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Seasonal Variations of Chemical Composition, Antibacterial and Antioxidant Activity of *Rosmarinus officinalis* L. Essential Oil from Southwestern Romania

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Abstract: Our study reports for the first time, over a 12-month period, the seasonal variations of chemical composition, antibacterial and antioxidant activity of *Rosmarinus officinalis* L. essential oil (RoEO) from southwestern Romania (Oltenia Region). To analyze the constituents of RoEO, a comprehensive gas chromatography/mass spectrometry (GC/MS) method was employed. The analysis aimed to identify and quantify the various components by comparing their mass spectra with reference spectra from the National Institute of Standards and Technology (NIST) Library 2020. *Staphylococcus aureus* minimum inhibitory concentration (MIC) values were determined using the microdilution method (96-well plates). The antioxidant activity was analyzed using 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and hydrogen peroxide (H_2O_2) radical scavenging assays. This analysis provided a detailed profile of the RoEO's constituents, revealing significant monthly variations. Key compounds, such as camphor, eucalyptol, α-pinene, camphene and α-myrcene, were quantified, alongside lesser-studied constituents like β-pinene, α-terpinene, linalool, terpinolene, and carvacrol. Comparisons were made with a reference sample from Tunisia. Correlations between specific compounds and their bioactivity were explored to understand their contributions to the overall efficacy of the RoEO. This comprehensive analysis provides valuable insights into the potential applications and seasonal variability of RoEO from Romania.

Keywords: *Rosmarinus officinalis* L.; Romania flora; essential oil; antibacterial activity; antioxidant activity; seasonal variations

1. Introduction

Rosmarinus officinalis L. is a sempervirent subshrub, with a height ranging from 60 to 150 cm, although under natural conditions it can reach up to 250 cm [1–3]. The leaves are sessile, coriaceous, acicular, and persistent, with revolute margins. The superior surface of the leaves is glabrous, while the inferior surface is tomentose, possessing branched protective hairs and glandular hairs. The flowers are pale blue, less commonly white or pink, and are arranged in lax, spicate inflorescences.

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The corolla is bilabiate and pubescent on the exterior, lacking a hairy ring on the interior, with a tube slightly longer than the calyx. The posterior stamens may be absent or represented by two rudimentary structures. At the base of the ovary, there is a nectary disc [2,3]. The plants typically flower in April–May and June–August, respectively. Rosemary grows spontaneously on the sunny slopes of the Mediterranean coast and is also cultivated in various regions, including the countries of the former Yugoslavia, along the Black Sea coast, in the USA, and Mexico. This species requires protection from cold and wind in temperate climate zones [1–3]. Although it does not have special humidity requirements due to the hairs on the dorsal surface of the leaves, which allow it to tolerate drought periods relatively well, it needs light, permeable, calcareous soil that warms easily and preferably has a southwest exposure [4].

In the Mediterranean basin, rosemary is extensively cultivated for culinary purposes [5]. Alongside lavender, it is also significant for its role as an insecticide and insect repellent, useful in deterring moths [2,3].

The name *Rosmarinus* originates from the Latin words *ros* (meaning "dew") and *marinus* (meaning "sea"), thus the name translates to "dew of the sea". The term *officinalis* refers to its medicinal use known since antiquity. Rosemary is mentioned in the medical practices of Ancient Egypt and in the writings of Hippocrates, Galen, and Dioscorides. It was also introduced to India by the English [6].

For *R. officinalis* L. species, the synonymous names *Salvia rosmarinus* Schleid. and *R. angustifolius* Mill. are also mentioned [7].

The systematic classification of this species includes the family *Lamiaceae*, order *Lamiales*, subclass *Asteridae*, and class *Magnoliopsida* [8]. Various cultivars and forms are mentioned based on leaf shape and flower color. In Romania, four forms are noted: *angustifolius*, *latifolius*, *albiflorus*, and *variegatus* [2,3]. For the species present in the flora of Tunisia, four varieties have been reported: var. *typicus* Batt., var. *laxiflorus* De Noé, var. *troglodytorum* Maire, and var. *lavandulaceum* Batt. [8].

The chemical composition of rosemary leaves includes essential oil (EO), flavonoids, phenolic acids (rosmarinic acid, caffeic acid, chlorogenic acid), tannin, diterpenes, pentacyclic triterpenes [9–12].

The EO from the glandular hairs on the dorsal side of the rosemary leaves exhibits variability depending on the region where the plant grows, the soil, climatic conditions, harvest period, and certain genetic characteristics. Four main chemotypes have been established based on a predominant component in the rosemary EO (RoEO), and they are named after the dominant constituent. The four chemotypes include the α-pinene chemotype, the 1,8-cineole chemotype, the camphor chemotype, and the myrcene chemotype. These chemotypes are characteristic of specific geographical regions: the α-pinene chemotype is found in France, Spain, Italy, Romania, and Iran; the 1,8-cineole chemotype is present in plants from Austria, Algeria, and Morocco; the camphor chemotype has been identified in India and Cuba; and the myrcene chemotype is noted for Portugal and Argentina. Despite the establishment of these four chemotypes, RoEO has numerous common components regardless of the region where the plants grew. These components include: α- and β-pinene, borneol, camphor, camphene, linalool, β-caryophyllene, β-myrcene, bornyl acetate, sabinene, verbenone, and limonene [6,7,12–17].

RoEO is used in traditional medicine to enhance physical and mental states and to treat headaches, stomach pain, rheumatic pain, epilepsy, dysmenorrhea, spasms, depression, hysteria, and nervous agitation. It is also utilized to improve memory capacity [7,13,17–22].

Numerous studies have investigated the pharmacological actions of RoEO and extracts obtained from rosemary leaves, reporting various properties, including antioxidant [5,12,15,23–25], anti-inflammatory [7,26,27], antihypertensive (rosmarinic acid) [10,17] or antihypotensive (RoEO) [28,29], antihypercholesterolemic [17,30], antihyperglycemic [17,31,32], antiglycative (mitigation of age-related pathology effects) [33,34], antibacterial [13,15,35,36], antifungal [15,36,37], antiviral [17,38], neuroprotective [11,17,18,25], antidepressant [39,40], hepatoprotective [30,41], nephroprotective [42,43], antitumor, antiproliferative [9,12,17,23,24], antiangiogenic [11,44], antiallergic (in cutaneous

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allergies) [45,46], cutaneous texture restoration (with applications in dermatocosmetics) [5,47,48], radioprotective–antimutagenic [17,49,50].

