), Caryophylene oxide (5.01%), α -pinene (4.65%), γ-Cadinene (4.21%), Phytol (3.85%) and Hexadecanoic acid (3%).

Table 1. Physicochemical composition of *Marrubium vulgare* essential oil

Specification	*Marrubium vulgare*
Yield	0,54%
Density D	0.813
Optical activity	+ 14.5
Solubility in ethanol 90%	1:2 (v/v)
Freezing Point (°C)	- 18
Refractive index N 20	1.4344
Acidity	0.50

Table 2. Chemical compositions and retention indices (RI) of the M. vulgare essential oil

No.	Identified components	RI	Composition (%)
1.	α -pinene	931	4.65
2.	Myrcene	970	0.2
3.	Limonene	1011	0.4
4.	p-cymene	1027	0.1
5.	1,8-cineol	1035	0.1
6.	2-nonanal	1074	0.1
7.	Linalool	1078	0.2
8.	Terpinolene	1089	0.3
9.	Camphor	1120	0.83
10.	Borneol	1212	Tr
11.	2-undecanone	1273	0.4
12.	Thymol	1284	1,1
13.	Carvacrol	1290	2,01
14.	δ-Elemene	1320	0.6
15.	α-Cubebene	1356	0.52
16.	3-Dodecanone	1401	0.21
17.	α-copeane	1379	0.15
18.	Tetradecane	1400	0.5
19.	**ß-Caryophylene**	1416	**13.1**
20.	**E-β-Farnesene**	1450	**6.4**
21.	**α-Humulene**	1455	**9.2**
22.	**Germacrene D**	1481	**5.95**
23.	α-Amorphene	1492	0.31
24.	Bicyclogermacrene	1494	2.5
25.	**β-Bisabolene**	1498	**16.5**
26.	α- farnesene	1501	0.2
27.	**γ-Cadinene**	1505	**4.21**
28.	δ-Cadinene	1512	2.1
29.	α- Cadinene	1528	0.4
30.	Spathulenol	1562	0.87
31.	**Germacrene D-4- ol**	1570	**5.31**
32.	**Caryophylene oxide**	1578	**5.01**
33.	α-Muurolene	1630	0.9
34.	β –Cubebene	1662	0.3
35.	Farnesol	1671	3.90
36.	Octadecane	1801	0.5
37.	Nonadecane	1891	0.2
38.	**Phytol**	1921	**3.85**
39.	**Hexadecanoic acid**	1978	**3.00**
40.	Tricosane	2300	0.1
41.	Pentacosane	2485	0.6
		Total	**97.78**

Antimicrobial activity

Paper disc diffusion method

Some microorganisms, which cause human health harm, exhibit drug resistance due to insufficient antibiotic use. Thus, the discovery of new substances from natural sources, including plants, is important.

The antimicrobial activity of essential oils from aromatic species used in Algeria was previously assessed in this work as defined by Abadi *et al*. [1], Benarba *et al*. [13], Chouitah *et al*. [19], Debib *et al*. [21], Djahra [23] and Larbi [45]. The *M. Vulgaris* essential oils were tested for their antimicrobial activity using a disc diffusion method by measuring inhibition zones. Interesting antimicrobial properties have been observed (Table 3), showing bacteriostatic activity at 2.5% disk concentration. This concentration demonstrated greater activity against *S. aureus*, *B. subtilis*, and yeast *C. albicans* with 16.0, 15.0 and 16.0 mm inhibition zones respectively. In addition, they had shown against bacterium *E. coli*, *S. typhimurium* and *P. aeruginosa* a weak activity of 13.0, 13.0 and 9 mm respectively. The highest concentration (20% of EO) showed strong antimicrobial activity for the majority of microorganisms.

Broth Microdilution Assay

The data of the study clearly indicated that the EO of *Marrubium vulgare* had antimicroberial activity against a number of bacteria. MIC of EO of *Marrubium vulgare* against *S. aureus*, *B. subtilis*, and yeast *C. albicans* was 0.31, 0.62, and 0.31 respectively and with MIC values of 2.5, 1.25, 1.25% against *P. aeruginosa, S. typhimurium*, *E. coli* respectively (Table 4).

