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Chemical profile and biological properties of the essential oil of Rosemary leaves (*Rosmarinus officinalis* L.)

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ABSTRACT

Rosemary leaf essential oil (RoEO) is extracted using steam distillation at 95 -100 °C for 60 min. The aim of study is to determine physicochemical characteristics of essential oil (EO), such as acid/saponification/esterification index, relative/absolute density, freezing point, and fragrance retention. The chemical composition of EOs was analyzed by gas chromatography-mass spectrometry (GC-MS) method and identified 50 volatile compounds, of which α-pinene (33.76%), 1,8-cineole (18.47%), and levoverbenone (6.11%) constituted the highest proportions in EO. The antioxidant capacity (AC) of the EO was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method with a half-maximum inhibitory concentration (IC_{50}) of 425.473 mg/mL. In particular, antibacterial activity (AA) by the paper plate diffusion method for susceptibility testing to essential oil showed that RoEO strongly inhibited the growth of four tested bacterial strains (*Staphylococcus aureus*, *Bacillus cereus*, *Salmonella typhimurium*, and *Escherichia coli*). Perhaps, it is possible to apply RoEO in the food industry and other fields owing to the good properties of RoEO.

Keywords: Antibacterial activity, antioxidant activity, composition, essential oil, GC-MS

INTRODUCTION

Rosemary (*Rosmarinus officinalis* L.) is a genus of the Lamiaceae family; it is a flowering plant with a very fragrant and pleasant smell. This plant is native to the Mediterranean, but is widely cultivated worldwide [1, 2]. Rosemary has only been grown in Vietnam in recent years, and the capital of rosemary thrives in the tropics. At the same time, Bac Giang province is located in the tropical monsoon climate region of Northeast Vietnam. The annual average temperature is approximately 23°C, with the relative humidity ranging from 74% to 87%. The amount of surface water, rainfall, and groundwater is enough to supply agriculture [3]. Bac Giang province has a mild climate compared to other regions in Vietnam and it is entirely favorable for cultivating and developing various crops, especially rosemary.

The major compounds common in rosemary leaf essential oil (RoEO) are terpenes which are divided into monoterpenes, sesquiterpenes, diterpenes, and triterpenes, depending on the number of carbon atoms. The terpenes in rosemary can be found as carnosol, epirosmanol, ursolic acid, carnosic acid, and oleanolic acid (triterpenes) [4]. The essential oil (EO) gained from the leaves had a major component of 1,8-cineole (35.8%), while caryophyllene (16.7%) was predominant in the EO extracted from the stem. For EO extracted from flowers, the main compound is caryophyllene oxide (11.9%) [5]. Of note, rosemary leaves are most commonly used for essential oil extraction [6]. Rosemary has the highest antioxidant activity among the natural antioxidants from plants [7]. According to pharmacological studies performed by extracting EO from rosemary, EO has some special biological effects, such as anti-diabetic, anti-infective, and anti-inflammatory [4]. According to research by Andrade *et al*., the yield of RoEO using steam distillation is up to 2.5%; the obtained RoEO is a pale yellow to colorless liquid, insoluble in water, and has a characteristic camphor aroma. The main components of RoEO are 1,8-cineole (15-55%), α-pinene (9.0-26%), camphor (5.0-21%), camphene (2.5-12%), and β-pinene. (2.0-9.0%); this ratio varies depending on the growth stage and local climatic conditions [4].

In addition, the composition of EO may vary according to the extraction method; the quality or quantity of the chemical composition of EO may vary according to the parts of the plant used for extraction, soil conditions, changes in climate, and the growth cycle of raw materials [8].

Many studies related to *Rosmarinus officinalis* L. already exist; however, to date, there are no studies on the chemical composition, physicochemical properties, antioxidant activity, and antibacterial potential of the RoEO grown in Bac Giang province. Therefore, the primary aim of this study was to evaluate all of the above properties of RoEO from Vietnam because it may possess some unique properties when compared to RoEO from various regions of the world.

EXPERIMENTAL

Plant material: Rosemary leaves were harvested in Bac Giang province (Vietnam) at maturity about 6-8 months after planting. RoEO was isolated using the steam distillation method (250 kg of fresh leaves/batch) at 95-100 °C for 60 min. The yield of EO was about 0.82% (v/w), and the RoEO was kept in a dark bottle at 4 °C until analysis.

Bacterial strains: The foodborne bacteria strains were selected for this study, including *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 11778), *Salmonella typhimurium* (ATCC 14028), and *Escherichia coli* (ATCC 25922).