The aim of the study was to report for the first time, over a 12-month period, the seasonal variations of chemical composition, antibacterial and antioxidant activity of *R. officinalis* EO from southwestern Romania (Oltenia Region).

2. Materials and Methods

2.1. Plant Material and Essential Oil Extraction

The plant material (leaves) of *R. officinalis* cultivated species were collected over a 12-month period (February 2022 to January 2023) from southwest Romania flora (Cârcea Village, Dolj County, Oltenia Region). The study site is situated at an elevation of 181.05 m above the mean sea level and is positioned between the following geographical coordinates: 44.26°N latitude, 23.90°E longitude. The plant remained in the flowering stage, during the entire harvesting period. All vegetal samples for analysis were collected in the middle of each month from the above-mentioned time interval and were deposited in the Herbarium of the Department of Pharmaceutical Botany, Faculty of Pharmacy, University of Medicine and Pharmacy of Craiova. The study did not involve endangered or protected species. Samples were cleaned and naturally air-dried in shaded, cool areas, and then grounded for extraction. In a NeoClevenger-type apparatus, 100 g of died and grounded leaves were hydro-distilled for four hours with 1000 mL of distilled water, in a round-bottom flask. The mixture was heated, and the released RoEO was collected in a graded column after passing through a condenser. After hydro-distillation, extracted RoEOs samples were collected in glass vials, dehydrated on anhydrous sodium and stored in dark, tightly sealed bottles, at 4°C, for further analysis. RoEO amounts were directly measured from extraction burette. The yield was calculated as volume (mL) of RoEO per 100 g dried weight (d.w.) of leaves.

2.2. GC/MS Analysis

To analyze the constituents of RoEO, a comprehensive gas chromatography/mass spectrometry (GC/MS) method was employed. The analysis aimed to identify and quantify the various components by comparing their mass spectra with reference spectra from the National Institute of Standards and Technology (NIST) Library 202038.

The instrumentation used for this analysis included a Thermo Scientific Focus GC (Norristown, PA, USA) equipped with an AI/AS 3000 autosampler and coupled with a DSQ II mass detector. The GC system was fitted with a TraceGOLD TG-624 column, which measured 60 m in length, 0.25 mm in internal diameter, and had a film thickness of 1.4 μm.

For the analysis, an injection volume of 1 μL was used. The flow rate was maintained at 1.4 mL per minute, and a split ratio of 1:50 was applied, using helium as the carrier gas. The oven temperature program was set to begin at 90°C. Subsequently, the temperature was increased at a rate of 3°C per minute until it reached 220°C, where it was maintained for an additional five minutes.

The MS conditions were carefully controlled to ensure accurate detection and analysis of the RoEO constituents. The MS transfer line temperature was maintained at 240°C, while the ion source temperature was set at 230°C. Electron impact ionization (EI) was employed at 70 eV, with the spectra recorded in full scan mode over a mass range of 50 to 450 *m*/*z*.

Throughout the analysis, performed in triplicate, all constituents' retention times (RTs) were meticulously recorded. The resulting mass spectra were then scrutinized and compared with reference spectra from the NIST Library 2020 to ensure accurate identification and quantification of each component [51,52].

This detailed GC/MS method allowed for a comprehensive profiling of the RoEO, providing valuable insights into their chemical composition and facilitating further applications and research in various fields.

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2.3. Assessment of the Antibacterial Activity

Staphylococcus aureus minimum inhibitory concentration (MIC) values were determined using the microdilution method in 96-well plates. Microbial suspensions were adjusted to a turbidity of 0.5 McFarland units. Each well received 100 µL of sterile nutrient broth supplemented with 0.5% Tween 80 and 100 µL of undiluted RoEO. Serial dilutions were performed, and 10 µL of microbial suspension was added to each well. The plates were incubated at 37°C. After incubation, 20 µL of resazurin solution (0.2 mg/mL) was added to each well, and the plates were incubated for an additional two hours. Wells showing blue color indicated inhibition of microbial growth (MIC). The assay was tested in triplicate [53,54].

2.4. DPPH Free Radical Scavenging Assay

For the antioxidant assay, 50 µL of each sample was added to a 96-well microplate. Then, 200 µL of 2 mM 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution was added to each well. Serial dilutions were performed to obtain a range of concentrations for analysis. The reaction mixtures were incubated in the dark for 30 minutes at room temperature (RT). The decrease in absorbance was measured at 517 nm using a FLUOstar Optima microplate reader (BMG Labtech, Offenburg, Germany). The antioxidant activity was assessed in triplicate and calculated based on the reduction in DPPH absorbance compared to a control (ascorbic acid). The half-maximal inhibitory concentration (IC_{50}) value, representing the concentration of the sample required to inhibit 50% of the DPPH free radicals, was determined from the dose–response curve generated [54].

2.5. ABTS Radical Scavenging Assay

The antioxidant activity of the RoEO was evaluated using the 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS•+) decolorization assay. Briefly, the ABTS•+ was produced by reaction of a 7 mM ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at RT for 12–16 hours before use. The ABTS•+ solution was then diluted with ethanol to an absorbance of 0.70±0.02 at 734 nm. Different concentrations of the RoEO were added to the ABTS•+ solution, and the reduction in absorbance was measured at 734 nm after six minutes of reaction, at RT, using a FLUOstar Optima microplate reader. The percentage inhibition of the ABTS radical was calculated, and the IC_{50} value, defined as the concentration of the EO required to reduce the initial ABTS concentration by 50%, was determined from the dose–response curve. The assay was performed in triplicate [55].

2.6. H_2O_2 Radical Scavenging Assay

The hydrogen peroxide (H_2O_2) scavenging activity of the RoEO was determined using a spectrophotometric method (FLUOstar Optima microplate reader). A solution of 40 mM H_2O_2 was prepared in phosphate buffer (pH 7.4). Various concentrations of the RoEO were added to the H_2O_2 solution, and the absorbance of the solution was measured at 230 nm after 10 minutes of incubation at RT. The percentage of H_2O_2 scavenged by the EO was calculated by comparing the absorbance of the test sample with that of a blank solution containing only the H_2O_2 solution. The IC_{50} value, which is the concentration of the EO required to scavenge 50% of the H_2O_2, was determined in triplicate from the plotted graph of scavenging activity against the concentration of the EO [56].

