Journal of Medicinal Plants and By-Products (2024) 4: 1137– 1148
https://doi.org/10.22034/jmpb.2024.365089.1663

Original Article

Exploring the Chemical Landscape and Biological Potentials of *Rosmarinus officinalis* Essential Oil: a GC Analysis Approach

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Article History

Received: 26 July 2024
Accepted: 20 September 2024

Keywords
Rosemary
Chemical composition
Antioxidant
Antimicrobe
Volatile oil

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ABSTRACT

This article presents an analysis of the chemical constituents, antimicrobial efficacy, and antioxidant activity of the essential oil extracted from leaves of *Rosmarinus Officinalis*, commonly referred to as Rosemary, originating from Hamedan province in Iran. The essential oil was obtained by hydrodistillation method from the sample purchased from the market (LRM) and those cultivated in the university garden (LRU). The research employed Gas Chromatography/Mass Spectrometry (GC/MS) analysis to examine the chemical composition of the obtained essential oils. The antimicrobial efficacy was evaluated by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) via a sequence of microdilutions, while the DPPH assay was used to assess the antioxidant activity. Standard methods determined the ash contents including total ash and acid-insoluable ash. The chemical composition of the essential oils showed some variations. 1,8-cineol, borneol, and camphor were the main components in LRM, while 1,8-cineol and α-pinene were the major constituents of LRU. In the case of microbial tests, LRM showed the best efficacy on *Pseudomonas aeroginosae(SA)* and LRU had the highest efficacy on *Methicillin-resistant Staphylococcus aureus (MRSA).* The research exhibited the comparable antioxidant activity of essential oils derived from different plant variations, noting that these activities were not as potent as the positive control. The total ash content of LRM was higher than that of LRU, which indicates impurities in the first sample. The study further explored the variations in these characteristics based on the plant's conditions (purchased or cultivated). The presence of different components in the essential oils from various sources may contribute to the observed variations in biological properties. The researchers recommend further extensive investigations into the chemical constituents and their relative abundance in *R. officinalis* essential oil to enhance understanding of its therapeutic potential and industrial applications.

Running Title: Investigations on *Rosmarinus Officinalis* Essential oil

INTRODUCTION

The therapeutic application of plant-derived compounds in disease management is a long-standing practice worldwide, particularly in developed nations. Over the past hundred years, there has been an increased interest in medicinal plants possessing antimicrobial and antioxidant properties. This interest is driven by issues such as the overuse of synthetic drugs and the escalating resistance of bacteria to these agents [1]. Plants, therefore, represent a reservoir of potentially beneficial chemicals that are yet to be fully exploited. These chemicals hold promise not only as therapeutic agents but also as unique precursors for the synthesis of pharmaceutical analogs and as valuable tools for understanding biological processes [2].

Rosmarinus officinalis, commonly known as rosemary, is a plant species with a rich history and wide-ranging applications in natural medicine. The first mention of rosemary is found on cuneiform stone tablets as early as 5000 BCE. Egyptians used it for embalming corpses starting in 3500 BCE. The ancient Greeks and Romans were among the first to recognize its potential [3]. This plant is native to the Mediterranean region but is now cultivated around

the world. It is a member of the sage family Lamiaceae, which includes many other medicinal and culinary herbs. The name rosemary derives from the Latin "rosmarinus", which translates to "dew of the sea" [3].

It has a long history of cultivation and has been found to have been used for medicinal, culinary, and cosmetic purposes in ancient civilizations such as Egypt, Mesopotamia, China, and India. The popularity of essential oils derived from medicinal plants has seen a surge in recent times. A notable example is the essential oil extracted from rosemary (*R. officinalis*), which is extensively utilized as a natural food preservative. This is primarily due to its antimicrobial, antiviral, and antimycotic properties, coupled with its antioxidant capabilities. The essential oil's affordability and easy accessibility further enhance its appeal. It is also used in traditional medicine for its choleretic, hepatoprotective, and antitumorigenic activities [4, 5]. The essential oil of rosemary is also known to enhance blood circulation in the limbs, exhibit antirheumatic effects, and alleviate neuralgic pain. In addition to its therapeutic applications, essential oil is extensively used in the cosmetic industry for the production of various products such as cologne waters, bathing essential oils, hair lotions, and shampoos. The leaf of rosemary is a staple spice in French, Italian, and Spanish cuisines [6, 7]. The composition of rosemary essential oil has been the subject of numerous studies, primarily in countries from the Mediterranean region, the Balkans, and South-Eastern regions [8].

