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Relevance of Carnosic Acid, Carnosol, and Rosmarinic Acid Concentrations in the in Vitro Antioxidant and Antimicrobial Activities of *Rosmarinus officinalis* (L.) Methanolic Extracts

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ABSTRACT: The importance of the diterpenic and rosmarinic acid content in the biological activities of rosemary extracts has been studied previously, but how the relationship between the concentration of these components affects their antioxidant and antibacterial activities has received little attention. Accordingly, from a total of 150 plants, 27 methanolic extracts were selected, for their similar diterpene contents but different ratios between carnosic acid and carnosol concentrations. In extracts with similar rosmarinic acid contents but differing proportions between carnosic acid and carnosol, the two diterpenes were seen to equally affect the in vitro antioxidant activity; however, and related with the antibacterial efficiency, this biological activity improved when carnosol was the major diterpene component.

KEYWORDS: *Rosmarinus officinalis L, carnosic acid, carnosol, rosmarinic acid, antioxidant activity, antimicrobial activity*

INTRODUCTION

Rosemary (*Rosmarinus officinalis*) is one of the most collected, transformed, and traded of aromatic plants.[1,2] According to the scientific literature, rosemary essential oil exhibits numerous biological activities. To name a few examples, rosemary essential oil has demonstrated antimicrobial[3,4] and cognition-improving[1,5−8] qualities. It has been shown to enhance the resistance of rat hepatocytes against DNA-damaging oxidative agents,[9] even in testicular cells.[10] It is also known that rosemary improves local blood circulation, relieves pain,[11] has anticancer activity,[12] and controls blood lipid and antilipid peroxidation,[13] among many other activities.

Furthermore, following extraction of the essential oil, the material remaining postdistillation is considered to be a natural source of antioxidants.[14]

Generally, solvent extraction is the most commonly used method to extract these natural antioxidants; however, the technique has several drawbacks, including low selectivity and elimination of solvent residues that are often prohibited by food regulations.[15] Given this situation, supercritical fluid extraction (SFE) has been proposed for the direct extraction of phenolics from rosemary leaves.[16−21]

The antioxidant activity of these extracts is related to the presence of phenolic abietane diterpenes, such as carnosic acid and its derivatives, carnosol, rosmadial, rosmanol, rosmanol isomers, and methyl carnosate, and phenolic acids such as rosmarinic acid.[14,22,23] Also, previous in vitro investigations have shown that the antioxidant activity of rosemary extracts is primarily associated with the amount of carnosoic acid and, subsequently, the total amount of phenolic diterpenes present.[24]

Recently, Pérez-Fons et al.[22] published that rosmarinic acid, carnosic acid, carnosol, rosmadial, and genkwanin are responsible for the antioxidant activity of rosemary leaf extracts, although the mechanisms by which these compounds act are quite different. For example, and in contradiction with previous works published, carnosol was demonstrated to be the strongest antioxidant compound in the thiobarbituric acid-reactive substances (TBARS) assay, whereas no significant differences in the Trolox equivalent antioxidant capacity (TEAC) values were obtained between the hydrophobic diterpenes carnosol and carnosic acid and the hydrosoluble compound rosmarinic acid.

Besides acting as antioxidants, rosemary extracts have also been seen to exhibit several physiological and medicinal activities.[1,25,26]

Along these lines, the search for natural substances with antimicrobial activity is of increasing intensity, and rosemary has been considered interesting by some researchers because of its use in popular medicine in the form of remedies for many infectious diseases.[4,27−31] Microbial contamination of food is an important public health and economic problem and, here again, the benefits of rosemary extract as a powerful antimicrobial agent in the prevention of food spoilage have been demonstrated. For example, Bernardes et al.,[4] Weerakkody et al.,[28] Klancnik et al.,[29] Tornuk et al.,[30] Vegara et al.,[31] and Klancnik et al.[32] described the minimum inhibitory concentration (MIC) and minimum bactericide concentration (MBC) of commercial rosemary extracts against Gram-positive (*Bacillus* and *Staphylococcus*) and Gram-negative (*Campylobacter* and *Salmonella*) bacteria. For these authors, the antimicrobial and

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antioxidant activities depended on the concentration and chemical nature of the phenolic compounds in the extracts. Gram-positive bacteria were more sensitive than Gram-negative bacteria, especially in the face of oil-soluble extracts, with carnosic acid being the major phenolic compound. These results agree with those published by Castano et al.,[33] because the antimicrobial activity of rosemary extracts against *Shigella sonnei*, *Salmonella* Typhimurium, and *Listeria monocytogenes* yielded MIC values of 1024 ppm.