2.7. Statistical Analysis

All statistical analyses were conducted to evaluate the variation in chemical compounds and biological activities (antioxidant and antimicrobial) across different months. The data were analyzed using a combination of two-way analysis of variance (ANOVA), principal component analysis (PCA), and Spearman's correlation.

A two-way ANOVA was performed to assess the influence of the month and compound/activity type, as well as their interaction, on the measured outcomes ($p<0.05$). Prior to analysis, the data were tested for normality using the Shapiro–Wilk test, and results indicated that the data did not follow a

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normal distribution. Therefore, non-parametric methods such as Spearman's correlation were used for pairwise associations.

To explore data patterns and identify variables contributing most to the observed variation, PCA was conducted. PCA reduced the dimensionality of the dataset, highlighting clustering and the relationships between compounds and activities across months. PCA loading plots were interpreted to identify key compounds or activities driving the variance.

The relationships between individual compounds and biological activities (antibacterial and antioxidant) were further examined using Spearman's correlation analysis. This non-parametric approach was used to assess the strength and direction of monotonic relationships, particularly when variables did not meet the assumptions of normality.

All analyses were performed using GraphPad Prism version 9.1.0. Graphical outputs, including bar graphs, PCA loading plots, and correlation heatmaps, were generated to visually represent significant patterns and relationships. The significance threshold of $p<0.05$ was used for all statistical tests.

3. Results

3.1. Extraction Yield and Chemical Profile of R. officinalis Essential Oil

The yields of RoEO extractions were expressed as volume (mL) of EO relative to the d.w. of plant material (leaves) (Figure 1). The highest extraction yield of RoEO was recorded in August 2022 (Ro_7; 1.25 mL/100 g d.w.), and the lowest was highlighted in January 2023 (Ro_12; 1.03 mL/100 g d.w.).

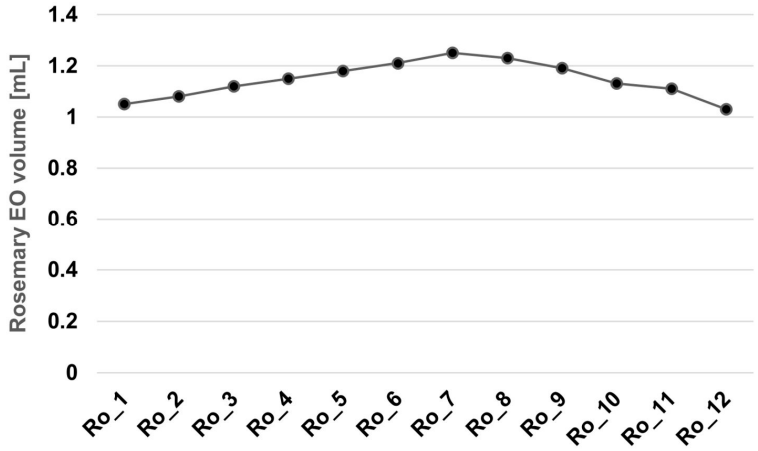

Figure 1. Extraction yield of rosemary EO, expressed as volume (mL) of EO per 100 g d.w. of leaves. d.w.: Dried weight; EO: Essential oil; Ro: *Rosmarinus officinalis*; Ro_1 to Ro_12: Samples of rosemary EO (February 2022 to January 2023).

The chemical composition of RoEO from Romania, collected over a one-year period, was thoroughly analyzed using GC/MS. This analysis provided a detailed profile of the EO's constituents, revealing significant monthly variations. Key compounds, such as camphor, eucalyptol, α-pinene, camphene, and α-myrcene, were quantified, alongside lesser-studied constituents like β-pinene, α-terpinene, linalool, terpinolene, and carvacrol. Comparisons were made with a reference sample from Tunisia (Table 1; Figures S1–S13).

Table 1. Monthly variation in chemical constituents (%) of rosemary EO from Romania and comparison with Tunisian reference.

No.	Compound	t_R (min)	RI (NIST)	Ro_1	Ro_2	Ro_3	Ro_4	Ro_5	Ro_6	Ro_7	Ro_8	Ro_9	Ro_10	Ro_11	Ro_12	Ro_Tunise
1.	Tricyclene	6.56	933	0.23	0.30	0.33	0.31	0.31	0.34	0.29	0.35	0.41	0.36	0.38	0.36	0.11
2.	**Camphene**	7.59	950	6.28	8.26	9.44	9.20	8.37	9.00	8.16	9.21	10.15	9.76	9.57	9.57	3.27
3.	2,4-Thujadiene	7.73	971	0.18	0.15	0.15	0.16	0.15	0.13	0.13	0.14	0.17	0.18	0.18	0.17	0.03
4.	β-Pinene	8.73	972	0.17	0.22	0.28	0.28	0.31	0.30	0.35	0.39	–	0.34	0.32	0.38	5.33