Research on the composition of essential oils from various chemotypes of rosemary (including α-pinene, 1,8 cineol, camphor, and verbenone chemotypes) has primarily centered around the analytical characterization of its phytochemical constituents. These include non-oxygenated monoterpene and sesquiterpene hydrocarbons, oxygenated monoterpenes and sesquiterpenes, phenolic derivatives, and non-isoprenoid components such as volatile alcohols, aldehydes, and ketones [9]. It is hypothesized that these hydrophobic compounds can disrupt the plasma and outer membranes of Gram-negative bacteria, leading to changes in membrane permeability and subsequent cell death [10, 11]. Research has also explored the potential of bioactive plant products to enhance antibiotic activity to increase effectiveness against multi-drug resistant (MDR) microorganisms [12]. Currently, the demand for *R. officinalis* is on the rise due to its applications in traditional medicine, pharmaceutical industries, and agribusiness. However, most of the material used is sourced from natural rosemary populations growing in areas characterized by low rainfall (<300 mm/year), repeated drought, poor soil quality, and overgrazing. These factors, coupled with increasing harvest, have led to the continuous degradation of these populations [13]. On the other hand, the determination of the ash content of plants can be used as one of the evaluation indicators for the purity and quality of plant samples.

The rosemary plant is native to the Mediterranean climate and exists in Iran as a cultivated plant. The plant has been cultivated in Iran for less than thirty years. All the specimens of this plant in Iran are as cultivated plants, and the wild type of Rosemary does not exist in this region [14]. In this study, two types of cultivated plants were examined. One was cultivated in the medicinal plants garden of the pharmacy faculty, and the other type was purchased from a market in downtown Hamedan, which was also cultivated but in a different environment from the first type.

This study will focus on the evaluation of the chemical composition, antioxidant, and antimicrobial of the *R. officinalis* cultivated in the garden of the Hamedan Faculty of Pharmacy and those purchased from the market in Hamedan province. Besides the determination of total and acid-insoluble ash contents is another aim of the study.

MATERIALS AND METHODS

Ethical Considerations

The research was approved by The Ethics Committee of Hamadan University of Medical Sciences under the code IR.UMSHA.REC.1400.882.

Preparation of the Plant Material

The aerial parts of *R. officinalis* (herbarium code: 324) were collected in May 2022 from the medicinal plant garden of the Hamedan Faculty of Pharmacy and the leaves of the plant were separated and placed away from the sun to dry. Also, dried leaves of *R. officinalis* were purchased from downtown Hamedan market and stored at 25° C shielded from sunlight until the day of utilization. The

authentication of plant specimens was conducted at the Herbarium of the Department of Pharmacognosy, School of Pharmacy, Hamadan University of Medical Sciences, Hamadan, Iran.

Preparation of the Essential Oil

The essential oil was extracted from leaves of *R. officinalis* samples individually using a hydro-distillation process facilitated by a Clevenger-type apparatus. This involved placing 100 g of the dried components of *R. officinalis* in a 2-liter flask, to which 1.5 liters of purified water was added. The system was then subjected to heating for 5 hours. Following this, the essential oil was separated and dried using anhydrous sodium sulfate, then preserved in sealed, dark-colored glass vials at 4 °C until use [15-17].

GC-MS Analysis

The essential oil derived from leaves of *R. officinalis* was subjected to analysis using a ThermoQuest-Finnigan TRACE MS gas chromatograph-mass spectrometer (GC-MS) equipped with a fused methyl silicon DB-5 column (30m*0.25mm*0.25mm film thickness). The carrier gas used was helium, flowing at a rate of 1.1 mL /min. The column temperature was initially held at 60°C for two minutes, then ramped up to 250°C at a rate of 5°C/min, and finally maintained at 250°C for an additional 2 minutes. The injector temperature was set at 250°C and the split ratio was adjusted to 1/10. The injection volume was 0.2 ml. The mass spectrometer was operated under the following conditions: an ionization potential of 70 eV and a source temperature of 200° C. The constituents were identified by comparing their Retention Index with that of C5-C24 n-alkanes, as well as by comparing the RI provided in the literature and the mass spectra recorded by the MAINLIB and Willey.

