Bulgarian Chemical Communications, Volume 52, Special Issue D (pp. 54-59) 2020

Chemical composition, antioxidant and antimicrobial activity of essential oils from leaves and flowers of *Rosmarinus officinalis* L.

D. G. Teneva[1*], B. G. Goranov[2], R. S. Denkova-Kostova[3], Y. M. Hristova-Ivanova[5], D. I. Klisurova[1], A. K. Slavchev[2], Z. R. Denkova[2], G. A. Kostov[4]

[1] *Laboratory of Biologically Active Substances, Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, 139 Ruski Blvd., 4000 Plovdiv, Bulgaria*
[2] *Department of Microbiology, University of Food Technologies, 26 Maritza Blvd., 4000 Plovdiv, Bulgaria*
[3] *Department of Biochemistry and Molecular Biology, University of Food Technologies, 26 Maritza Blvd., 4000 Plovdiv, Bulgaria*
[4] *Department of Wine and Brewing, University of Food Technologies, 26 Maritza Blvd., 4000 Plovdiv, Bulgaria*
[5] *Department of Food Technologies, Institute of Food Preservation and Quality-Plovdiv, Agricultural Academy of Bulgaria, 154 Vasil Aprilov Blvd., 4000 Plovdiv, Bulgaria*

Received February 7, 2020; Accepted March 13, 2020

The chemical composition, antioxidant and antimicrobial activity of essential oils obtained from leaves and flowers of rosemary (*Rosmarinus officinalis* L.) were examined. The essential oils were extracted by steam distillation and their chemical composition was determined by GC/MS. The antioxidant activity was studied by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. The main components of the essential oils were ρ-cymene, linalool, bornanone, α-pinene and β-pinene. Regarding the DPPH radical scavenging ability, the essential oil from leaves showed slightly higher activity than that obtained from flowers. The antimicrobial activity of the essential oils against pathogenic (*Staphylococcus aureus* NBIMCC 3703, *Salmonella* sp. (clinical isolate), *Pseudomonas aeruginosa* NBIMCC 1390, *Bacillus subtilis* NBIMCC 1208, *Escherichia coli* NBIMCC 3702) microorganisms was examined by the disc-diffusion method. Gram-positive bacteria were more sensitive to the oils (inhibition zones being between 12.00 and 12.50 mm) and the minimum inhibitory concentration was 60 ppm; Gram-negative bacteria were less sensitive. The results demonstrated that essential oils could be used as a biopreservative agent.

Keywords: rosemary (*Rosmarinus officinalis* L.), essential oil, antioxidant activity, chemical composition, antimicrobial activity

INTRODUCTION

Essential oils are commonly used as natural preservatives and fragrances in cosmetic products. More recently, mainly thanks to their antimicrobial properties, new applications as food preservatives, natural pesticides in organic agriculture and insecticides are emerging [1]. Rosemary (*Rosmarinus officinalis* L.), which belongs to mint family is widely spread and cultivated in the Mediterranean region. The chemical composition of the oil of rosemary from various geographical origins has been extensively studied. Rosemary essential oil includes phenolic constituents in its composition. Due to this composition which mainly involves monoterpenes like 1,8-cineole, α-pinene, camphor, camphene, rosemary essential oil has many therapeutical indications [2]. The effects of growth location, environmental characteristics, soil properties, micronutrients and vegetative stage have been pointed out. The essential oil and various extracts of rosemary have provoked interest as sources of natural products. They have been screened for their potential uses as alternative remedies for the treatment of many infectious diseases [3]. Particularly, the antimicrobial and antioxidant activities of plant oils and extracts have formed the basis of applications, including raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies. Because of the possible multiple resistances and side effects of the synthetic antimicrobial, increasing attention has been directed towards natural antimicrobial.

The purpose of the present study was to determine and compare the chemical composition, antioxidant and antimicrobial activity of essential oils obtained from leaves and flowers of *Rosmarinus officinalis* L. against some pathogens.

MATERIALS AND METHODS

Chemicals

All solvents and reagents were purchased from Sigma-Aldrich (Steinheim, Germany).

Essential oil extraction and chemical substances

Essential oils from leaves and flowers of *Rosmarinus officinalis* L. were used for the conduction of the experiments.

* To whom all correspondence should be sent:
E-mail *desi_gerinska@yahoo.com*

The essential oil content was determined by steam distillation [4]. Steam distillation, the method used for essential oil extraction, takes advantage of both the volatility of a compound to evaporate when heated with steam and the hydrophobicity of the compound to separate into an oil phase during condensation.