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5.	**α-Pinene**	8.74	939	11.36	14.97	15.46	15.14	16.07	18.14	17.55	18.35	19.46	17.40	18.61	19.33	11.11
6.	1-Octen-3-ol	8.82	1078	0.16	0.15	0.13	0.12	0.14	0.17	0.18	0.15	0.15	0.14	0.17	0.17	0.02
7.	3-Octanone	9.07	1121	0.51	0.21	0.18	0.16	0.17	0.23	0.26	0.24	0.23	0.20	0.27	0.28	0.03
8.	**α-Myrcene**	9.27	991	1.65	2.24	2.38	2.49	2.33	2.62	2.34	2.63	2.63	2.87	2.83	3.08	0.86
9.	β-Thujene	9.36	964	0.63	–	0.15	–	–	–	–	0.19	–	–	–	0.17	0.23
10.	3-Octanol	9.61	1126	0.06	–	–	–	–	0.04	0.06	–	–	–	–	–	–
11.	α-Phellandrene	10.04	1015	–	0.14	–	–	0.32	0.23	0.41	–	0.14	0.16	0.15	–	0.11
12.	3-Carene	10.14	1030	0.03	–	–	–	0.04	0.03	0.04	0.05	0.05	0.03	0.03	0.04	0.11
13.	α-Terpinene	10.56	1016	0.95	0.40	0.46	0.68	0.66	0.59	–	0.60	–	0.63	–	–	0.36
14.	**β-Cymene**	10.95	1018	4.27	2.49	2.60	2.69	2.59	2.36	2.40	2.15	2.07	2.02	1.95	2.12	1.57
15.	**D-Limonene**	11.19	1031	4.13	3.75	3.87	3.95	3.79	3.99	3.90	3.89	3.75	3.59	3.63	3.85	1.86
16.	**Eucalyptol**	11.35	1034	16.16	15.42	14.16	14.31	13.92	13.42	13.70	14.23	13.07	14.29	14.22	14.47	52.77
17.	β-*cis*-Ocimene	12.12	1049	–	–	–	–	–	–	–	–	–	–	–	–	0.03
18.	γ-Terpinene	12.72	1062	0.08	0.12	0.22	0.33	0.31	0.31	0.32	0.48	0.52	0.62	0.55	0.39	0.60
19.	Linalool	15.15	1095	0.54	0.82	0.77	0.80	0.85	0.89	0.88	0.87	0.74	0.77	0.80	0.84	0.57
20.	Crysanthenone	16.31	1102	–	0.03	0.05	0.07	0.06	0.05	0.06	0.06	0.08	0.08	0.07	0.10	–
21.	Terpinolene	17.47	1088	0.10	0.14	0.17	0.25	0.25	0.30	1.09	0.37	0.93	0.38	0.95	0.82	0.26
22.	**Camphor**	17.90	1146	40.03	34.23	30.85	32.21	33.13	31.22	34.24	29.62	29.29	29.69	29.86	29.41	9.27
23.	Camphenilanol	18.31	1151	0.08	0.11	0.09	0.08	0.03	0.07	0.08	0.06	0.04	–	0.03	–	–
24.	Sabinone	18.76	1162	0.07	–	–	–	–	0.02	–	–	–	0.05	–	0.06	–
25.	Pinocarvone	18.76	1191	–	0.04	–	–	0.04	–	–	–	–	–	0.05	–	–
26.	D-Pinocamphone	18.85	1206	0.33	0.04	0.14	–	0.12	0.13	–	–	0.07	0.09	0.08	0.07	0.02
27.	***endo*-Borneol**	19.40	1163	3.87	6.40	6.09	4.79	4.63	4.22	3.83	5.01	4.78	4.70	4.98	5.32	2.60
28.	Terpinen-4-ol	19.95	1176	0.64	0.75	0.71	0.65	0.65	0.69	0.61	0.66	0.61	0.59	0.60	0.69	0.58
29.	**α-Terpinyl propionate**	20.91	1333	1.83	1.91	1.64	1.53	1.66	1.74	1.66	1.65	1.51	1.58	1.58	1.74	1.78
30.	**Verbenone**	21.56	1204	2.86	2.77	2.68	2.18	2.64	3.20	3.37	2.97	2.94	3.19	3.08	3.33	0.04
31.	*trans*-Shisool	23.76	1326	–	–	–	–	–	–	–	–	–	–	0.03	0.03	–
32.	(+)-Borneol acetate	25.45	1330	0.34	0.72	2.72	3.05	2.88	2.97	2.23	3.22	3.55	3.48	2.16	0.86	0.81
33.	(+)-*cis*-Verbenol acetate	25.68	1351	–	–	–	–	–	–	–	–	–	–	–	–	0.01
34.	Thymol	25.91	1290	0.09	–	–	–	–	–	0.04	–	–	0.07	0.04	–	–
35.	Piperitenone	26.92	1303	0.03	0.05	0.06	0.06	0.05	0.07	0.03	0.04	0.05	0.05	0.02	0.03	–
36.	α-Cubebene	27.61	1374	–	–	–	–	–	–	–	0.03	–	–	–	0.04	0.02
37.	Ylangene	27.68	1395	0.05	0.07	0.17	0.16	0.15	0.06	0.04	0.05	0.06	0.06	0.07	0.05	0.05
38.	Copaene	27.82	1415	0.04	0.03	0.07	0.07	0.07	0.04	0.03	–	0.03	0.04	0.05	–	0.22
39.	Carvacrol	28.04	1298	0.07	0.15	0.08	–	0.07	0.06	0.04	0.06	0.05	0.05	0.04	0.04	–
40.	Methyleugenol	28.34	1396	–	–	–	–	–	–	–	–	–	–	–	–	0.01
41.	**Caryophyllene**	28.71	1420	0.68	1.14	1.33	1.43	1.29	1.01	0.60	1.12	1.20	1.47	1.56	1.10	3.77
42.	Humulene	29.36	1454	–	–	–	0.24	0.26	–	0.16	0.13	0.14	–	0.18	0.13	0.38
43.	*p*-Thymol	29.58	1465	–	–	0.08	0.15	0.07	0.06	–	0.07	0.04	–	–	0.05	–
44.	7-*epi*-α-Cadinene	29.76	1491	0.05	0.05	0.11	0.11	0.10	0.04	0.03	0.04	0.03	0.04	0.03	0.03	0.02
45.	α-Bisabolene	30.20	1506	–	–	–	0.08	0.05	–	–	–	–	–	–	–	0.05
46.	(-)-δ-Cadinene	30.36	1520	0.09	–	0.19	0.18	0.18	0.11	–	–	0.09	0.11	0.12	0.08	0.23
47.	*trans*-Calamenene	30.41	1542	0.04	–	–	–	–	–	–	0.03	–	0.02	–	0.02	–
48.	α-Calacorene	30.72	1561	0.04	0.06	0.14	0.12	0.11	0.05	0.04	0.04	–	0.04	0.04	0.03	0.01
49.	(+)-Sativen	30.94	1583	0.02	–	–	0.05	–	0.03	–	–	0.01	–	–	–	–
50.	Cubenol	32.01	1600	0.05	0.07	0.11	–	0.10	–	0.03	–	–	–	0.02	–	–
51.	α-Bisabolol	32.62	1621	0.03	0.04	–	0.01	0.08	0.03	–	0.02	0.01	0.01	0.02	0.01	0.01
52.	Levomenthol	32.62	1632	–	–	0.06	0.05	–	–	0.02	–	–	0.02	–	–	–
53.	Caryophyllene oxide	38.74	1652	0.03	0.06	0.05	0.07	0.05	0.03	0.02	0.02	0.01	0.02	0.02	0.02	0.10
Total No. of compounds identified				41	36	37	36	41	40	37	37	36	38	40	39	38
Total (%)				98.85	98.50	98.26	98.21	99.05	98.99	99.22	99.19	99.06	99.19	99.34	99.28	99.21
Monoterpene hydrocarbons (%)				30.06	33.18	35.51	35.48	35.50	38.34	36.98	38.80	40.28	38.34	39.15	40.28	25.84
Oxygenated monoterpenes (%)				66.94	63.44	60.18	59.93	60.80	58.81	60.79	58.52	56.82	58.70	57.64	57.04	68.46
Sesquiterpene hydrocarbons (%)				1.01	1.35	2.10	2.44	2.21	1.34	0.90	1.44	1.56	1.78	2.05	1.48	4.75
Oxygenated sesquiterpenes (%)				0.11	0.17	0.16	0.08	0.23	0.06	0.05	0.04	0.02	0.03	0.06	0.03	0.11
Other compounds (%)				0.73	0.36	0.31	0.28	0.31	0.44	0.50	0.39	0.38	0.34	0.44	0.45	0.05