DPPH Assay

The free radical scavenging ability of the essential oil derived from *R. officinalis* leaves was assessed using a slightly modified version of the method proposed by Motaghed *et al* [18]. This method involves the decolorization of a purple-colored methanol solution of DPPH, which signifies the antioxidant or electron donation capacity of the plant material. In brief, 750 µL of plant extract and essential oil at various concentrations (ranging from 25000 µg/mL to 50 µg/mL) were combined with 300 µL of 0.3 mM DPPH radicals in methanol, maintaining a ratio of 5:2. The mixture was allowed to stand at room temperature for 30 minutes. The absorbance of the solution was then measured against a blank at 518 nm, with all measurements being performed in triplicate. The percentage of antioxidant activity was calculated using the formula:

$$AA\% = [(AbsControl - AbsSample) / AbsControl] \times 100$$

Here, AbsControl represents the absorbance of the control reaction (which contains all reagents except for the test compound), and AbsSample represents the absorbance of the test compound. BHT was used as a positive standard. The essential oil concentrations that provided 50% inhibition (IC50) were determined from the graph of inhibition percentage plotted against their concentration.

MIC and MBC Evaluation

The serial dilution method was utilized to ascertain the minimum inhibitory and lethal concentration. The broth microdilution technique (CLSI 2009) was applied to establish the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of rosemary essential oils for the organisms being studied. To determine the minimum inhibitory and lethal concentration of the essential oil from *R. officinalis* leaves, the bacteria were initially thawed and cultivated on the Soybean Casein Digest broth (SCDB) culture medium in a 37°C incubator for 24 hours. They were then transferred to the Soybean Casein Digest Agar (SCDA) culture medium using a sterile swab and incubated for another 24 hours. A portion of the grown bacteria was diluted in a 0.9% sodium chloride solution to achieve 0.5 McFarland turbidity (equivalent to 1.5 × 108 colony-forming unit (CFU)/ml), which was then diluted tenfold, and 1 mL of it was inoculated into 100 mL of SCDB culture medium. 1 mL of the inoculated culture medium was introduced into 10 separate test tubes, and 0.5 mL of essential oil and 0.5 mL of DMSO were added. Ten two-fold dilutions from 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.2, 0.1, and 0.05 mg/mL of the essential oil were used. To examine the antimicrobial effects of DMSO instead of essential oil, 0.5 mL of normal saline was added, and dilution was performed in the same manner. The concentrations for which no turbidity was observed after 24 hours of incubation were considered as MIC. Finally, from the last 4 dilutions

without bacterial growth, 10 microliters were taken and cultured in the SCDA medium and placed in a 37°C incubator for 24 hours. The first concentration for which no bacterial growth was observed after 24 hours of incubation was considered as the minimum bactericidal concentration (MBC). The test was repeated three times for each microorganism.

Ash Content Detemination

Total Ash Content

Each plant sample was powedred to reach the particle size below 24 meshes. It was poured in a tared crucible, weighed accurately, and put in the oven at 570°C. After turning into white ash with constant weight, again, it was weighed. The percentage of total ash content was calculated using the formula: ash weight *100/ plant sample weight.

Acid- insoluable Ash Content

The mixture of 10 ml of diluted hydrochloric acid and the ash from the previous step, was heated for 10 minutes. After cooling, the filtrate was rinsed with boiling water. Then, the filter paper and ash residue were put in the oven. After the complete burning of the filter paper, the residue was cooled and weighed. The percentage of acid-insoluble ash content was calculated according to the formula: ash weight *100/ plant sample weight.

RESULTS

The total essential oil yield from *R. officinalis* leaves, sourced from the university garden and purchased from the Hamedan market, was found to be 1.53% and 0.95%, respectively. The GC-MS analysis of *R. officinalis* from Hamedan, Iran, disclosed the existence of numerous compounds (Table 1). The primary constituents were identified as Linalool, 1,8-Cineol, Borneol, and Camphor. Other compounds such as α-Pinene, Camphene, β-Pinene, n-Decane, p-Cymene, limonene, γ-Terpinen, linalool, α-Terpineol, and bornyl acetate were also detected in different quantities (Table 1).

As can be noticed from Table 1, certain components could only be found in one of the samples of the essential oil from *R. officinalis* and are absent in the other essential oil samples. The following compounds were only found in the essential oil from university garden: Thuja-2,4(10)-diene, 1-Octen-3-ol, 3-Octanone, β-Myrcene, alpha-Phellandrene, α-Terpinene, α-Terpinolene, Filifolone, Chrysanthenone, Pinocamphone, cis-pinocamphone, Homomyrtenol, Citronellol, Geraniol, β-Caryophyllene.

