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Antioxidant and Antibacterial Properties of *Rosmarinus officinalis* Essential Oil

Duaa Y. Khalil , Omar M. Hassan*

Biology department, College of Science, University of Anbar,Ramadi, Iraq;

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ABSTRACT

The essential oil extracted from *Rosmarinus officinalis* L. is gaining attention for its therapeutic uses. The rosemary essential oil (REO) was extracted from the plant leaves using steam distillation, and the chemical composition of the essential oil was analyzed using a GC-MS apparatus. 30 compounds were identified in the essential oil, dominated by 1,8-cineole (21.24%), camphor (10.81%), and α-pinene (7.41%). The antibacterial activity was screened using the agar disc diffusion method against four bacterial strains, including *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Klebsiella pneumonia*, and the minimum inhibitory concentration (MIC) of rosemary essential oil was determined. The rosemary essential oil showed good antimicrobial activity against all microbial strains. MIC values ranged from 1.56 to 25 mg/L. Based on MIC results; *Pseudomonas aeruginosa* was inhibited more than *Staphylococcus aureus*. The essential oils were evaluated for their antioxidant activity using a ABTS radical inhibition test. Rosemary essential oil showed high antioxidant activity, with 86.92% radical inhibition. The biological activities demonstrated by *R. officinalis* essential oils suggest potential applications in various fields, including medicine, food, and cosmetics, making our findings useful for future studies and applications..

Introduction

Essential oils (EOs) are volatile mixtures of organic substances with an oily consistency. They are isolated from plants using different methods, including distillation and organic solvent extraction. Essential oils are primarily classified into two categories, based on the chemical structure of their components. One group contains mostly hydrocarbons, comprising terpenes (monoterpenes, diterpenes, and sesquiterpenes), as well as oxygenated terpenes, are usually alcohols, phenols, aldehydes, ketones, esters, and oxides [1].

Essential oils have a variety of biological and medical functions, including antibacterial, antiviral, insecticidal, and antifungal activities. Essential oils can easily penetrate the cell membranes and combine with lipid components of mitochondria due to their hydrophobic nature. This property cause disrupts the cell structure, increases permeability, and releases essential molecules and ions, ultimately leading to cell death [2].

Rosmarinus officinalis L. (Rosemary) is an evergreen shrub, native to the Mediterranean region and grown for aromatic and medicinal properties. The aerial parts of the plant are traditionally used in medicine for relieve stomach disorders, inflammation, and pain. The plant essential oil (EO) mainly contains 1,8-cineole, alpha-pinene, camphor, borneol, and camphene as main components. The essential oil has anti-inflammatory, antirheumatic, and antispasmodic properties, and can be used to relieve renal colic, and dysmenorrhea and to enhance carminative and choleretic effects [4].

Rosemary exhibits various advantageous properties, including antidiabetic, anticancer, antinociceptive, and antitumor [5]. The European Union has approved rosemary extract (E392) as a safer and more effective natural antioxidant for food preservation [6]. Aromatic and medicinal plants containing essential oils can possess antibacterial, antifungal, and antioxidant properties [7]. Many researchers have investigated the antimicrobial effects of plant extracts and essential oils

*Corresponding author at : Biology department, College of Science, University of Anbar, Iraq
ORCID:https:// https://orcid.org/0000-0002-2026-8114 ,
Tel: +964 781 084 7800
Email: sc.omerhasan@uoanbar.edu.iq

against different microbial pathogens to develop safe and effective alternatives to standard antibiotics [8]. The main components of essential oils, such as camphor, 1,8-cineole, and alpha-pinene, show inhibitory activity against the growth of bacteria and fungi [9].

The study aimed to determine the phytochemical composition, evaluate the antioxidant properties, and assess the antibacterial activity of *R. officinalis* essential oil against various pathogenic bacterial isolates.

Materials and Methods

Plant materials

R. officinalis was gathered in August 2022 from Rawa city, Anbar, Iraq. The fresh branches, 35-50 cm long, were harvested from the front of the bushes during the flowering season. After harvesting, the samples were transported to the laboratory and fresh rosemary leaves were air-dried at room temperature in the shade to extract the essential oil. The plant was identified by Prof. Dr. Mohammed Othman Musa, director of the Natural Plant Museum at the Center for Desert Studies at Anbar University.