Dried leaves and flowers of *Rosmarinus officinalis* L. were used for the extraction of essential oils, respectively. The extraction was carried out during 4 h from the first drop of distillate until the amount of essential oils stabilized. The oils were collected in glass tubes and were kept at 4°C till further use.

Gas chromatography/ mass spectrometry (GC/MS) analysis

The composition of the oils was determined by gas chromatography with mass selective detector (GC/MS) [5]. The GC/MS analyses were performed on a 7890A gas chromatograph (Agilent Technologies) coupled to an 5975C quadrupole mass spectrometer (Agilent Technologies). The analytes were separated on a HP-5MS capillary column (30 m×0.25 mm with a phase thickness of 0.25 μm). The split/splitless injector temperature was set at 250 °C and the temperature program was 60 °C for 3 min, 6 °C min^{-1} ramp rate to 250 °C and held constant for 3 min. The carrier gas was helium (99.999 %) at a 1 ml min^{-1} flow rate. In the SPME analysis, splitless injection (3 min) was used at 250 °C. The mass spectrometer was operated in the electron-impact mode (EI) at 70 eV. The identified components were arranged according to the retention time and their quantity is given in percentages.

The obtained mass spectra were analyzed using 2.64 AMDIS (Automated Mass Spectral Deconvolution and Identification System, National Institute of Standardization and Technology, NIST, Gaithersburg, MD, USA). The separated polar and nonpolar compounds were identified by comparison of their GC-MS spectra and Kovach retention index (RI) with referent compounds in the NIST 08 database (NIST Mass Spectral Database, PC-Version 5.0, 2008). The RIs of compounds were recorded with a standard *n*-hydrocarbon calibration mixture (C_{10}–C_{40}, Sigma-Aldrich) using the 2.64 AMDIS software.

DPPH radical scavenging assay

The ability of essential oils to scavenge free radicals was assayed with the use of a synthetic free radical scavenger compound 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma-Aldrich), according to the method employed in [6]. Briefly, essential oils were serially diluted (0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL (w/v)) in methanol. A solution of DPPH (0.004% (w/v)) was prepared in the same solvent. Then 300 μL of each dilution were mixed with 2700 μL of DPPH solution. The reaction was performed at 37°C in darkness and the absorption at 517 nm was recorded after exactly 15 min against methanol. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Sigma-Aldrich) was used as standard. Each test was performed in triplicate.

The antioxidant activity was calculated as follows:

$AA\% = [(Abs - Abs)/Abs] * 100$

AA: antioxidant activity

Abs: absorbance.

Determination of the antimicrobial activity against pathogenic microorganisms

- *Test microorganisms*: *Staphylococcus aureus* NBIMCC 3703, *Salmonella* sp. (clinical isolate), *Pseudomonas aeruginosa* NBIMCC 1390, *Bacillus subtilis* NBIMCC 1208, *Escherichia coli* NBIMCC 3702. All strains are deposited in the culture collection of the Institute of Food Preservation and Quality-Plovdiv, Agricultural Academy of Bulgaria.
- *Preparation of the suspensions of the test pathogenic microorganisms*: The test pathogenic microorganisms were cultured on LBG-agar (Luria Bertani Medium with glucose – agar medium, LB Broth, Miller-Novagen, Merck, Germany) at 37±1°C for 24-48 h. Using sterile loop biomass of the developed test pathogenic microorganisms were suspended in sterile saline solution in order to obtain suspensions of the test pathogenic microorganisms.
- *The antimicrobial activity was studied by the disc-diffusion method*: Agar disc-diffusion testing developed in 1940 [7], is the official method used in many clinical microbiology laboratories for routine antimicrobial susceptibility testing. Nowadays, many accepted and approved standards are published by the Clinical and Laboratory Standards Institute (CLSI) for bacteria and yeasts testing. In this well-known procedure, agar plates are inoculated with a standardized inoculum of the test microorganism. Then, filter paper discs (about 6 mm in diameter), containing the test compound at a desired concentration, are placed on the agar surface. The Petri dishes are incubated under suitable conditions. Generally, the antimicrobial agent diffuses into the agar and inhibits germination and growth of the test microorganism and then the diameters of inhibition growth zones are measured.