β-Caryophyllene: β-Caryophyllene, more formally (−)-β-caryophyllene, (BCP) , is a natural bicyclic sesquiterpene that is a constituent of many essential oils, especially clove oil, the oil from the stems and flowers of *Syzygium aromaticum* (cloves), the essential oil of *Cannabis sativa*, copaiba, rosemary, and hops. It is used extensively in the fragrance industry due to its woody, spicy, and cooling aroma [19].

β-Myrcene: β-Myrcene, also known as Myrcene, is a monoterpene that manifests as a colorless oil. It is predominantly found in essential oils and is primarily produced semi-synthetically from a plant species known as Myrcia. It serves as an intermediate in the synthesis of various fragrances. β-Myrcene has been identified to possess antioxidative properties and is frequently utilized in numerous food flavorings and additives. Additionally, it is postulated to offer a multitude of health and medicinal benefits [20].

α-Terpinene: α-Terpinene is a monoterpene that is industrially synthesized via the acid-catalyzed rearrangement of α-pinene. While it possesses perfume and flavoring properties, its primary application is to impart a pleasant odor to industrial fluids. The hydrogenation of α-Terpinene yields the saturated derivative p-menthane [21].

Pinocamphone: Pinocamphone is a naturally occurring bicyclic monoterpene ketone found in hyssop oil. It constitutes up to 70 percent of the composition of hyssop oil. Pinocamphone and compounds with similar structures are implicated in the toxic effects of hyssop oil [22].

In contrast, the following compounds were uniquely detected in the essential oil of *R. officinalis* from Hamedan market: O-Cymene, trans-Linalool oxide, Trans-p-mentha-2,8-diene-1-ol, trans-Pinocarveol, p-Cymen-8-ol, Cryptone, cis-Carveol, m-Cumenol, Isobornyl formate, Cumin aldehyde, D-Carvone, Carvenone, p-Cymen-7-ol, γ-Cadinene, Caryophyllene oxide, α- Cadinol.

O-Cymene: a naturally occurring monoterpene, is a significant component in the essential oil of *Lepechinia meyeni*. The mass spectra of the o-, m-, and p-cymenes are similar, making it challenging to unambiguously identify the three cymene isomers. Further research is needed to develop more precise

methods for the identification and quantification of O-Cymene in essential oils [23].

Cryptone: is a bioactive compound found in the essential oil of Cymbopogon martini, also known as ginger-grass. The essential oil, rich in cryptone, exhibits significant inhibition of pro-inflammatory markers in activated HaCat cells without a cytotoxic effect. This suggests potential applications of Cryptone-rich essential oils in anti-inflammatory therapies [24].

Cumin aldehyde: is a major component in the essential oil of cumin (*Cuminum cyminum* L.). The essential oil extracted by supercritical carbon dioxide extraction (SCE), subcritical butane extraction (SBE), and traditional solvent extraction (SE) methods showed significant differences in the content of Cumin aldehyde. This highlights the importance of extraction methods in determining the chemical composition of essential oils [25].

Trans-Linalool oxide: is a terpene found in the essential oil of various plant species. It has been detected in the essential oil of *Cymbopogon martini* and Lavandula species. The presence of trans-Linalool oxide contributes to the overall aroma and potential biological activities of these essential oils [26].

Following the eradication of free radicals, the absorbance value obtained via the spectrophotometric method signifies the quantity of DPPH free radicals. A higher value indicates a lower capacity of the essential oil to remove free radicals. The IC50 values of the essential oils from *R. officinalis* leaves are depicted in Table 2. The IC50 value in BHT is reported as a positive control. Our study indicated that an elevation in essential oil concentrations results in an increase in free radical-scavenging activity. The activity of the essential oil of *R. officinalis* leaves sourced from the university garden was superior to the plant purchased from the Hamedan market, but it was not comparable to BHT (IC50= 11.16 ± 1.05 μg/ml) used as a positive control.

The results of MIC and MBC in terms of the concentration of essential oil in the test tubes on the bacteria are given in Table 3.