Evaluation of the physical properties of RoEO: The freezing point (FP), relative density (RD), absolute density (AD), acid index (AI), ester index (EI), and saponification index (SI) of RoEO were evaluated according to ISO 1041; 279; 1242; and 7660, respectively [9-12].

Evaluation of fragrance retention (FR) of the RoEO: Fragrance retention (FR) was determined by EO concentration and scent duration, according to Mahajan with some small adjustments [13]. The EOs were prepared at concentrations at 10, 20, 30, 40, and 50% (v/v) in ethanol 96%. Then, samples were prepared by adding 3 drops to the odor test paper, allowing a few seconds to penetrate the paper. The time it took to lose the EO scent entirely under normal conditions was recorded.

Gas chromatography-mass spectrometry (GC-MS) analysis: The constituents of RoEO were investigated by the GC-MS. 1 µL of EO was injected into a gas chromatography machine (Shimadzu Nexis GC-2030, Japan) with a versatile capillary column (Rtx-5sil-MS, 30 m × 0.25 mm × 0.25 µm, Restek Technologies, USA) equipped with a quadrupole mass analyzer (Shimadzu GC-MS-QP2020 NX, Japan). Helium was used as a carrier gas at a constant flow rate of 3.0 mL/min, and a split ratio of 10:1. The injection temperature was 250 °C and the temperature program was set as follows: initial temperature of 50 °C, held for 2 min, increased until 250 °C at a rate of 10 °C/min, and held for 5 min, increased until 280 °C at a rate of 10 °C/min, and held for 3 min. The chemical compositions of the RoEO were identified based on the comparison between their mass spectra with NIST 2017 library.

Evaluation of the antioxidant capacity (AC) of the RoEO: The determination of the antioxidant capacity (AC) of EO using 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging of free radicals ($DPPH_{RSC}$) assay was performed as described by Gounder and Lingamallu with some minor corrections [14]. RoEO was dissolved in ethanol (96%, v/v) to achieve different concentrations (100, 200, 300, 400, 500, and 600 mg/mL). Then, 0.3 mL of RoEO was mixed with 3.7 mL of 0.1 mM DPPH solution in ethanol. This solution was kept in the dark for 30 min at 26-28 °C and the absorbance of mixture was recorded at 517 nm. Ascorbic acid was used as a standard to compare with the AC of the RoEO. Percent inhibition plotted against EO concentration to reckon concentration gives 50% inhibition (IC_{50}). The AC was calculated as the following formula:

$$DPPH_{RSC}(\%) = \frac{A_0 - A_s}{A_0} \times 100\% \quad (1)$$

where A_0 is the absorbance of control and A_s is the absorbance of RoEO samples in the presence of DPPH solution at 517 nm.

Evaluation of the antibacterial activity (AA) of RoEO: The antibacterial activity (AA) of RoEO was evaluate based on the paper disc diffusion assay with some minor modifications [15]. 100 µL of bacterial suspension (0.5 McFarland standard, approximately 1.5×10^8 CFU/mL) was inoculated into Mueller Hinton Agar medium. Then, EO impregnated the sterile paper discs placed on the agar surface (5 µL EO/disc). In this study, ampicillin (10 µg/disc) and dimethyl sulfoxide (DMSO) solution (5%, v/v) were considered as positive and negative antibacterial control. The petri dishes were incubated for 24 h at 37°C and the AA was evaluated for the bacterial inhibitory zone by measuring the diameter of the antibacterial ring, including the disc diameter of 6 mm.

Data analysis: All the experimental results were analyzed using Statgraphics Centurion software (Version 15.1.02). Every assay was done in triplicate. Analysis of variance with Fisher's least significant difference procedure was used to determine the significant differences ($p < 0.05$) between means.

RESULTS AND DISCUSSIONS

Physicochemical properties of RoEO: RoEO is a light yellow, very clear liquid with a characteristic aroma and a slightly sharp taste. The pH value of EO is about 4.713 (Table 1), similar to many EOs extracted from different plants; typically, *Ageratum conyzoides* L. leaf EO (pH=4.46) and *Ceratonia siliqua* seed EO (pH=5.2) [15, 16]. This finding shows that the pH value is influenced by the chemical ingredients of materials.

Table 1 shows that when the temperature is lowered to -38 °C, RoEO still does reach the freezing point (FP).

Table 1. Physicochemical properties of the RoEO.