However, the bibliography consulted concerning the antioxidant and antimicrobial activities of rosemary refers only to pure compounds or rosemary (hydrophobic or hydrophilic) leaf extracts, without taking into consideration the relative concentrations of the major diterpenic components responsible for this biological activity.

In light of the above, the main goal of the present study was to evaluate the relevance of the concentration ratio of the two major diterpenes (carnosic acid and carnosol), along with rosmarinic acid, on the antioxidant and antimicrobial activities of rosemary extracts.

MATERIALS AND METHODS

Plant Material. A total of 27 samples of wild rosemary plants (with very similar diterpene contents) were selected from among 151 shrubs collected from 31 wild populations located at different altitudes (from sea level to 1500 m above sea level) in the province of Murcia between July and August 2009. Cuttings from new shoots of individual plants were collected at the phenological stage of fruit maturation. Before essential oil extraction, the plant material was dried in a forced-air dryer at 35 °C for 48 h, until it reached a constant weight. The air-dried aerial parts of each sample were them submitted to hydrodistillation for 3 h using a Clevenger-type apparatus.

Chemicals. 2,2-Diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$), 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt [ABTS-$(NH_4)_2$], 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and potassium persulfate were purchased from Sigma-Aldrich (Madrid, Spain). Methanol, acetonitrile, petroleum ether, formic acid, ethanol, glacial acetic acid, hydrochloric acid, $FeCl_3 \cdot 6H_2O$, sodium acetate, and anhydrous sodium sulfate were supplied by Scharlau Chemie S.A. (Sentmenat, Spain). 2,4,6-Tripyridyl-*s*-triazine (TPTZ) was obtained from Fluka (Madrid, Spain). Methanol and acetonitrile were of HPLC grade, and all other reagents were of analytical grade.

High-purity standards, carnosic and rosmarinic acids and the lactone carnosol, were purchased from Sigma-Aldrich.

Extraction of Polyphenolic Compounds. Distilled plant material was dried in a forced-air dryer at 35 °C for 48 h (until it reached a constant weight) and then ground to pass through a 2 mm mesh. Dried samples (0.5 g) were first extracted with 20 mL of petroleum ether while stirring and taken to dryness at room temperature. Second, they were extracted using 150 mL of methanol in a Soxhlet extractor (B-811) (Buchi, Flawil, Switzerland), for 2 h under a nitrogen atmosphere. Methanolic extracts were taken to dryness at 40 °C under vacuum conditions in an evaporator system (Syncore Polyvap R-96) (Buchi). The residue was redissolved in methanol and made up to 5 mL.[34] The yield of the extracts was expressed in terms of milligrams of dry methanolic extract per gram of dry plant weight. Final extracts were kept in vials at −80 °C until their corresponding analysis.

HPLC Analysis. For the HPLC analysis, a method adapted from Zheng and Wang et al.[35] was followed using a reverse phase Zorbax SB-C18 column (4.6 mm × 250 mm, 5 μm particle size, Hewlett-Packard, USA) with a guard column (Zorbax SB-C18 4.6 mm × 125 mm, 5 μm pore size, Hewlett-Packard, USA) at ambient temperature. Extracts were passed through a 0.45 μm filter (Millipore SAS, Molsheim, France), and 20 μL was injected into a Hewlett-Packard (Germany) system equipped with a G1311A quaternary pump and G1315A photodiode array UV−vis detector. The mobile phase was acetonitrile (A) and acidified water containing 5% formic acid (B). The gradient was as follows: 0 min, 5% A; 10 min, 15% A; 30 min, 25% A; 35 min, 30% A; 50 min, 55% A; 55 min, 90% A; 57 min, 100% A, held for 10 min before returning to the initial conditions. The flow rate was 1.0 mL/min, and the wavelengths of detection were set at 280 and 330 nm. The identification of the phenolic components was made by comparison of retention times and spectra with those of commercially available standard compounds. For quantification, linear regression models were determined using standard dilution techniques. Phenolic compound contents were expressed in grams per kilogram of dry methanolic extract.

DPPH$^{\bullet}$ Radical Scavenging Activity. The ability of the 27 methanolic extracts to scavenge DPPH$^{\bullet}$ free radicals was determined according to the method described by Brand-Williams et al.[36] Briefly, 500 μL of methanolic extracts at different concentrations (2.4−10 μL/mL) were added to 1 mL of DPPH$^{\bullet}$ methanolic solution (0.1 mM). Decolorations were measured using a Shimadzu (UV-2401PC, Japan) spectrophotometer at 517 nm after incubation for 20 min at room temperature in the dark. Absorbance was measured against a blank of 500 μL of sample plus 1 mL of methanol. The absorbance of the control consisting of 500 μL of methanol and 1 mL of DPPH$^{\bullet}$ solution was measured daily against a blank of 1.5 mL of methanol. Measurements were performed in triplicate.