As per the results, DMSO does not have a significant inhibitory impact on 4 strains of bacteria (E. Coli, S. Aureus, S. Aeruginosa, and MRSA) and is incapable of eliminating any bacteria even at its maximum concentration, i.e., 25 mg/mL. The MIC values for the essential oil of leaves from the plant purchased from the Hamedan market and the one sourced from the university garden were alike and equal to 0.16-1.25mg/mL. The MBC values for the purchased leaves from the Hamedan market and the ones collected from the university garden were 0.16-1.25 mg/mL and 0.31-5 mg/mL, respectively. The most effective antimicrobial effect was observed in the essential oil of leaves purchased from the Hamedan market on *S. aeruginosa* where both MBC and MIC were 0.16 mg/mL. As seen in Table 3, leaves cultivated in a university garden showed the best efficacy on MRSA with MIC and MBC of 0.16 AND 0.31, respectively.

The results of total ash contents were 9.5 and 10 percent for the *R. officinalis* leaves sourced from the university garden and the plant purchased from the Hamedan market, respectively. Determination of acid-insoluble ash content results presents 1.1 and 1.08 percent for the *R. officinalis* leaves sourced from the university garden and the plant purchased from the Hamedan market, respectively.

Table 1 Chemical composition of the essential oil from leaves of *R. officinalis.*

Compounds	RLU[a]	RLM[b]	RI
α-thujene	0.20	0.11	924
α-Pinene	18.76	1.75	935
Camphene	4.82	1.44	950
Thuja-2,4(10)-diene	0.78	ND	955
1-Octen-3-ol	0.15	ND	975
β-Pinene	0.63	1.19	979
3-Octanone	1.26	ND	984
β-Myrcene	1.89	ND	990
n-Decane	0.54	0.67	998
alpha-Phellandrene	0.23	ND	1006
α-Terpinene	0.72	ND	1017
O-Cymene	ND	0.43	1022

p-Cymene	1.78	1.52	1025
Limonene	4.10	0.82	1029
1,8-Cineol	18.87	29.24	1033
γ-Terpinen	0.68	0.23	1058
trans-Linalool oxide	ND	0.35	1088
α-Terpinolene	0.79	ND	1089
Linalool	4.28	0.47	1099
Filifolone	0.74	ND	1105
Trans-p-mentha-2,8-diene-1-ol	ND	0.79	1123
Chrysanthenone	0.62	ND	1127
trans-Pinocarveol	ND	1.56	1142
Camphor	7.05	13.51	1148
pinocamphone	1.07	ND	1163
Pinocarvone	0.43	0.52	1166
Borneol	5.58	25.91	1169
cis-pinocamphone	0.66	ND	1178
4-Terpineol	1.10	0.86	1180
p-Cymen-8-ol	ND	0.45	1188
Cryptone	ND	3.24	1191
α-Terpineol	2.99	0.97	1194
Myrtenal	0.76	1.29	1200
Homomyrtenol	0.75	ND	1207
Verbenone	8.63	0.57	1215
cis-Carveol	ND	0.59	1221
m-Cumenol	ND	0.27	1226
Citronellol	0.12	ND	1228
Isobornyl formate	ND	0.90	1232
Cumin aldehyde	ND	1.87	1243
D-Carvone	ND	0.94	1247
Carvenone	ND	0.37	1254
Geraniol	2.62	ND	1255
Bornyl acetate	5.22	1.84	1289
p-Cymen-7-ol	ND	0.78	1292
Geranyl acetate	0.64	1.77	1383
β-Caryophyllene	0.53	ND	1424
γ-Cadinene	ND	0.33	1518
Caryophyllene oxide	ND	1.28	1589
α-Cadinol	ND	1.15	1645
Oxygenated monoterpenes	62.13	89.06	-
Monoterpene hydrocarbons	35.38	7.49	-
Oxygenated sesquiterpenes	ND	1.15	-
Sesquiterpene hydrocarbons	0.53	1.61	-
Alkenes	0.54	0.67	-
Alcohol	0.15	-	-
Ketone	1.26	-	-
Yield of essential oil	1.53	0.95	-

[a]: *R. officinalis* Leaves collected from university garden . [b]: *R. officinalis* Leaves purchased from Hamedan Market. RI: Retention index.

Table 2 Antioxidant activity on 2.2-diphenyl-1-picrylhydrazy defined as IC_{50} values.