Physicochemical properties	Value
Freezing point (FP, °C)	<-38°C
pH	4.713 ± 0.097
Relative density (RD)	0.896 ± 0.008
Absolute density (AD, g/mL)	0.895 ± 0.008
Acid index (AI, mg KOH/g)	1.852 ± 0.029
Saponification index (SI, mg KOH/g)	14.259 ± 0.890
Ester index (EI, mg KOH/g)	12.407 ± 0.840
Fragrance retention (FR, h):	
10% EO (v/v)	3.9 ± 0.140
20% EO (v/v)	6.5 ± 0.000
30% EO (v/v)	9.08 ± 0.140
40% EO (v/v)	12.33 ± 0.280
50% EO (v/v)	16.5 ± 0.000

So, its FP is very low compared to other EOs, such as *A. conyzoides* leaf EO (-10.33℃) and *Eucalyptus camaldulensis* EO (0-1℃) [15, 17]. However, up to now, studies have not researched the FD of RoEO, so this result cannot be compared with those of different studies. The RD and AD are a characteristic and important index for each type of EO. In this study, RD (0.896) and AD (0.895 g/mL) of RoEO were similar to those in previous studies; for example, RoEO from Lam Dong province - Vietnam (AD: 0.8978 g/mL) [18] and Campestre da Serra province – Brazil (AD: 0.8887 g/mL) [19].

The AI, SI, and EI are three important indices used to assess the quality of an EO, and their values in RoEO were 1.852, 14.259, and 12.407 mg KOH/g, respectively. The AI low value indicates that the essential oil is of good quality, with little change or oxidation over time. The AI in this study is slightly higher than that of RoEO originating from Lam Dong province (AI: 1.122 mg KOH/g), whereas the EI is smaller than that of RoEO originating from Lam Dong province (EI: 15.708 mg KOH/g) [18]. Thereby, it can be seen that these indices will depend on many factors, such as chemical composition, harvest time, age of material, distillation method, etc.

The FR of RoEO is quite long, reaching 16.5 h at 50% concentration but only 4 h at 10% concentration. The results demonstrate that RoEO offers high potential for applications in the perfume and food technology.

Chemical profile of RoEO: Table 2 shows the volatile compounds of RoEO. Many chemical compounds were detected in RoEO using GC-MS analysis, including 50 different components, with retention times (RTs) ranging from 5.255 to 15.62 min; these components account for 100% of the EO.

The main ingredients in RoEO are α-Pinene (33.76%), 1,8-Cineole (18.47%), and Levoverbenone (6.11%). The results were different from rosemary in different regions, for example, for RoEO in Lam Dong province, Vietnam, 23 compounds were found, including α-Pinene (35.54%), Eucalyptol (20.902%), Bicyclo[3.1.1]hept-3-en-2-one (7.794%) [18], while RoEO from Campestre da Serra province, Brazil, possessed 20 volatile compounds, such as α-pinene (40.55-45.10%), 1,8-cineole (17.40-19.35%), camphene (4.73-6.06%), and verbenone (2.32-3.86%) [19]. This difference in chemical components may be explained due to various climatic conditions, soils, extraction methods, as well as experimental conditions, etc.

Generally, α-pinene is dominant in RoEOs from various regions, and α-pinene has wide potential due to anticoagulant, antitumor, antimicrobial, antimalarial, antioxidant, anti-inflammatory, and analgesic effects [20, 21].

Table 2. Chemical profile of the RoEO.