Sterile melted LBG-agar medium was poured in Petri dishes and after the hardening of the agar, the

dishes were spread-plated with suspensions of the test pathogenic microorganisms. Decimal dilutions of the essential oil in saline solution containing 1 % (v/v) Tween 80 were prepared. The experiments were conducted with dilutions of 1×, 10× and 100× in order to determine the MIC (minimum inhibitory concentration). The used paper discs were 6 mm in diameter. Six μL of the corresponding dilution were pipetted on the corresponding paper discs. Paper discs soaked in distilled water were used as blanks. The results were recorded as diameters of the clear zones around the paper discs, in millimeters, after 24-48 hours of incubation of the Petri dishes at optimal temperature for the growth of the corresponding test-microorganism 37°C [8]. The MIC was defined as the lowest concentration of the essential oil at which the microorganism does not demonstrate visible growth [9].

Statistical analysis

The experiments were performed in triplicate. The mean values and the standard deviations were calculated using MS Office Excel 2010. The MICs, in ppm, were calculated on the basis of the obtained results.

RESULTS AND DISCUSSION

Chemical composition

An essential oil is a concentrated hydrophobic liquid containing volatile chemical compounds from plants. Oil content in plants is a very small part (less than 5% of the dry matter content of the plant) and consists primarily of hydrocarbon terpenes [10]. Results obtained by GC/MS analysis of the essential oils of *Rosmarinus officinalis* L. are presented in Table 1.

Table 1. Chemical composition of *Rosmarinus officinalis* L. essential oils (leaves and flowers)

№	Compounds	RT	RI	Leaves	Flowers
				Area %	Area %
1	β-Thujene	5.62	928	0.47	0.21
2	α-Pinene	5.84	940	9.68	6.65
3	Camphene	6.27	955	4.44	3.24
4	Sabinene	6.78	967	0.16	0.16
5	β-Pinene	6.96	980	7.16	3.16
6	β-Myrcene	7.10	991	0.41	0.61
7	ρ-Cymene	8.16	1010	42.95	42.35
8	D-Limonene	8.26	1026	1.97	1.02
9	Eucalyptol	8.38	1029	4.27	4.31
10	cis-4-Thujanol	9.42	1031	0.11	-
11	Linalool	10.14	1037	6.48	20.40
12	L-Pinocarveol	11.50	1049	0.13	-
13	(+)-2-Bornanone	11.74	1068	10.40	6.40
14	δ-Terpineol	12.31	1086	0.29	2.19
15	endo-Borneol	12.40	1098	2.35	2.65
16	L-4-terpineneol	12.59	1155	0.64	0.66
17	α-Terpineol	13.00	1182	1.81	0.86
18	Myrtenal	13.11	1194	0.13	-
19	Bornyl acetate	15.40	1220	1.93	1.63
20	α-Copaene	17.83	1228	0.23	0.13
21	Caryophyllene	19.04	1230	1.40	2.20
22	Humulene	19.92	1238	0.21	-
23	γ-Muurolene	20.30	1246	0.20	0.23
24	Caryophyllene oxide	22.43	1253	1.55	0.56
25	Humulene-1,2-epoxide	22.89	1273	0.12	-
Total				99.49	99.62

*RT, retention time; RI, retention index (Kovach's index)