EO: Essential oil; NIST: National Institute of Standards and Technology (USA); RI: Retention index; Ro: *Rosmarinus officinalis*; Ro_1 to Ro_12: Samples of rosemary EO (February 2022 to January 2023); Ro_Tunise: Rosemary EO Tunisian reference; t_R: Retention time.

In the RoEO from Romania, between 36 and 41 compounds were identified and also quantified from a percentage point of view of the total identified components for each EO sample, as follows: 36

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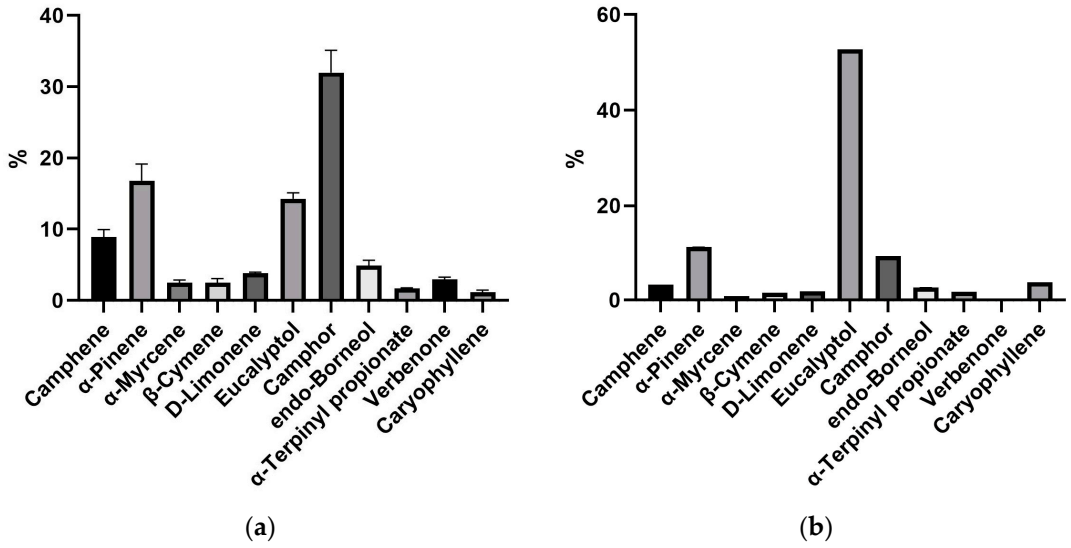
compounds for Ro_2, Ro_4, and Ro_9 (98.50%, 98.21% and 99.06%, respectively), 37 compounds for Ro_3, Ro_7 and Ro_8 (98.26%, 99.22% and 99.19%, respectively), 38 compounds for Ro_10 (99.19%), 39 compounds for Ro_12 (99.28%), 40 compounds for Ro_6 and Ro_11 (98.99% and 99.34%, respectively), and 41 compounds for Ro_1 and Ro_5 (98.85% and 99.05%, respectively) (Table 1).

From the point of view of the variation of terpenoid content (maximum; minimum) in the RoEO, the following considerations can be made. Oxygenated monoterpenes reach the highest concentration (66.94% in February 2022 – Ro_1; 56.82% in October 2022 – Ro_9), followed by monoterpene hydrocarbons (40.28% in October 2022 and January 2023 – Ro_9 and Ro_12, respectively; 30.06% in February 2022 – Ro_1), sesquiterpene hydrocarbons (2.44% in May 2022 – Ro_4; 0.90% in August 2022 – Ro_7) and oxygenated sesquiterpenes (0.23% in June 2022 – Ro_5; 0.02% in October 2022 – Ro_9). Among oxygenated monoterpenes, the highest concentration was recorded for camphor (40.03% in February 2022 – Ro_1; 29.29% in October 2022 – Ro_9), followed by eucalyptol (16.16% in February 2022 – Ro_1; 13.07% in October 2022 – Ro_9), endo-borneol (6.40% in March 2022 – Ro_2; 3.83% in August 2022 – Ro_7), verbenone (3.37% in August 2022 – Ro_7; 2.18% in May 2022 – Ro_4), α-terpinyl propionate (1.91% in March 2022 – Ro_2; 1.51% in October 2022 – Ro_9). The highest concentration among monoterpene hydrocarbons was evidenced for α-pinene (19.46% in October 2022 – Ro_9; 11.36% in February 2022 – Ro_1), followed by camphene (9.76% in November 2022 – Ro_10; 8.16% in August 2022 – Ro_7), β-cymene (4.27% in February 2022 – Ro_1; 1.95% in December 2022 – Ro_11), D-limonene (4.13% in February 2022 – Ro_1; 3.59% in November 2022 – Ro_10), α-myrcene (3.08% in January 2023 – Ro_12; 1.65% in February 2022 – Ro_1). Among sesquiterpene hydrocarbons, the highest amount was highlighted for caryophyllene (1.56% in December 2022 – Ro_11; 0.60% in August 2022 – Ro_7) (Table 1; Figure 2, a and b).



(**a**) (**b**)

Figure 2. Main compounds in Romanian (**a**) *vs.* Tunisian (**b**) rosemary essential oil.