Plant samples	DPPH (IC_{50}µg/ml)
LRM	11134 ± 905.864
LRU	10326 ± 887.024
BHT (Butylated hydroxytoluene)	11.16 ± 1.05

Table 3 antimicrobial activity of *R. officinalis* defined as MIC and MBC values

sample / Essential oil	*MRSA*[a]		*S. aeruginosa*		*S.aureus*		*E.Coli*	
	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)
LRM	0.31	0.31	0.16	0.16	1.25	1.25	0.63	0.63
LRU	0.16	0.31	0.31	1.25	0.63	0.63	1.25	5
DMSO	20	>20	10	>20	20	>20	10	>20

[a]: Methicillin-Resistant Staphylococcus Aureus

DISCUSSION

Chemical Composition

In the essential oils derived from the leaves of *R. officinalis*, 1,8-Cineol, and camphor were the predominant constituents. The essential oil from the leaves cultivated in the university garden was distinguished by a greater concentration of Camphene and lesser quantities of camphor, in contrast to the essential oil from the plant purchased from the Hamedan market, which had elevated levels of camphor and almost no significant presence of camphene. The plant sourced from the Hamedan market demonstrated a higher concentration of borneol compared to the other sample, whereas Linalool and Limonene were detected in larger quantities in the plant from the university garden. Interestingly, the essential oil from the plant cultivated in the university garden contained a relatively high quantity of Verbenone (8.63%), but this component was a minor component in the sample from the Hamedan market. Although monoterpene oxygenated compounds have the highest percentage of compounds in the essential oil of the garden, the amount of monoterpene hydrocarbon compounds is also significant. In contrast, in the essential oil of market, monoterpene oxygenated compounds constitute a very high percentage (about 90%) and monoterpene hydrocarbon compounds constitute a lower percentage. Also, the amount of sesquiterpenes in both essential oils is much lower than in monoterpenes. Due to the popularity of *R. officinalis*, there numerous research focused on the analysis of the constituents of the plant's essential oil [27-38].

HCINI1 *et al*. evaluated the Chemical Composition of the Essential Oil of *R. officinalis* of Tunisian Origin in their study [27]. The Essential oils were extracted from three populations of rosemary using water distillation, yielding 1.8%, 1.6%, and 1.4% based on the dry weight of the samples from Beja, Sidi Bouzid, and Gabes, respectively. These authors noted that the primary constituents of the rosemary oils were found to be 1,8-cineole, camphor, α-pinene, α-terpineol, borneol, camphene, and p-cymene, with varying percentages for each population. This study revealed that Tunisian rosemary includes chemotypes of 1,8-cineole and camphor, similar to those reported for the species from other Mediterranean countries. Previous reports have indicated the presence of α-pinene, 1,8-cineole, camphor, verbenone, and borneol, which constitute about 80% of the total *R. officinalis* oil. These major components were also reported in the essential oil of Sardinian *R. officinalis*. The major constituents of rosemary oil, including cineole, borneol, pinene, and camphor, make up about 28%, 18%, 12%, and 10% of the oil, respectively. When compared with other rosemary oils, Brazilian oils were found to be more similar to those of French origin due to their 1,8-cineole and camphor contents. Another research by Sienkiewicz *et al*. [28] has indicated that the primary constituents of rosemary essential oil are 1,8-cineole (46.4%), camphor (11.4%), and β-pinene (11.0%). In a separate study, Jiang *et al*. [29] utilized rosemary essential oil primarily composed of 1,8-cineole (26.54%) and β-pinene (20.14%). Furthermore, an investigation by Bendeddouche *et al*. [30] revealed that the tested essential oil's main components were

camphor (37.6%), 1,8-cineole (10.0%), p-cymene-7-ol (7.8%), and borneol (5.4%).

A study by Ladan Moghadam *et al*. performed The GC-MS analysis of the essential oil derived from *R. officinalis* L. which delivered a yield of 1.4% [31]. The analysis identified 20 volatile compounds, which accounted for 92.95% of the total composition, in the aerial parts of the oils. The primary constituents of the *R. officinalis* oils were identified as α-pinene (43.12%), camphene (10.50%), limonene (6.12%), 1,8-cineole (10.02%), camphor (8.07%), and linalool (8.09%). Other components were present in quantities less than 2%. The total oil composition of primary compounds (α-pinene, camphene, limonene, 1,8-cineole) was 81.45% which was lower than other studies on *R. officinalis* in Iran (99.74%)[32] and Algeria (98.2%)[33]. Their findings align with those of Chalchat *et al*., [34] and Aidi Wannes *et al*. [35], reported that these organs yield the highest essential oil.