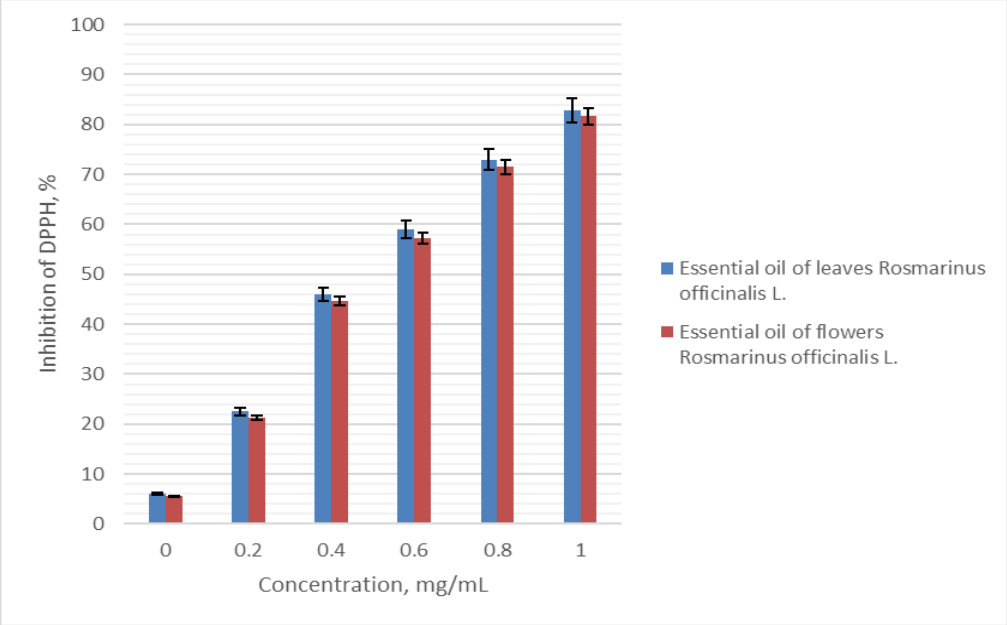

Fig. 1. Free radical-scavenging ability of *Rosmarinus officinalis* L. essential oils (leaves and flowers). The biological function of the chemical components of the *Rosmarinus officinalis* L. essential oil is not limited to their antioxidant and antimicrobial activity. Some of them also have antitumor (linalool, borneol), anti-inflammatory (pinene) and analgesic function [10, 16-18].

Twenty-five compounds were identified in the essential oil of leaves of *Rosmarinus officinalis* L. The main compounds were α-pinene (9.68%), β-pinene (7.16%), eucalyptol (4.27%), ρ-cymene (42.95%), camphene (4.44%), linalool (6.48%) and 2-bornanone (10.40%). Twenty compounds were identified in the essential oil of flowers of *Rosmarinus officinalis* L. The main compounds were the same, but linalool content was 20.40%. Linalool is contained in the flower of the aromatic plants, so the percentage in the essential oil of flowers of *Rosmarinus officinalis* L. is higher than the oil obtained from the leaves [11]. The chemical characterization by gas chromatography/ mass spectrometry (GC/MS) analysis of rosemary oils revealed the presence of many compounds, among them the most represented was ρ-cymene (42.95%) (leaves) and (42.35%) (flowers). This compound shows a variety of biological activities which include antioxidant, antinociceptive, anti-inflammatory, anxiolytic, anticancer and antimicrobial activities. Gema *et al.* [12] reported the presence of α-pinene, camphor and 1,8-cineole, constituting about 80% of the total *Rosmarinus officinalis* L. essential oil. Moreover, the major components α- and β-pinene, camphene, thimol, linalool were also reported to be in *Rosmarinus officinalis* L. essential oil [13]. The differences in chemical compositions of the *Rosmarinus officinalis* L. essential oils could be attributed to climatic effects on the plant.

These variations probably occur due to factors related to the oil extraction method, genetic characteristics of the species and environmental conditions in which they were grown.

Total antioxidant capacity based on the DPPH radical-scavenging assay

The antioxidant effect of rosemary is due to the polyphenols present in the leaves (mainly rosmarinic acid, carnosol and carnosic acid), which accumulate in the fatty membranes of cells where the antioxidant effect is required. One of the most significant aspects of the antioxidant activity of rosemary is the relationship between diterpenes and radical-scavenging activity. In this regard, the study by Munné-Bosch and Alegre [14] describes the antioxidant capacity of diterpenes in rosemary. The most important elements in the rosemary structure are the aromatic ring (C_{11}–C_{12}) in the catechol group together with the conjugation of the three basic rings. Fig. 1 shows the free radical-scavenging potential of different concentrations in the essential oils of leaves and flowers of *Rosmarinus officinalis* L., as determined by the DPPH assay. It could be seen from the results that, as the concentration of rosemary essential oils increased, the percentage of DPPH inhibition increased. The highest percentage of DPPH inhibition was for 1 mg/ml of leaves rosemary essential oil - 82.8% and of flowers rosemary essential oil - 81.7%. The antioxidant activity of both oils (leaves and flowers) is identical.

Wang *et al.* [15] reported that it is very difficult to attribute the antioxidant effect of a total essential oil to one or a few active principles (1,8-cineole, α-pinene and camphor), because an essential oil always contains a mixture of different chemical compounds. They also added that, in addition to the major compounds, also minor compounds may make a significant contribution to the oil activity. The results of antioxidant activity of *Rosmarinus officinalis* L. essential oil are needful by the food industry. *Rosmarinus officinalis* L. essential oil showed interesting results, being one of the best performing ones in terms of ability to neutralize free radicals.

Antimicrobial activity

Plant extracts and their secondary metabolites are rich sources of antimicrobial substances, including coumarins and psoralens, acetylenes, flavonoid and non-flavonoid polyphenols, and terpenes [19-23]. Monoterpenes (i.e., eucalyptol, borneol, camphor, bornylacetate, carvacrol, menthol, γ-terpinene, α-pinene, β-pinene, and p-cymene) are the most important constituents of essential oils produced through liquid extraction and steam distillation of edible and medicinal plants. In addition to the antibacterial and anti-biofilm activities of terpenes, in particular of p-cymene, an anti-inflammatory activity was also proved. It was concluded that limonene had an antibacterial effect weaker than the antifungal activity.