3.2. Antibacterial Activity

The antibacterial activity (MIC values) of RoEO against *S. aureus* was also assessed (Table 2; Figure 3). Comparisons were made with a reference sample from Tunisia.

Table 2. Antibacterial (*S. aureus* MIC) and antioxidant (DPPH, ABTS and H_2O_2 IC_{50}) activity of rosemary EO from Romania and Tunisian reference.

Activity	Ro_1	Ro_2	Ro_3	Ro_4	Ro_5	Ro_6	Ro_7	Ro_8	Ro_9	Ro_10	Ro_11	Ro_12	Ro_Tunise
S. aureus MIC (μg/mL)	115.6	194.4	228.8	131.5	178.2	249.7	244.9	186.8	220.2	236.7	137.1	53.87	32.63
DPPH IC_{50} (μg/mL)	3.351	3.307	3.447	6.158	4.556	2.763	2.692	3.008	5.033	4.517	3.917	4.233	3.456

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ABTS IC_{50} (µg/mL)	4.283	4.812	4.941	8.672	6.348	3.926	3.281	4.738	6.591	5.316	5.423	5.679	4.190
H_2O_2 IC_{50} (µg/mL)	5.317	5.824	5.265	9.398	6.137	4.579	4.764	4.448	7.289	6.642	5.756	6.919	5.054

ABTS: 2,2′-Azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid); DPPH: 2,2-Diphenyl-1-picrylhydrazyl; EO: Essential oil; H_2O_2: Hydrogen peroxide; IC_{50}: Half-maximal inhibitory concentration; MIC: Minimum inhibitory concentration; Ro: *Rosmarinus officinalis*; Ro_1 to Ro_12: Samples of rosemary EO (February 2022 to January 2023); Ro_Tunise: Rosemary EO Tunisian reference.

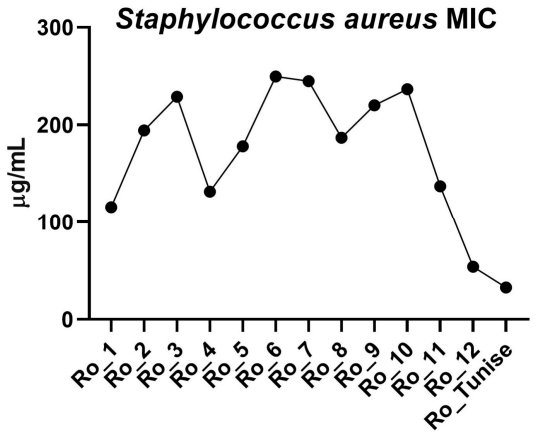

Figure 3. Antibacterial activity of rosemary EO. EO: Essential oil; MIC: Minimum inhibitory concentration; Ro: *Rosmarinus officinalis*; Ro_1 to Ro_12: Samples of rosemary EO (February 2022 to January 2023); Ro_Tunise: Rosemary EO Tunisian reference.

Camphor was found in high concentrations ranging from 29.41% to 40.03%, but this did not consistently correlate with enhanced antibacterial activity. For instance, while February 2022 (Ro_1) had the highest camphor content (40.03%), its antibacterial activity (115.6 µg/mL) was not the most potent. Conversely, January 2023 (Ro_12), with a lower camphor content (29.41%), exhibited the best antibacterial activity (53.87 µg/mL), suggesting that other compounds or synergistic effects might play a role. Camphor is known for its antimicrobial and anti-inflammatory properties. A study showed that RoEO exhibited significant antimicrobial properties against *Bacillus cereus*, *Escherichia coli*, and *Pseudomonas* spp., with camphor being a major component contributing to this activity [7,57].

Eucalyptol (1,8-cineole) was another dominant compound, ranging from 13.07% to 16.16%, significantly lower compared to the Tunisian reference (52.77%). Eucalyptol is a major constituent with antimicrobial, expectorant and anti-inflammatory effects, significantly contributing to the medicinal properties of RoEO.

α-Pinene and α-myrcene also showed considerable variations. α-Pinene ranged from 11.36% to 19.33%, while α-myrcene ranged from 1.65% to 3.08%. α-Pinene, another primary compound in RoEO, is known mainly for its anti-inflammatory and bronchodilator effects. RoEO demonstrated antimicrobial and antibiofilm activity against different bacteria, including *S. aureus* and *Listeria monocytogenes*, supporting its potential as a natural preservative in meat products. Recognized for its analgesic and anti-inflammatory properties, α-myrcene is a valuable component of RoEO. The research on RoEO microemulsion applied in tomato paste found that it exhibited significant antibacterial activity against *E. coli* and *Salmonella typhi* [7,58]. Also, natural antimicrobials based on *R. officinalis* leaf distillation by-products can be used as partial nitrite replacers in meat products (bacon) [59].

β-Pinene has antimicrobial and anti-inflammatory properties and contributes to the overall therapeutic profile of RoEO. Linalool is well-regarded for its calming, anti-anxiety effects, and antimicrobial properties. Exhibiting antimicrobial activity, terpinolene is another valuable compound found in RoEO. Known for its strong antimicrobial properties, carvacrol enhances the antibacterial efficacy of RoEO. Caryophyllene has anti-inflammatory and analgesic properties, contributing to the medicinal value of the RoEO. For example, *Trichilia monadelpha* EO contains β-caryophyllene as the

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major constituent and exhibits moderate inhibition against various microorganisms [60]. Although present in smaller amounts, 14-hydroxycaryophyllene also contributes to the anti-inflammatory and antimicrobial properties of RoEO.

3.3. Antioxidant Activity

The antioxidant capacity (DPPH, ABTS and H_2O_2 assays) of RoEO was also evaluated (Table 2; Figure 4). Comparisons were made with a reference sample from Tunisia.