No	RT (min)	Compounds	Molecular Formula	Content (%)
1	5.255	4-Carene	$C_{10}H_{16}$	0.18
2	5.307	α-Thujene	$C_{10}H_{16}$	0.25
3	5.450	α-Pinene	$C_{10}H_{16}$	**33.76**
4	5.593	Hydroperoxide, 1-ethylbutyl	$C_{6}H_{14}O_{2}$	0.35
5	5.751	Camphene	$C_{10}H_{16}$	3.57
6	5.861	2,4(10)-Thujadiene	$C_{10}H_{14}$	1.11
7	5.931	Octadecanoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester	$C_{28}H_{46}O_{4}$	0.46
8	6.254	(-)- β-Pinene	$C_{10}H_{16}$	2.00
9	6.431	β-Myrcene	$C_{10}H_{16}$	1.44
10	6.465	(-)-Carvyl acetate	$C_{12}H_{18}O_{2}$	0.12
11	6.754	1-Phellandrene	$C_{10}H_{16}$	0.40
12	6.947	α-Terpinolene	$C_{10}H_{16}$	0.55
13	7.076	Cymene	$C_{10}H_{14}$	1.19
14	7.170	L-Limonene	$C_{10}H_{16}$	3.27
15	7.225	1,8-Cineole	$C_{10}H_{18}O$	**18.47**
16	7.440	β-trans-Ocimene	$C_{10}H_{16}$	0.13
17	7.677	γ-Terpinene	$C_{10}H_{16}$	1.28
18	8.161	Terpinolene	$C_{10}H_{16}$	0.95
19	8.228	2-Methyl-1-phenylpropene	$C_{10}H_{12}$	0.11
20	8.362	Linalool	$C_{10}H_{18}O$	2.29
21	8.410	Filifolone	$C_{10}H_{14}O$	0.20
22	8.770	Chrysanthenone	$C_{10}H_{14}O$	0.29
23	8.853	2,3,3-Trimethyl-3-cyclopentene acetaldehyde	$C_{10}H_{16}O$	0.14
24	9.124	trans-Verbenol	$C_{10}H_{16}O$	0.14
25	9.180	Umbellulol	$C_{10}H_{16}O$	0.18
26	9.222	(+)-2-Bornanone	$C_{10}H_{16}O$	2.23
27	9.442	trans-3-Pinanone	$C_{10}H_{16}O$	0.14
28	9.475	Pinocarvone	$C_{10}H_{14}O$	0.12
29	9.586	α-Terpineol	$C_{10}H_{18}O$	0.40
30	9.631	endo-Borneol	$C_{10}H_{18}O$	3.05
31	9.702	3-Pinanone, cis	$C_{10}H_{16}O$	0.73
32	9.763	Terpinene-4-ol	$C_{10}H_{18}O$	1.20
33	9.825	8-Hydroxy-p-cymene	$C_{10}H_{14}O$	0.26
34	9.983	Terpineol	$C_{10}H_{18}O$	2.21
35	10.099	Nopol	$C_{11}H_{18}O$	0.59
36	10.195	Levoverbenone	$C_{10}H_{14}O$	**6.11**
37	10.360	trans-Carveol	$C_{10}H_{16}O$	0.12
38	10.426	β-Citronellol	$C_{10}H_{20}O$	0.22
39	10.632	β-Citral	$C_{10}H_{16}O$	0.23
40	10.728	(1S,3S,4S,5R)-1-Isopropyl-4-methylbicyclo[3.1.0]hexan-3-ol	$C_{10}H_{18}O$	0.21
41	10.801	cis-Geraniol	$C_{10}H_{18}O$	2.96
42	11.075	Z-Citral	$C_{10}H_{16}O$	0.22
43	11.389	α-Fenchyl acetate	$C_{12}H_{20}O_{2}$	2.35
44	12.540	β-Terpinyl acetate	$C_{12}H_{20}O_{2}$	0.26
45	12.677	Neryl acetate	$C_{12}H_{20}O_{2}$	0.37
46	12.002	Methyl Eugenol	$C_{11}H_{14}O_{2}$	0.27
47	12.445	trans-Caryophyllene	$C_{15}H_{24}$	2.10
48	13.670	Geranylacetone	$C_{13}H_{22}O$	0.23
49	13.937	α-Humulene	$C_{15}H_{24}$	0.43
50	15.620	(-)-Caryophyllene oxide	$C_{15}H_{24}O$	0.16
	Total			100%

In fact, most of the components in RoEO possess biological properties. Some chemical compounds account for a small proportion of RoEO, but they also have some specific characteristics. For example, Myrcene (1.44%) was used as a aroma agent in the food industry, as well as cosmetic products [22], while Limonene exhibited anti-inflammatory, antioxidant, antinociceptive, anticancer, antidiabetic, antihyperalgesic, and antiviral effects in health [23]. Therefore, these ingredients contribute to the RoEO potential in medicine and the food and cosmetic industries.