These findings indicated that such essential oils may be powerful than the natural products antibacterial activities. Essential oils from *Marrubium vulgare* can be shown to have a stronger antibacterial effect than Streptomycin. For very low amounts the essential oils from *Marrubium vulgare* inhibited the growth of all miro-ororganisms.

Table 3. Antibacterial activity of essential oil extract of whole plant of *Marrubium vulgare*

Bacteria	Zone of inhibition (mm)* (2,5% of EO concentration)	Streptomycin
Pseudomonas aeruginosa	09 ± 0.30	13
Salmonella typhimurium	13 ± 0.15	12
Staphylococcus aureus	16 ± 0.22	12
Escherichia coli	13 ± 0.18	11
Bacillus subtilis	15 ± 0.32	10.5
Candida albicans	16 ± 0.14	12.5

*Zone of inhibition (mm) is average of triplicate experiments. Disc diameter = 6 mm

Table 4. Minimum inhibitory concentration data obtained by the broth microdilution method

Microorganism	MIC (%)
Pseudomonas aeruginosa	2.5
Salmonella typhimurium	1.25
Staphylococcus aureus	0.31
Escherichia coli	1.25
Bacillus subtilis	0.62
Candida albicans	0.31

Extract of essential oil from the whole *M. vulgare* plant in comparison with Streptomycin norm, demonstrated moderate to strong antibacterial activity against five bacterial species tested and one yeast. The study revealed that crude oil extract from essential drugs was highly effective against *B. subtilis*, *S. aureus* (the gram positf bacteria) and *C. albicans*, and moderately successful against *S. typhimurium, E. coli* and *P. aeruginosa* (the gram negative bacteria) [63].

DISCUSSION

Many authors have worked on the composition of essential oils of *Marrubium vulgare* in different regions in the world. The evaluation of our result with literature shows some qualitative and quantitative differences in compositions of essential oils of *Marrubium vulgare*. This plant has many species and varieties and their essential oil composition has been determinate before [2, 16-19, 30, 33, 38, 42, 43, 50, 63, 73-77].

The essential oil is constituted essentially by various amounts of oxygenated sesquiterpenes, diterpenes, monoterpenes and hydrocarbons [35-37]. Our findings follow those mentioned by Semnani *et al.* [63], Abadi [2] Boudjelal *et al.* [14], and Asadipour [11]. There were some similarities in the chemical composition between our results and Bokaeian [16], Masoodi *et al.* [47], Khanavi *et al.* [41], Morteza [51]. Large number of studies such as Soković [66], Grassia *et al.* [35] and Abadi *et al*. [2] confirmed that essential oils of *Marrubium vulgare* contained almost the same amount of sesquiterpene compounds present in quite different percentage.

In our results, major components of essential oils (EOs) of *M. vulgare* were β-Bisabolene (16.50%), followed by ß-Caryophylene (13.1%) , α-Humulene (9.2%), E-β-Farnesene (6.4%), germacrene (5.95%). Study In Iran, conducted by Morteza-Semnani, *et al.* [51], they reported that major constituents of EOs of *M. vulgare* , were β-bisabolene (20.4%), δ-cadinene (19.1%) and isocaryophyllene (14.1%). Others works, in North center (Rezazi, *et al.* 2017), and eastern (Belhattab,*et al*. 2006) part of Algeria. Eugenol was the most representative compounds at proportion of 21.5 and 50.1% respectively followed by β-Bisabolene (10.3 & 10.9%) β-Caryophyllene 11.5 & 3.9 % Germacrene D (6.7 & 0.3%) in essential oils of *M. vulgare* [73].In our study Eugenol was absent in EOs. Such differences are probably attributed to geographical origin of plants collected from different regions (North center, eastern or western part of Algeria). In Turkey, Bayir *et al*. [12]observed that, major constituents of *M. vulgare* leaves essential oil were identified as α-Pinene (28.85%), β-Pinene (18.31%), β-Phellandrene (17.40%), 2- hexenal (14.80%).

Moreover, many studies suggested that EOs from *Marrubium vulgare* showed important variability in their chemical composition according to the site and stages of their development [56, 64, 65, 72].