Table 2. Antimicrobial activity of *Rosmarinus officinalis* L. essential oils (leaves and flowers)

Test-microorganism	Leaves		Flowers	
	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm
Staphylococcus aureus NBIMCC 3703	12.50±0.40	6	12.00±0.40	60
Pseudomonas aeruginosa NBIMCC 1390	9.00±0.47	600	9.00±0.47	600
Salmonella sp.	10.00±0.47	60	9.00±0.47	60
Escherichia coli NBIMCC 3702	9.00±0.47	>600	10.00±0.47	>600
Bacillus subtilis NBIMCC 1208	12.00±0.40	6	12.00±0.40	6

* IZ, inhibition zones; MIC, minimum inhibitory concentration

The results showed that both essential oils (from leaves and flowers of *Rosmarinus officinalis* L.) are active against all the pathogenic microorganisms (Table 2). Gram-positive bacteria were more sensitive to the activity of the essential oil, the measured zones of inhibition were 12.5 mm, the minimum inhibitory concentration was 60 ppm. The tested Gram-negative bacteria showed zones of inhibition between 9 and 10 mm, with a minimum inhibitory concentration of more than 600 ppm. The observed difference in the sensitivity of the different test microorganisms to the examined essential oil was due to the difference in the cell wall structure and composition of the two groups of bacteria. The inhibitory effect of rosemary is the result of the action of rosmarinic acid, rosmaridiphenol, carnosol, epirosmanol, carnosic acid, rosmanol and isorosmanol. They interact with the cell membrane, causing changes in genetic material and nutrients, altering the transport of electrons, leakage of cellular components and production changes in fatty acid. In addition, they also produced an interaction with the membrane of proteins that caused the loss of membrane functionality and its structure [12]. The presence of an outer membrane in Gram-negative bacteria hinders the diffusion of the essential oil through the membrane to the cytoplasm of the cell, making them more resistant to the action of the oil. Other investigations have shown the antibacterial activity of rosemary oil against *E. coli*, *Bacillus cereus*, *Staphylococcus aureus* [24], *Clostridium perfringens*, *Aeromonas hydrophila*, *Bacillus cereus* and *Salmonella choleraesuis*. Zaouali *et al.* [25] reported that, compared with *S. aureus*, the antimicrobial activity improves with the presence of α-pinene as a major component. This effect can be correlated with the fact that terpenes can disorganize the cell membrane, and therefore promote the lysis. The effectiveness of the essential oil of rosemary against *E. coli* is related to the combined action of the different minor components present in its volatile fraction and should not be associated with the action of any particular component, agreeing with the conclusions published by Zaouali *et al.* [25]. The results obtained for the different resistance of Gram-positive and Gram-negative bacteria to inhibitors of microbial growth were consistent with literature data [9, 26].

Antioxidant and antimicrobial activity of rosemary essential oil depend on the fruiting stage,

mode of extraction, presence of a synergistic effect with other components, and concentration of active components. If these aspects are taken into account, the application of this natural product can be complimented in different food systems such as sausages, vegetable, meat and fish canned food, chutneys, mayonnaise, ketchup, salad dressings, processed cheese and more. In view of its application, rosemary essential oil could be used in functional foods, pharmaceutical products, plant products and for food preservation.

CONCLUSIONS

Natural extracts can be obtained from leaves, flowers, peel and seeds. The studied rosemary essential oils (obtained from leaves and flowers of *Rosmarinus officinalis* L.) can act as antioxidant and antimicrobial agents in food products to replace synthetic additives. The chemical composition (determined by GC/MS), antioxidant ability (measured with 2,2-diphenyl-1-picrylhydrazyl (DPPH)) and their antimicrobial force (using a diffusion disk method with *Staphylococcus aureus* NBIMCC 3703, *Salmonella* sp., *Pseudomonas aeruginosa* NBIMCC 1390, *Bacillus subtilis* NBIMCC 1208, *Escherichia coli* NBIMCC 3702) were determined. The results show that both extracts are good antioxidant and antimicrobial agents *in vitro*, and their antioxidant and antimicrobial capacity can also be used in food products, acting as natural preservatives.

Acknowledgements: *This work was supported by the Bulgarian National Fund "Scientific Research" under the project DM 17/4 from 12.12.2017 "Biopreservation of salad dressings and sauces with aromatic components and probiotic bacteria" and KP-06-Rila/2 from 20.12.2018 "Bio-preservation by synergistic action of probiotics and plant extracts".*

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