Extraction of essential oils

The essential oil was extracted from *R. officinalis*, using steam distillation. 100 grams of dried plant leaves was used to distill the samples in water for 3 hours. To isolate the oil from the water, the water layer was drained by opening the faucet until the meniscus was slightly higher than the calibration point. Finally, the distilled oil was stored in an airtight glass vial at 4°C until ready to use in the assays.

GC/MS Analysis of Essential Oils

Rosemary essential oil was analyzed using an Agilent 7890A GC-MSD system with an HP-5 MS capillary column (30m × 0.25 mm; 0.25 µm film thickness). A flow rate of 1 mL/min of nitrogen was employed as the carrier gas. The temperature of the GC oven was adjusted to 60 °C for three minutes, then to 280 °C for five minutes at a rate of 15 °C per minute, and lastly to 300 °C. The analysis involved injecting a 1 µl sample diluted in ethanol (1:100, v/v) at a constant temperature of 250 °C for one minute using a split ratio of 1:20. The retention time and content of each component were obtained from the area percent of the GC-MS chromatogram. All analyses were run in triplicate [10].

Analysis of ABTS Radical Scavenging

The total antioxidant capacity (ABTS technique) was used to measure the antioxidant activity of rosemary essential oil in vitro using the ABTS radical scavenging test [11]. Briefly, 10 microliters of Rosemary Essential Oil were diluted with 15 milligrams per milliliter of ethanol. In a 96-well plate, 190 µL of the reaction solution was combined with tertiary butylhydroquinone (TBHQ) as a positive control. The absorbance at 734 nm was measured within 10 minutes. The inhibition rate was calculated using the following formula:

$$\text{Inhibition (\%)} = (\text{OD}_{\text{blank}} - \text{OD}_{\text{sample}})/\text{OD}_{\text{blank}} \times 100\%$$

Bacterial strains and cultures

Rosemary Essential Oil was tested for its antimicrobial properties on four different microbes. These included Gram-positive bacteria (*Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*). These bacterial strains were obtained from the microbiology laboratory at Ramadi Teaching Hospital in Iraq and were stored at −80 °C in Tryptic Soy Broth (TSB) with 15% v/v glycerol. The frozen stock cultures were activated by streaking onto a nutrient agar medium and incubating at 37°C for 24 hours. To make the bacterial inoculum, four or five colonies were chosen and mixed with 10 ml of normal saline to make a slurry with a density of 10^8 CFU ml^{-1} [12].

Evaluation of antibacterial activity

The antibacterial activity of REO was examined using the agar disc diffusion method against the studied bacterial strains [12]. 0.5 grams of rosemary essential oil were weighed and dissolved in 10 mL of 5% dimethyl sulfoxide (DMSO) to achieve a concentration of 50 mg/L of REO. Discs with a diameter of 6 mL were impregnated with REO. Negative control was prepared using DMSO (5%), while positive control was prepared using ciprofloxacin (10 µg/ml). Bacterial inoculum was

spread on Mueller-Hinton agar plates using cotton swabs, and then the discs were placed on the inoculated agar plates using sterile forceps. Using a digital vernier, the diameters of each disc's inhibitory zone were measured after a 24-hour incubation period at 37°C. Each experiment was performed in three replicates.

The minimum inhibitory concentration (MIC) values of bacterial strains were determined through the broth microdilution method [14], which was carried out in 96-well microplates using Resazurin as a viability indicator. A sterile 96-well microplate was prepared by dispensing Mueller-Hinton broth (95 µL) and bacterial inoculum (5 µL) into each well. Then, 100 µL of the highest oil dilution was added to the first well, followed by 100 µL of each subsequent dilution to the consecutive wells. As a sterility control, 200 µL of Mueller-Hinton broth supplemented with 5% DMSO was used with no bacteria added. 195 µL of Mueller Hinton broth supplemented with 5% DMSO and 5 µL of bacterial inoculums devoid of essential oil were utilized to control development. Every well's growth was contrasted with that of the control well. After sealing the plates with parafilm, they were incubated for a day at 37°C. To determine the minimum inhibitory concentration (MIC), 30 µL of resazurin (0.02% (w/v)) was introduced into each well, and then it was incubated for a further 1-2 hours. Changing resazurin from blue/purple to pink resorufin showed that bacteria were growing, and the MIC value was found to be the lowest amount of REO that inhibited bacterial growth.

Statistical analysis

IBM® SPSS® Statistics 28 software was used for statistical analysis. Each experiment was replicated three times. Means and standard deviation were calculated, followed by ANOVA and Tukey's HSD test ($P \leq 0.05$).