DPPH$^{\bullet}$ radical scavenging activity:

$$\%\ \text{decoloration} = [1 - (\text{absorbance sample}/\text{absorbance control})] \times 100$$

The results were expressed as the inhibitory concentration of the extract needed to decrease DPPH$^{\bullet}$ absorbance by 50% (IC_{50}). Concentrations are expressed in micrograms of dry plant methanolic extract per milliliter of methanol.

ABTS$^{\bullet+}$ Radical Cation Decoloration Assay. The ABTS free radical scavenging activity of each sample was determined according to the method described by Re et al.[37] ABTS$^{\bullet+}$ radical cation was produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 16 h before use. A working solution was diluted with ethanol to an absorbance of 0.70 (±0.02) nm (constant initial absorbance value used for standard and samples) at 734 nm and 30 °C. An aliquot (15 μL) of each sample (with appropriate dilution) or Trolox standard was mixed with the working solution (1.5 mL) of ABTS$^{\bullet+}$, and the decrease of absorbance was measured after 6 min at 734 nm using a Shimadzu (UV-2401PC, Japan) spectrophotometer. Measurements were performed in triplicate. The ABTS$^{\bullet+}$ scavenging rate was calculated to express the antioxidant ability of the sample, and results were expressed in terms of Trolox equivalent antioxidant capacity (TEAC, micromolar Trolox equivalents per milligrams of dry plant methanolic extract).

Ferric Reducing Antioxidant Power (FRAP). The ability to reduce ferric ions was measured using the method described by Benzie and Strain et al.[38] The FRAP reagent was freshly prepared from 300 mM acetate buffer, pH 3.6, and 10 mM 2,4,6-tripyridyl-*s*-triazine (TPTZ) made up in a 40 mM HCl and 20 mM $FeCl_3 \cdot 6H_2O$ solution. All three solutions were mixed together in the ratio of 10:1:1 (v/v/v). An aliquot of 40 μL of each sample (with appropriate dilution) was added to 1.2 mL of FRAP reagent. The absorption of the reaction mixture was measured at 593 nm after 2 min of incubation at 37 °C. Measurements were performed in triplicate. Fresh working solutions of known Fe(II) concentrations ($FeSO_4 \cdot 7H_2O$) (0−12 mM) were used for calibration. The antioxidant capacity based on the ability to reduce ferric ions in samples was calculated from the linear calibration curve and expressed as millimolar $FeSO_4$ equivalents per milligram of dry plant methanolic extract.

Bacterial Strains and Growth Conditions. The antimicrobial activity of the 27 rosemary methanolic extracts was tested against four common foodborne pathogens including two Gram-positive strains, *Listeria monocytogenes* serovar 4b (CECT 935) and *Staphylococcus aureus* (CECT 240), and two Gram-negative strains, *Salmonella* Typhimurium (CECT 443) and *Escherichia coli* serovar O157:H7 (CECT 4267). All of the strains were obtained from the Spanish

Table 1. Quantitative Composition of Rosemary Extracts

carnosic acid/carnosol ratio: extract yield (g/kg):[a]	60:40 137.2 ± 32.29	50:50 128.8 ± 21.92	40:60 134.1 ± 24.98
	Phenolic Acids (g/kg)[b]		
gallic acid	1.1 ± 0.1	0.7 ± 0.2	0.6 ± 0.2
caffeic acid	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
ferulic acid	0.4 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
p-coumaric acid	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
rosmarinic acid	14.3 ± 9.9	7.6 ± 2.9	14.6 ± 4.6
	Flavonoids (g/kg)		
apigenin	0.5 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
genkwanin	4.0 ± 0.4	2.9 ± 0.5	2.9 ± 0.4
hesperidin	12.1 ± 0.9	8.4 ± 1.3	7.6 ± 1.0
luteolin	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
	Diterpenes (g/kg)		
carnosol	28.0 ± 2.7	31.4 ± 1.9	41.7 ± 4.5
carnosic acid	39.4 ± 3.4	30.4 ± 2.4	27.9 ± 3.4
diterpenes (total content)	67.4 ± 5.2	61.8 ± 3.8	69.6 ± 7.9

[a]Methanolic extract yield expressed as grams of extract per kilogram of distilled dry rosemary leaves. [b]Grams of polyphenol per kilogram of methanolic extract.