Other research by Santoyo et al. in Spain has reported the presence of α-pinene, 1,8-cineole, camphor, verbinone, and borneol, which made up approximately 80% of the total *R. officinalis* essential oil [36]. The essential oils of *R. officinalis* have been reported to contain major components such as α-pinene, borneol, camphene, camphor, verbinone, and bornyl acetate. Kadr *et al*. found that α-pinene, 1,8-cineole, camphor, verbenone, and borneol constituted about 77.32% of the total *R. officinalis* oil [37]. The variability in the qualitative and quantitative composition of the essential oil can be attributed to intrinsic factors such as genetics and plant age, as well as extrinsic factors like climate, cultivation conditions, and extraction methods [38]. Significant variations in the chemical composition of the oil have been reported in relation to geographic origin. Factors such as harvest times, condition of the twigs and leaves, distillation equipment, and management practices also play a crucial role in the overall quality of the oil [36].

Antimicrobial

Interestingly, the essential oil from the plant cultivated in the university garden demonstrated a higher antimicrobial potency against *S. Aureus*, while its effect on *E. coli* was less pronounced. Conversely, the antimicrobial effects of the essential oil from the plant purchased from the Hamedan market were more pronounced against *E. coli* and less significant against *S. Aureus*. This variation could be attributed to various factors. For instance, Cineol has a better efficacy to damage the cell membrane in *E. coli* than α-pinene. The variation of antimicrobial effects of *R. officinalis* essential oils on *E. coli* bacteria could be due to differences in the amounts of Cineol and α-pinene in the essential oils.

As indicated in Table 3, the antimicrobial characteristics of the essential oil extracted from the leaves of *R. officinalis* display a similar potency against gram-negative bacteria such as *S. aeruginosa*, with their MIC ranging from 0.16 to 0.31 mg/mL, when compared to gram-positive bacteria like *S. Aureus* and *MRSA*. The findings from our investigation into the antimicrobial characteristics of *R. officinalis* essential oil present some discrepancies in comparison to previous studies where the efficacy of the essential oil was higher against *E. coli* bacteria.

The antibacterial properties of rosemary have been evaluated through various types of assays, primarily based on either the Minimum Inhibitory Concentration (MIC) or the Minimum Bactericidal Concentration (MBC). In this context, Sienkiewicz *et al*. have demonstrated the antibacterial effects of basil (*Ocimum basilicum*, L.) and rosemary (*R. officinalis* L.) [28]. The authors reported that both essential oils inhibited microbial growth, as indicated by the MIC values. Antibiotic susceptibility was assessed using disc diffusion, and the results revealed that both tested essential oils were active against all clinical strains of *Escherichia coli*.

Makonnen and colleagues investigated the effects of a sample of rosemary essential oil that contained a high amount of alpha-pinene (50.8%). They found that this essential oil possessed a MIC of 36.33 μg/mL against *Pseudomonas aeruginosa*. Apart from the difference in the composition of the essential oil, the difference in the strain used in the studies can also explain the difference in antimicrobial effects. In the study by Alvarez and colleagues, rosemary essential oil had a MIC of 0.65 and 0.91 μg/mL against *E. coli* and *Staphylococcus aureus*, respectively. This study is consistent with the findings of the study by Alvarez on possessing greater effects of *R. officinalis* on *E. coli* than on *S. aureus*.

In another study, Mihajilov-Kristev *et al*. showed that essential oils, primarily composed of carvacrol

(67.0%) and γ-terpinene (15.3%), were effective against Gram-negative strains, including *Escherichia coli* [39]. The MIC values ranged from 0.025 µL/mL to 0.78 µL/mL, as determined by the broth microdilution method. Probuseenivasan *et al*. further confirmed that rosemary essential oil strongly inhibits E. coli ATCC 25922, with the minimal inhibitory concentration of rosemary oil against *E. coli* exceeding 6.4 mg/L [40]. Zaouali *et al.* reported that the antimicrobial activity of rosemary essential oil, when compared with *S. aureus*, improves with the presence of β-pinene as a major component [41]. This effect can be associated with the ability of terpenes to disrupt the cell membrane, thereby promoting lysis, as suggested by Bjapai *et al* [42]. The efficacy of rosemary essential oil against *E. coli* is attributed to the synergistic action of the various minor components present in its volatile fraction, rather than the action of any specific component, aligning with the conclusions drawn by Zaouali *et al* [41]. Several authors have asserted that *E. coli*, *L. monocytogenes*, and *S. aureus* are highly resistant bacteria, emphasizing the significance of the chemical composition and the proportion of oil components in determining their antimicrobial efficacy [43, 44].