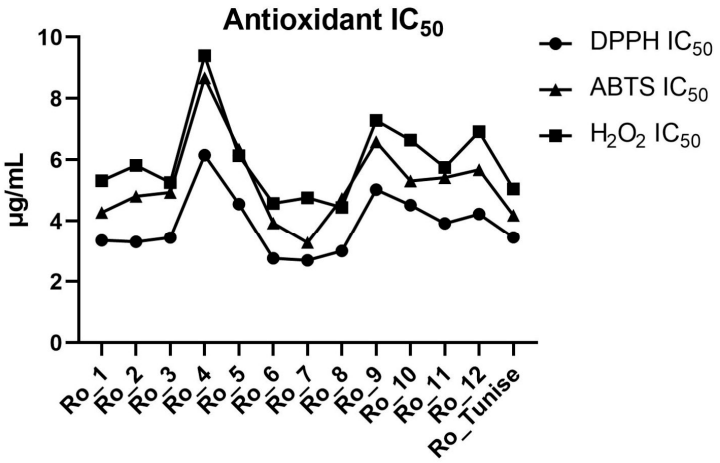

Figure 4. Antioxidant activity of rosemary EO. ABTS: 2,2′-Azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid); DPPH: 2,2-Diphenyl-1-picrylhydrazyl; EO: Essential oil; H_2O_2: Hydrogen peroxide; IC_{50}: Half-maximal inhibitory concentration; Ro: *Rosmarinus officinalis*; Ro_1 to Ro_12: Samples of rosemary EO (February 2022 to January 2023); Ro_Tunise: Rosemary EO Tunisian reference.

The research on RoEO microemulsion applied in tomato paste found that α-myrcene exhibited antioxidant properties [60]. Known for their antioxidant properties, α-terpinene and terpinolene add to the RoEO's ability to combat oxidative stress. Humulene is another important constituent of RoEO, with antioxidant and anti-inflammatory effects. *trans*-Calamenene has also antioxidant properties, adding to the RoEO's overall efficacy in combating oxidative stress.

4. Discussion

4.1. Chemical Profile of R. officinalis Essential Oil

The chemical composition of RoEO shows quite a large variability, depending on the origin. The main producer of RoEO is Spain. The best EOs come from France and North Africa (Morocco, Tunisia, Algeria). The quality of RoEO varies greatly depending on the proportion of branches from the plant material subjected to distillation. Spanish EOs are characterized by a high content of α-pinene, 1,8-cineole and camphor, while French EOs are rich in α-pinene, 1,8-cineole and bornyl acetate. Moroccan EOs are richer in 1,8-cineole than European ones. European EOs are also characterized by the presence of verbenone in concentrations >1%, while in African EOs it is found only in very small quantities [61–66].

4.2. Antibacterial–Antioxidant Activity Correlation

The PCA loading plot provides a comprehensive overview of how the main compounds contribute to the antibacterial activity and antioxidant activity (DPPH values were selected since all three antioxidant data sets occupied the same influence zone on the plot), illustrating distinct roles for each compound. The horizontal axis, PC1, captures the majority of the variability in the dataset and highlights two main clusters of compounds, separating those primarily associated with antibacterial activity from those driving antioxidant activity (Figure 5).

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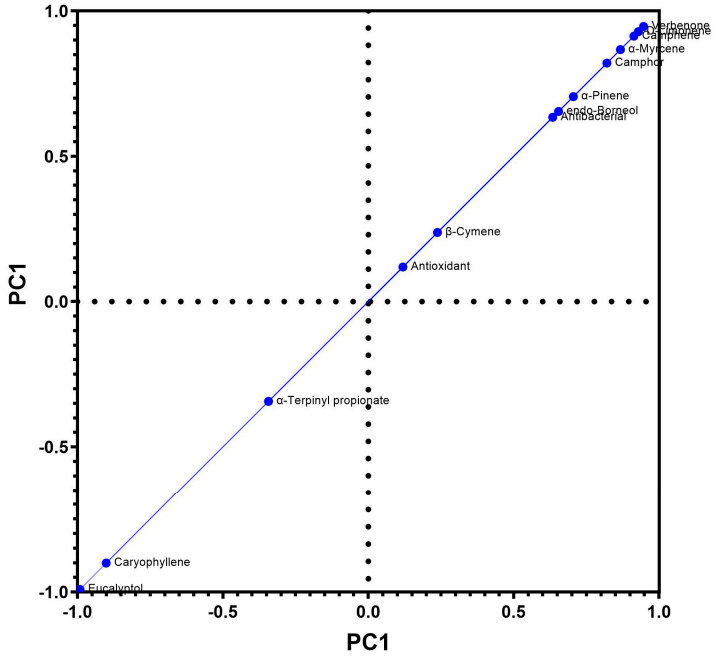

Figure 5. Principal component analysis (PCA) loading plot illustrating the relationships between key compounds and biological activities (antibacterial and antioxidant activities). Principal component 1 (PC1) represents the axis that captures the largest amount of variance in the dataset, indicating the most dominant patterns or trends in the relationships between the compounds and biological activities, such as their primary contributions to antibacterial or antioxidant effects.

Compounds like verbenone, camphene, α-pinene, α-myrcene, D-limonene, *endo*-borneol and camphor are positioned strongly on the positive side of PC1, indicating their significant contribution to antibacterial activity. These compounds emerge as key drivers of antimicrobial efficacy, making them highly relevant for applications targeting microbial inhibition. Among these, verbenone, D-limonene and camphene show the strongest alignment, suggesting they play a dominant role in enhancing antibacterial properties.

In contrast, the antioxidant activity, representing radical scavenging potential, is associated with compounds like caryophyllene, eucalyptol, and α-terpinyl propionate, which are positioned on the negative side of PC1. These compounds contribute minimally to antibacterial activity but could influence antioxidant applications. Caryophyllene and eucalyptol stand out as possible contributors to the antioxidant activity.

β-Cymene occupies a more central position along PC1, showing moderate contributions to both activities. This suggests that while this compound is not the primary driver of either antioxidant or antibacterial activity, it might still play a supportive role in enhancing the overall bioactivity of the oil.

The distinct separation of compounds along PC1 underscores their specialization. Compounds like verbenone, camphene, and α-pinene are critical for antimicrobial efficacy, whereas caryophyllene and eucalyptol might induce antioxidant activity. α-Terpinyl propionate, while positioned closer to antioxidant activity, shows a weaker correlation, indicating a more moderate role in radical scavenging.

This analysis highlights the importance of selecting specific compounds based on the intended application. For products targeting oxidative stress, such as dietary supplements or cosmetics, caryophyllene and eucalyptol should be prioritized. On the other hand, for antimicrobial applications, verbenone, camphene, and α-pinene are essential for achieving optimal efficacy. The PCA plot offers valuable insights for tailoring extraction and formulation strategies to meet the desired bioactivity goals, ensuring that the compounds are utilized to their full potential.