In our study, five bacterial organisms and one fungal strain were used; streptomycin was used as a standard drug. Our results suggest that Gram (+) bacteria are more sensitive to the *M. vulgare* essential

oil than Gram (-) bacteria. This was compatible with the preceding studies on other extract of plants [32, 43, 60, 74].

Most studies reporting that essential oils are more active against Gram+ than Gram− bacteria [63, 65, 66, 67, 74]. The *P. aeruginosa* are proving a lot of work. Like all other gram negative bacteria it is more resistant to essential oils, due to cell wall composition. Gram-negative bacteria have an outer lipopolysaccharide wall, as defined by Gaunt *et al.* [34] and Friedman [31], which can serve as a shield against toxic agents.

In Gram (+), the absence of this barrier bacteria permit the direct passage of the essential oil's hydrophobic compounds with the phospholipids bilayer of the cell membrane, generating either an increase of ion permeability and exudation of crucial intracellular constituents, or destabilisation of bacterial enzymatic activity [73, 74].

There was an inhibitory effect of *Marrubium vulgare* oil. It can be observed from the results obtained that oils from *Marrubium vulgare* had a greater antibacterial activity than streptomycin.

It seems that many constituents of the essential oils play a significant role in determining the characteristics of oils, lipophobicity or hydrophilicity proprieties, and cellular extermination. This feature is very important because, depending on their component, the diffusion of the oil in the cell defines various forms of biological activities like antibacterial, and antifungal [16, 55, 74]. High contents of oxygenated compounds of essential oils from aerial part of *M. vulgare* are supposed responsible for the antimicrobial properties. It is normal to believe that their antimicrobial activity might be correlated to the large quantity of phenolic compounds.

The association between composition of EOs of *M. vulgaris* and his antimicrobial activity was investigated by Sahpaz *et al.* [61], Zhao [74] and Zawiślak [77].

Statistical experience and the traditional use of plants as medicinal products provide the basis for suggesting growing essential oils and plant extracts could be useful for specific medical conditions. Historically, many plant oils and extracts have been used as topical antiseptics, such as *Marrubium vulgare*, or have been reported to have antimicrobial properties. It is important to research scientifically those plants which have been used as potential sources of novel antimicrobial compounds in traditional medicines.

Our objective of this study is to find out the chemical composition of *Marrubium vulgare* oils and their antimicrobial properties. Even though many researchers were worked on this plant, very few researchers were reported about the chemical composition and the antimicrobial activity in Algeria. To our knowledge this is the first report in our region (Mascara province).

Many researchers compared the chemical composition of *Marrubium vulgare* oils from various origins in their work. Though there are similar works, but in the present work other compound are obtained like thymol and borneol, farnesol, phytol, hexadecanoic acid.

In this study the antimicrobial compounds identified as the most active against microorganisms of major pathogens, they may replace conventional antimicrobial chemicals. Due to their very high specific activity essential oils can also be used for the prevention and treatment of large number of diseases in animals and humans caused by pathogens microorganism at low and non-toxic concentrations.

Finally, this work can be considered as the initial report on the composition and antimicrobial effect of *M. vulgaris* volatile oil in our region. The synergy or antagonism between the different constituents of essential oils may be responsible for the significant antimicrobial effect. Further phytochemical and clinical studies are needed to identify active constituents responsible for the observed activity.

Acknowledgments. The authors are thankful to traditional healers for giving out their inherited information during this work. This paper consider the relevance of the work of Doumir M. (DVM, Tebessa, Algeria) and Soltani K. (MD, Tebessa, Algeria) for language help, and proof reading of manuscript. The authors would like to thank the University Mustapha Stambouli of Mascara for their counsel during this research.

Conflict of interest: There is no actual or potential conflict of interest in relation to this article.

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Received: June 29, 2021
Accepted: November 8, 2021
Published Online: November 10, 2021
Analele Universităţii din Oradea, Fascicula Biologie
https://www.bioresearch.ro/revistaen.html
Print-ISSN: 1224-5119
e-ISSN: 1844-7589
CD-ISSN: 1842-6433
University of Oradea Publishing House