Antioxidant

As it can be observed from Table 2., the antioxidant properties of the essential oil of *R. officinalis* cultivated in Hamedan province are not significant compared to BHT as a positive control. Nevertheless, it is still noteworthy that the essential oil from this plant contains some levels of antioxidant potency. This factor could be due to the geographical location of cultivation, the stage of plant development at the time of oil extraction, and the extraction method employed. Other research noted a much higher antioxidative effect for this plant, some even comparable to the positive control. For instance, Ladan Moghadam *et al.* reported an IC50 of 13.00±0.51 µg/ml for the essential oil of *R. officinalis* collected from Kermanshah province in Iran [31]. Furthermore, Bozin *et al.* conducted an analysis of the antioxidative effects of *R. officinalis* collected from Vojvodina province in the Republic of Serbia [9]. These researchers observed that the essential oil of this plant exhibited an IC50 of 3.82 ±0.05 µg/ml. Mariem Ben Jemia and colleagues conducted a study in 2014 to investigate the antioxidant effect of 8 samples of rosemary essential oil by the DPPH method in Tunisia. These samples showed antioxidant activity by inhibiting the DPPH radical with IC50 values ranging from 343.1 to 592.8 µg/mL [45]. In a study by Maliheh Karamat and colleagues, it was shown that rosemary essential oil from Shiraz significantly reduced the absorption of DPPH solution. They also reported the IC50 value of rosemary essential oil as 330±3670 µg/mL. This essential oil contained alpha-pinene 17.07%, camphor 9.62%, bornyl acetate 9.42%, 1,8-cineole 8.81%, verbenone 7.99% and borneol 6.33%. The plant in this study was obtained from a cultivated population in Shiraz, southern Iran [46]. In a study by Spyridoula D. Christopoulou and colleagues, they investigated the antioxidant effect of rosemary essential oil by the DPPH method in Greece and reported the IC50 value of rosemary essential oil as 12320 µg/mL. They also analyzed the composition of the essential oil. 1,8-cineole (40.1%), camphor (12.4%), alpha-pinene (12.94%), beta-pinene (8.94%), and camphene (6.38%) were reported as the major components of this essential oil. This study was conducted in Greece and the plant used for the study was cultivated [47]. It seems that the extent of antioxidant effects is not solely dependent on the amount of major components of the essential oil and other components are also influential.

Ash Content

Total ash content represents physiological and non-physiological ash the first is from the plant tissue, and the second is often derived from environmental contaminations such as soil and sand. The total ash content is insufficient to illustrate the quality of plant samples when the plant contains considerable amounts of physiological ash, particularly calcium oxalate. Considering that the acid-insoluble ash content is used as another index to reflect the quality of the plant. In the present study, the differences in the amount of total ash may explain the impurities associated with the market specimen, however, in the case of acid-insoluble ash, there are no significant differences.

CONCLUSION

This study highlighted the variations in the chemical composition, antimicrobial, and antioxidant properties of the essential oil extracted from the leaves of *R. officinalis*, depending on its cultivation environment. The findings suggest that the essential oils of *R. officinalis* from Hamedan province, Iran,

exhibit superior efficacy against *S. aeruginosa* and *meticillin-resistant staphylococcus aureus* bacteria. Furthermore, the antioxidant activity of the essential oil derived from both variations of the plant was similar, and not comparable to the positive control. It was also observed that certain components are exclusively present in the essential oil from either the plant from the university garden or the plant from the Hamedan market and vice versa. This could potentially contribute to the observed variations in the biological properties of the different essential oils derived from this plant. Considering the widespread use of *R. officinalis* in natural medicine, aromatherapy, perfume, and the cosmetic industry, conducting more comprehensive and extensive research on each chemical constituent and their relative abundance in the essential oil would be beneficial. This could further enhance our understanding of the plant's therapeutic potential and its applications in various industries.

Acknowledgments

We express our profound gratitude to the Vice Chancellor of Research and Technology, Hamadan University of Medical Sciences for their financial support in this article (Grant Number: 140011199764).

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