Collection of Type Cultures, and the culture was kept frozen at −80 °C in cryovials. Bacteria were grown in trypticase soy broth (Merck, Darmstadt, Germany) supplemented with 0.6% of yeast extract (Merck) and incubated at 37 °C.

Antimicrobial Assay (Disk Diffusion Technique). A filter paper disk (Whatman no. 1, 6 mm diameter) containing 20 μL of each methanolic extract was placed on the agar surface (previously seeded by spreading 0.1 mL of overnight culture). Disks of streptomycin (0.025 g/L) and methanol were used as negative and positive control, respectively. The plates were incubated overnight at 37 °C, and the diameter of the inhibition zone and the diameter of the disk were calculated. Results are expressed as the percentage of inhibition growth compared with the streptomycin (0.025 g/L) assay (100% of inhibition). All of the data collected are the averages of three determinations.

Statistical Analysis. All data were reported as the mean ± standard deviation of at least three experiments. Data were analyzed by an analysis of variance ($p < 0.05$), and the means were separated by Duncan's multiple-range test (ANOVA procedure). Results were processed by Excel and Statistica software (1998).

RESULTS AND DISCUSSION

Chromatographic analysis of 150 individual rosemary polyphenolic extracts pointed to the wide intraspecific variability that may be found among plants growing in the same geographical area. It is known that intrinsic (genetic and plant age) and extrinsic (edaphoclimatic) factors affect the quantitative chemical composition of wild rosemary plants.[39] This led us to wonder about a possible optimal relationship between the concentrations of the major components that would define the antioxidant and antimicrobial activities of these plant extracts. From the scientific bibliography, it is well-known that these biological properties are related to the major or minor presence of carnosic acid, its lactone carnosol, and rosmarinic acid in the polyphenolic extracts.[22] Aruoma et al.[40] published that approximately 5% of the dry weight of rosemary leaves is made up of carnosol and carnosic acid, although the same fraction was estimated to account for >90% of the antioxidant activity.

On the basis of these statements, and bearing in mind the major polyphenolic components quantified in the methanolic extracts (Table 1), a total of 27 rosemary extracts with very similar polyphenolic contents, but different proportions of carnosic acid and carnosol (40:60, 50:50, and 60:40; estimated by considering the total amount of these two diterpenes in the methanolic extracts), were selected, from 150 extracts, to determine the effect of the carnosic acid/carnosol ratio on the in vitro antioxidant and antimicrobial activities of these plant extracts. Of particular note was the low concentration at which rosmarinic acid and carnosic acid were detected. In this respect, it is important to clarify that, according to Almela et al.,[41] the hydrodistillation process to which rosemary leaves are submitted before methanolic extraction reduces by >10-fold the concentration of these two polyphenolic components.

Antioxidant Activity. To describe the antioxidant properties, the free radical scavenging activity was assessed by using the DPPH$^{\bullet}$ test, the ABTS$^{\bullet+}$ radical cation decoloration assay, and the FRAP test.

It is interesting that, despite the different techniques applied, with their different mechanisms and different specificities for some antioxidants, rosmarinic acid was seen to play an important role in the antioxidant capacities of the extracts containing very similar diterpene concentrations. As shown in Table 2, the extracts with a lower rosmarinic acid concentration

Table 2. Antioxidant Activities of Rosemary Extracts[a]

carnosic acid/carnosol	DPPH$^{\bullet}$ (IC_{50}, μg/mL)	ABTS$^{\bullet+}$ (mM Trolox/mg)	FRAP (mM Fe^{2+}/mg)
40:60	29.4 ± 2.8a	385.9 ± 30.3b	11.6 ± 1.7b
50:50	51.9 ± 6.3b	274.8 ± 44.1a	7.9 ± 1.3a
60:40	32.9 ± 5.4a	372.4 ± 94.9ab	12.1 ± 2.8b

[a]Values followed by different letters share significant differences at 95% (Duncan's test); values are the mean of at least three independent replicates ± SD.

(50:50) exhibited a poorer antioxidant capacity. Although Wellwood et al.[42] affirmed that the rosmarinic acid content did not correlate well with the measured antioxidant activity, our results agree with the recent publication of Pérez-Fons et al.,[22] who found that the TEAC (mM Trolox equivalents/g of extract needed to neutralize the ABTS$^{\bullet+}$ radical) values of hydrophilic rosemary extracts containing rosmarinic acid showed higher antioxidant activity than diterpene-enriched lipophilic extracts. Because the ABTS$^{\bullet+}$ radical is soluble in water and organic

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solvents, this technique allows the antioxidant capacity of both hydrophilic and lipophilic extracts to be determined,[43] and therefore the presence of rosmarinic acid in the rosemary extracts enhances their antioxidant capacity.