Results and Discussion

Chemical composition of REO

The yield of essential oils extracted by distillation from the aerial parts of *R. officinalis* was 1.8% of dry matter. The chemical compositions of hydrodistilled essential oils were analyzed by GC-MS. A total of 30 compounds were identified in Rosemary essential oils, representing 99.20% of the total essential oils. GC-MS analysis showed that monoterpenes and sesquiterpenes were the major chemical groups in *R. officinalis* essential oils. Oxygenated monoterpenes were the most abundant, accounting for approximately 52.90% of the rosemary essential oil. The main compounds in this chemical group were 1,8-cineole (21.24%), camphor (10.81%), and α-terpineol (5.14%). The second most abundant compounds are sesquiterpene hydrocarbons, which counted 20.37% of REO and consist mainly of δ-cadinene (4.75%), α-copine (3.55%) and α-Humulene (3.11%). The ratio of monoterpene hydrocarbons in REO was 16.24%, mainly consisting of α-Pinene (7.41%), β-Myrcene (2.86%), and camphene (2.51%). Oxygenated sesquiterpenes constituted 8.47% of the total oil components, with tau-Cadinol (5.96%) as its main component. In addition, the rosemary essential oil contained compounds from other chemical groups, such as 1-octen-3-ol and norcarane (Table 1).

Table 1. Chemical composition of rosemary essential oil

No.	Compounds	RT	Content%
	Monoterpene Hydrocarbons		**16.24**
1	α-Pinene	5.554	7.41
2	β-Myrcene	6.412	2.86
3	Camphene	5.845	2.51
4	β-Pinene	6.266	1.17
5	β-Ocimene	9.664	0.77
6	p-Cymene	7.905	0.51
7	Tricyclene	5.365	0.35
8	3-Carene	6.721	0.35
9	2-Carene	7.819	0.31
	Oxygenated Monoterpenes		**52.90**
10	Eucalyptol (1,8-Cineole)	7.038	21.24
11	Camphor	8.609	10.81
12	α-Terpineol	9.287	5.14
13	Borneol	9.003	3.87
14	Bornyl acetate	10.059	3.31
15	Carvacrol	10.771	3.19
16	methoxybenzenethiol	10.308	2.67
17	Linalool	8.111	2.18
18	fenchol	8.420	0.49
	Sesquiterpene		**20.37**
19	δ-Cadinene	12.513	4.75
20	α-Copaene	11.003	3.55
21	α-Humulene	11.870	3.11
22	β-Bisabolene	12.281	2.94
23	γ-Muurolene	12.033	2.13
24	Caryophyllene	11.492	1.65
25	Isocaryophyllene	15.620	1.32
26	Alloaromadendrene	11.363	0.92

	Oxygenated sesquiterpene	**8.47**	
27	**tau-Cadinol**	**14.341**	**5.96**
28	**Caryophyllene oxide**	**13.439**	**2.51**
	Other	**1.22**	
29	**1-Octen-3-ol**	**6.944**	**0.61**
30	**Norcarane**	**9.836**	**0.61**
	Total compounds %	**99.20**	

RT, retention times.

Previous research indicates that rosemary essential oils include varying concentrations of borneol and verbenone in addition to α-pinene, 1,8-cineole, and camphor [15]. Our results agree with those of recent investigations [1, 9, 16]. More than forty percent of Tunisian, Turkish, Moroccan, and Italian oils are made up mostly of 1,8-cineole. Meanwhile, the percentages of 1,8-cineole, α-pinene, and camphor (20–30%) in French, Spanish, and Greek oils are roughly identical [15].
According to Wang et al. [17], 1,8-cineole (27.23%), α-pinene (19.43%), camphor (14.26%), camphene (11.52%), and β-pinene (6.71%) are the primary components of the oil. High concentrations of 1,8-cineole (44.42%), α-pinene (12.57%), and borneol (8.52%) have been found in rosemary essential oil [18]. Ecological and meteorological factors, as well as management techniques like harvesting timing, affect the chemical makeup and yield of essential oils [19].

Antioxidant properties of REO

The ABTS radical-scavenging activity (%) of REO is shown in Figure 1. TBHQ was used as a reference standard compound because of its antioxidant properties. The results showed a high ability of rosemary essential oil to scavenge free radicals with an inhibition rate of 86.92 ± 2.85%, which was close to the ability of TBHQ to scavenge free radicals (89.82 ± 3.06%) and without a significant difference.