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To further clarify the relationships between compounds and biological activities, we conducted a Spearman's correlation analysis to quantify the associations between individual compounds and each activity. The Spearman's correlation analysis largely supports the insights from the PCA plot, providing further precision in understanding the relationships between specific compounds and their biological activities. For antibacterial activity, the significant negative correlation observed with eucalyptol (r=−0.7253, p=0.0067) confirms the PCA finding that eucalyptol contributes minimally to antimicrobial efficacy and may even inhibit it at higher concentrations. For DPPH antioxidant activity, α-terpinyl propionate showed a significant negative correlation (r=−0.5766, p=0.0421), confirming its strong contribution to radical scavenging. Similarly, caryophyllene, which might present antioxidant activity from the PCA plot, displayed a significant positive correlation (r=0.5934, p=0.0360), suggesting a more complex role, where higher concentrations may reduce antioxidant activity. For ABTS antioxidant activity, both camphene and α-terpinyl propionate demonstrated significant correlations, further validating their contributions as indicated in the PCA. Camphene showed a significant positive correlation (r=0.6245, p=0.0254), aligning with the PCA suggestion of its role in influencing ABTS activity. Conversely, α-terpinyl propionate exhibited a significant negative correlation (r=−0.6152, p=0.0281), reinforcing its strong role in enhancing antioxidant activity in this assay. For H_2O_2 antioxidant activity, neither the PCA nor the Spearman's correlation revealed any significant compound-activity relationships (p>0.05), suggesting that the measured compounds play minimal roles in H_2O_2 scavenging.

Spearman's correlation analysis complements the PCA findings by identifying specific compounds with statistically significant contributions to biological activities. However, the lack of significant correlations for many compounds and activities underscores the multifactorial nature of these interactions, suggesting that additional studies are needed to explore the synergistic or antagonistic effects between compounds. These insights can guide the targeted extraction and formulation of EOs for specific applications, whether focused on antimicrobial or antioxidant properties.

4.3. Importance of Studying the Dynamics Over a Year

Studying the dynamics of chemical constituents in RoEO over a year is crucial for several reasons. EOs are widely used in pharmaceuticals, cosmetics, and food industries, where consistent quality and efficacy are paramount. Understanding how the chemical composition varies with seasons can help in standardizing the production process and ensuring that the EOs meet the required quality standards throughout the year.

Seasonal variations can significantly impact the therapeutic properties of EOs. For instance, certain compounds with strong antimicrobial or antioxidant properties might be more abundant in specific seasons. By identifying these variations, producers can optimize harvesting times to yield EOs with desired properties for specific applications.

Additionally, this temporal analysis provides insights into the plant's metabolic processes and how they are influenced by environmental factors. Such knowledge can guide agricultural practices, such as the timing of fertilization and irrigation, to enhance the yield and quality of EOs. It also has implications for the storage and preservation of EOs, as understanding seasonal peaks in bioactive compounds can inform better storage practices to maintain efficacy.

Lastly, from a scientific perspective, studying these dynamics contributes to a deeper understanding of plant biochemistry and ecology. It helps elucidate how plants interact with their environment and how these interactions influence their secondary metabolite production. This can lead to the discovery of new compounds with potential health benefits and further the development of natural product-based therapies.

4.4. Limitations of the Study

This study, while comprehensive in its approach to analyzing the chemical constituents of RoEO over a year, has several limitations. Firstly, the study relied on GC/MS analysis, which, although highly accurate, might not detect all compounds present in the RoEO, particularly those in very low

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concentrations. Additionally, the seasonal variations in environmental factors such as temperature, humidity, and soil conditions were not controlled or recorded, potentially influencing the chemical profiles of the RoEO. The sampling process, despite being conducted monthly, might not capture rapid or short-term fluctuations in the chemical composition, potentially missing transient changes.

Another limitation is the geographical specificity of the study. The RoEO samples were collected from a single region in Romania, and while this provides a detailed local profile, it might not be representative of RoEO from other regions with different climatic and soil conditions. The comparison with the Tunisian reference is valuable, but it also underscores the regional variability that could affect the generalizability of the findings.

Furthermore, the study's focus on specific compounds means that other potentially bioactive but less studied compounds might have been overlooked. The correlations identified, while informative, do not establish causation. Additional biological assays and mechanistic studies would be needed to confirm the functional roles of these compounds in antimicrobial and antioxidant activities.

5. Conclusions

For the first time, over a 12-month period, the seasonal variations of chemical composition, antibacterial and antioxidant activity have been reported for *R. officinalis* EO from southwestern Romania (Oltenia Region). A comprehensive GC/MS method was used for the analysis of RoEO. This analysis provided a detailed profile of the EO's constituents, revealing significant monthly variations. The study underscores the importance of a comprehensive and dynamic approach to understanding the chemical profiles of EOs. Such knowledge is indispensable for both practical applications in various industries and advancing scientific research in plant biology and natural product chemistry.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1: Gas chromatogram of Ro_1 (February 2022) sample of *R. officinalis*; Figure S2: Gas chromatogram of Ro_2 (March 2022) sample of *R. officinalis*; Figure S3: Gas chromatogram of Ro_3 (April 2022) sample of *R. officinalis*; Figure S4: Gas chromatogram of Ro_4 (May 2022) sample of *R. officinalis*; Figure S5: Gas chromatogram of Ro_5 (June 2022) sample of *R. officinalis*; Figure S6: Gas chromatogram of Ro_6 (July 2022) sample of *R. officinalis*; Figure S7: Gas chromatogram of Ro_7 (August 2022) sample of *R. officinalis*; Figure S8: Gas chromatogram of Ro_8 (September 2022) sample of *R. officinalis*; Figure S9: Gas chromatogram of Ro_9 (October 2022) sample of *R. officinalis*; Figure S10: Gas chromatogram of Ro_10 (November 2022) sample of *R. officinalis*; Figure S11: Gas chromatogram of Ro_11 (December 2022) sample of *R. officinalis*; Figure S12: Gas chromatogram of Ro_12 (January 2023) sample of *R. officinalis*; Figure S13: Gas chromatogram of Ro_Tunise reference sample of *R. officinalis*.

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