Another interesting point is whether the relative carnosic acid and carnosol concentrations in the extracts with very similar rosmarinic acid contents affect the antioxidant capacity. According to the bibliography consulted, controversy exists concerning the greater or lesser antioxidant power of carnosic acid compared with its lactone carnosol. For instance, Del Baño et al.[44] mentioned that the high antioxidant activities of hydroalcoholic rosemary extracts in both lipophilic and hydrophilic media are directly related to the presence of carnosic acid and carnosol. Pérez-Fons et al.[22] revealed that a membrane-based antioxidant assay (TBARS) showed that the hydrophobic diterpene carnosol exhibited the highest antioxidant activity (4.4 and 5.7 times higher than those of rosmarinic acid and carnosic acid, respectively).

Nevertheless, and in agreement with Del Baño et al.,[44] the results obtained in the present work show that the greater or lesser presence of carnosic acid in relation with carnosol (in extracts with the same amount of rosmarinic acid) did not affect the in vitro antioxidant activities of the methanolic rosemary extracts. The total major diterpene content defines the antioxidant capacity of rosemary extracts, because no statistically significant differences were detected between the groups 60:40 and 40:60 (carnosic acid/carnosol ratio).

Antimicrobial Activity. The in vitro inhibitory activity of the rosemary methanolic extracts was more effective against the Gram-positive than the Gram-negative foodborne pathogens assayed. For this reason, the results presented here reflect only the antibacterial activity against *S. aureus* (Figure1) and *L. monocytogenes* (Figure2).

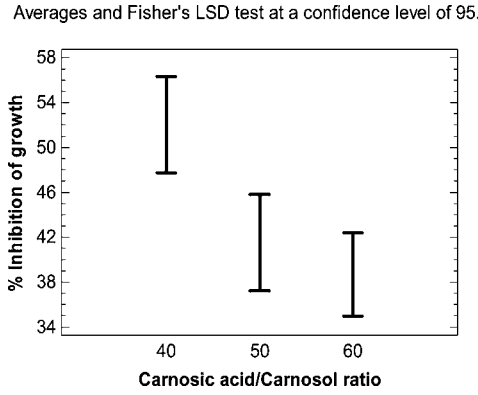

Figure 1. Antibacterial activity of rosemary methanolic extracts against *Staphyloccocus aureus*.

Others researchers have pointed to the low antimicrobial activity exhibited by methanolic rosemary extracts from Turkey.[45] Also, and in relation with rosemary essential oil, Bozin et al.[46] and Klancnik et al.[32] concluded that, in general, the Gram-positive strains of bacteria tested seemed to be more sensitive to the essential oil, although this study also recorded a notable susceptibility of the Gram-negative pathogenic bacteria examined.

According to the results obtained in the present work, the rosmarinic acid concentration of the rosemary extracts with very similar diterpene contents does not affect the antibacterial activity. Nevertheless, the relationship between the diterpenes

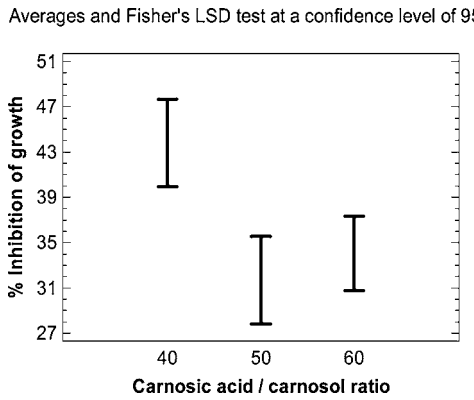

Figure 2. Antibacterial activity of rosemary methanolic extracts against *Listeria monocytogenes*.

carnosic acid and its lactone carnosol seems to affect the effectiveness of the extracts. As shown in Figures 1 and 2, a higher concentration of carnosol in relation to carnosic acid improves to a statistically significant degree the antibacterial activities of the rosemary extracts against *L. monocytogenes* and *S. aereus* strains. These results highlight the important role played by carnosol in the antibacterial activity of rosemary extracts, in contrast to the findings of different authors such as Klancnik et al.[32] and Bubonja-Sonje et al.,[47] who found that the biological activities of rosemary extracts are directly related to the presence of carnosic acid as the major phenolic component.

On the basis of the results obtained in the present study, it can be concluded that carnosic acid and carnosol (in extracts with similar rosmarinic acid contents) play a similar role in in vitro antioxidant activity; however, and with regard to antibacterial efficiency, this biological activity is improved by the presence of carnosol as the major diterpene content.

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Notes

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