The chemical structure of these constituents may be responsible for the antioxidant action seen in REO. According to reports, the presence of hydroxyl group substitution in the phenolic aromatic rings is associated to the antioxidant activity of REO. This enhances their capacity to provide hydrogen. The antioxidant activity is also strongly connected with REO main components such as 1,8‑cineole, or a‑pinene according to [20]. However, as some investigations have shown, the minor molecules can interact directly to produce a variety of biological functions [21]. It's worth noting that essential oil from *R. officinalis* can prevent lipid peroxidation, which is a harmful process that occurs due to oxidative stress [22]. Additionally, rosemary has been found to boost the body's antioxidant enzyme activity while reducing the number of reactive species present in the body [23]. All of these effects help to improve the body's defenses against oxidative damage caused by harmful reactive species [24].

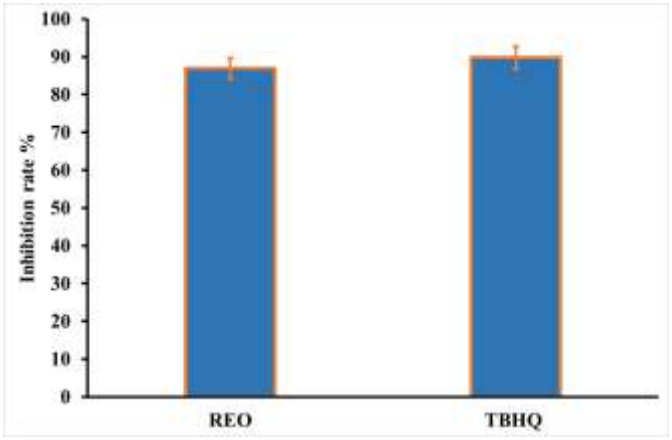

Figure 1. Antioxidant activity for rosemary essential oil and TBHQ

Antibacterial activity of REO

In this work, the antibacterial properties of locally sourced rosemary essential oil were examined against four strains of bacteria, namely *S. aureus*, *E. coli*, *P. aeruginosa*, and *K. pneumoniae*. The disc diffusion method was used to test the effectiveness of the oil. The results, shown in Table 2, indicate that the essential oil had the highest level of inhibition against *P. aeruginosa*, with a maximum inhibition zone of 22mm and an average diameter of 20.67mm. The oil also showed moderate to high activity against *E. coli* (with an inhibition zone of 19.33mm) and *K. pneumoniae* (with an inhibition zone of 18.67mm). Although the zone of inhibition of REO was lower than that of the positive controls used (ciprofloxacin 10 µg/disc), the results suggest that REO could be utilized as a natural anti-bacterial component. However, this oil displayed low to moderate effectiveness against S. aureus(15 mm). Based on the relevant research in this area, it can be inferred that REO typically has moderate efficacy against the tested group of microorganisms, which is consistent with

the outcomes of several previous studies [25, 26, 27]. The essential oil's chemical composition is primarily composed of monoterpenoids that exhibit potent antimicrobial properties[28]. REO's biological activities are mainly attributed to its main compounds, as per Bakali et al[29]. The top two major compounds, 1,8-cineole, and camphor, are known for their antimicrobial effects. Additionally, α-pinene has a strong potential for antimicrobial activity due to the presence of an oxygen function, which enhances the antimicrobial properties of terpenoids[30]. However, additional research highlights the significance of EOs' secondary components and the complementary role terpenoids and phenolic chemicals play in disrupting cell membranes, blocking cell respiration, and impairing the ion transport mechanism [26]. As a result, the increased antibacterial activity of REO may be due to the synergistic interaction between its numerous components [31].

Figure 2. Inhibition zones of REO against the studied bacteria

Table 2. Antimicrobial activity of rosemary essential oil expressed as inhibition zone diameters (mean ± standard deviation)

Tested bacteria	Diameter of the inhibition zone (mm)		
	REO	Ciprofloxacin	DMSO
P. aeruginosa	**20.67±0.57^{a}**	**36.33±1.15^{c}**	**nd***
E. coli	**19.33±1.15^{a}**	**35.00±1.00^{c}**	**nd**
K. pneumoniae	**18.67±1.52^{a}**	**25.33±0.57^{d}**	**nd**
S. aureus	**15.00±1.00^{b}**	**29.66±1.52^{e}**	**nd**

Significant differences in means are indicated by different lowercase superscripts based on Tukey's HSD test ($p \leq 0.05$). *nd: not detected.