Antioxidant capacity (AC) of RoEO: Fig. 2 displays that the antioxidant capacity (AC) depends on EO concentrations. RoEO decolorises the DPPH solution, and the AC increases with increasing concentration of EO. The IC_{50} value of RoEO was 425.473 (mg/mL), much higher than the control sample (ascorbic acid) with IC_{50} = 27.73 (μg/mL) (Fig. 1). This finding also proves that the AC of RoEO is very weak. Compared to RoEOs from different places, the IC_{50} in this study was much higher than that of RoEO in Lam Dong province, Vietnam (IC_{50} = 75.7 μg/mL) [24] and in Belgrade, Serbia (IC_{50} = 77.6 μL/mL) [6]. In addition, the AC of RoEO is also lower than that of some EOs from other materials, such as *A. conyzoides* leaves (IC_{50} = 8 mg/mL) [15], *C. griffithii* (IC_{50} = 82.4 μg/mL) and *C. macrocarpum* leaves (IC_{50} = 99.3 μg/mL) [25]. From this, it can be seen that the difference in AC can be caused by different chemical composition. Although the low AC of RoEO is a disadvantage, RoEO can apply and study more in typical cases.

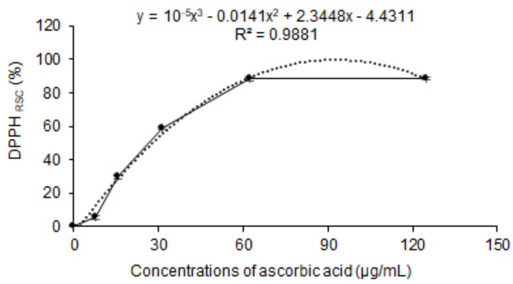

Fig. 1. Antioxidant capacity of ascorbic acid.

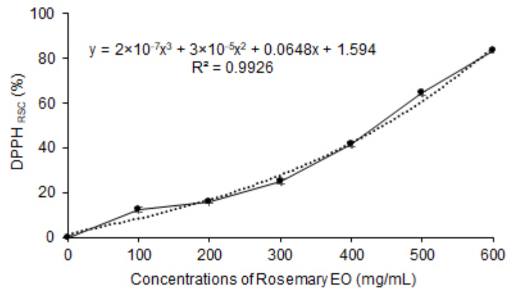

Fig. 2. Antioxidant capacity of RoEO.

Antibacterial activity (AA) of RoEO: Table 3 shows the antibacterial activity (AA) of RoEO against four strains of foodborne bacteria. The AA of RoEO were arranged in the following order: *B. cereus* and *S. typhimurium* < *S. aureus* < *E. coli*, while the AA of the positive control (Ampicillin) was listed in order: *B. cereus* and *S. aureus* < *E. coli* and *S. typhimurium*. With the obtained antibacterial diameter of RoEO, it can be concluded that the AA of RoEO is stronger than that of Ampicillin for three tested bacteria, excluding *S. typhimurium*. These results prove that RoEO is a very promising material that can be applied in food preservation to inhibit harmful bacteria.

Table 3. Antibacterial diameter of RoEO.

Tested bacteria	**Antibacterial diameter of RoEO (mm)**	**Antibacterial diameter of Ampicilin (mm)**
B. cereus	$12.667^{Ba} \pm 0.577$	$8.000^{Aa} \pm 0.000$
S. aureus	$17.333^{Bb} \pm 0.577$	$9.333^{Ab} \pm 0.577$
E. coli	$26.000^{Bc} \pm 1.000$	$24.667^{Ac} \pm 0.577$
S. typhimurium	$13.333^{Aa} \pm 0.577$	$24.333^{Bc} \pm 0.577$

Within a row (A-B) or a column (a-c), various letters indicate significant differences ($p < 0.05$)

The AA of the RoEO can be explained in different ways. According to Al-Harrasi *et al*., the AA of RoEO can follow the pathway of cell membrane disruption or cell wall degradation can change osmotic pressure, decrease intracellular ATP synthesis, etc. [26]. In addition, Ojeda-Sana *et al*., showed that RoEO contained α-pinene, 1,8-cineole, myrcene, etc.; these are bioactive compounds that can inhibit both Gram-positive and Gram-negative harmful bacteria [27]. Note that, the AA of RoEO is not only induced by a specific main ingredient but is also due to the interacting effects of many bioactive compounds.

CONCLUSIONS

The RoEO collected in Bac Giang province showed that its AC was insignificant compared to other EOs. However, AA against four bacterial strains *B. cereus*, *S. typhimurium*, *S. aureus*, and *E. coli* is more potent than other EOs. By GC-MS analysis, 50 volatile components of EO were identified prominently as α-Pinene (33.76%), 1,8-Cineole (18.47%), etc. In addition, the physicochemical properties were also determined, noting especially the excellent ability to retain the flavour of EO. This is an inexpensive natural preservative and can be used in foods.

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