The minimum inhibitory concentrations (MICs) of rosemary essential oil (REO) against the microorganisms under study are shown in Table 3. According to MICs which range from 1.56 to 25 µg/mL, the broth microdilution assay results show that REO is more efficient against gram-negative bacteria. When comparing the minimum inhibitory concentrations (MICs) of rosemary essential oil with those of ciprofloxacin, a potent antibacterial antibiotic. The tested essential oil's MICs were lower than the control, as was to be predicted. The test plate also had a negative control, which held the DMSO solution that was utilized for the experiment.

In comparison to S. aureus, the data indicate that REO had reduced MIC values against *P. aeruginosa*, *E. coli*, and *K. pneumoniae*. This could be because these two kinds of bacteria have different cell walls [32]. Previous investigations with similar circumstances have revealed a wide range of MIC values, extending from 1 to 300 mg/mL against various bacteria [33, 34, 35]. In conclusion, variations in MIC values may be the result of modifications to the chemical makeup of rosemary essential oil or variations in the methodology employed. Overall, rosemary essential oil has encouraging antibacterial qualities as a natural material.

Table 3. MICs of rosemary essential oil by broth microdilution method

Group	Tested bacteria	MIC (µg/mL)
G^{-ve} bacteria	***P. aeruginosa***	**1.56**
	E. coli	**3.12**
	K. pneumoniae	**6.25**
G^{+ve} bacteria	***S. aureus***	**25.0**

Conclusions

In the present study, the antibacterial and antioxidant activities of the essential oils of wild *Rosmarinus officinalis* from Rawa City, Iraq, were detected. The results indicated that rosemary essential oils have high antioxidant properties and moderate antibacterial activity. Rosemary essential oil is effective in inhibiting bacterial strains, especially *Pseudomonas*

aeruginosa, which is known to be multidrug resistant. Therefore, natural materials derived from rosemary can be appropriately used for applications in the food, cosmetic, and pharmaceutical industries due to their antibacterial and antioxidant properties.

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Conflict of Interest

The authors declare that there are no conflicts of interest, either financially or by personal association, with respect to the publication of this paper

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الخصائص المضادة للأكسدة والمضادة للبكتيريا للزيت العطري لنبات *Rosmarinus officinalis*

دعاء ياسين خليل، عمر محمد حسن*

قسم علوم الحياة، كلية العلوم، جامعة الأنبار، الرمادي، العراق
email: sc.omerhasan@uoanbar.edu.iq

الخلاصة:

يحظى الزيت العطري المستخرج من نبات *Rosmarinus officinalis* L. بالاهتمام لاستخداماته العلاجية. تم استخلاص الزيت العطري لإكليل الجبل من الأجزاء الهوائية للنبات باستخدام التقطير بالبخار، وتم تحليل التركيب الكيميائي للزيت العطري باستخدام جهاز GC-MS. تم تحديد 30 مركبًا في الزيت العطري، يهيمن عليها 1,8–سينيول (21.24%)، والكافور (10.81%)، وألفا–بينين (7.41%). تم فحص النشاط المضاد للبكتيريا باستخدام طريقة انتشار قرص أجار ضد أربع سلالات بكتيرية، بما في ذلك *Escherichia coli* و*Staphylococcus aureus* و *Pseudomonas aeruginosa* و*Klebsiella pneumoniae*. وتم تحديد التركيز المثبط الأدنى (MIC) لزيت إكليل الجبل العطري. أظهر زيت إكليل الجبل الأساسي نشاطًا جيدًا مضادًا للميكروبات ضد جميع السلالات الميكروبية. تراوحت قيم MIC من 1.56 إلى 25 مجم / لتر. استنادا إلى نتائج MIC، كان التأثير المثبط ضد *Pseudomonas aeruginosa* أعلى من تأثير المكورات العنقودية الذهبية. تم تقييم الزيوت الأساسية لنشاطها المضاد للأكسدة باستخدام اختبار تثبيط جذري ABTS. أظهر زيت إكليل الجبل العطري نشاطًا عاليًا مضادًا للأكسدة، مع تثبيط جذري بنسبة 86.92%. تشير الأنشطة البيولوجية التي أظهرتها الزيوت العطرية *R. officinalis* إلى تطبيقات محتملة في مختلف المجالات، بما في ذلك الطب والغذاء ومستحضرات التجميل، مما يجعل النتائج التي توصلنا إليها مفيدة للدراسات والتطبيقات المستقبلية.