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Seasonal variations of rosmarinic and carnosic acids in rosemary extracts. Analysis of their *in vitro* antiradical activity

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Abstract

Rosemary plants were analysed using HPLC and eight different compounds (vanillic acid, caffeic acid, rosmarinic acid, naringin, hispidulin, cirsimaritin, carnosol and carnosic acid) were identified and quantified. The analysis of the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity revealed that rosmarinic and carnosic acids were the best rosemary scavengers with IC_{50} values of 27 and 32 μM, respectively. Environmental influences on rosmarinic and carnosic acids content in rosemary plants were studied over a period of one year under southern UK conditions. Carnosic acid reached the maximum concentrations in December, decreasing by 50% during the summer months, while rosmarinic acid showed a constant concentration during the year. The significance of these results has been discussed later in this paper.

Additional key words: antioxidant, diterpene, DPPH, phenolic acids, rosemary.

Resumen

Variación estacional de los ácidos rosmarínico y carnósico en extractos de romero. Análisis de su actividad antirradicalaria *in vitro*

Se analizaron plantas de romero usando HPLC, hallando ocho compuestos diferentes los cuales fueron identificados y cuantificados. El análisis de la capacidad antioxidante, analizada usando el radical DPPH, reveló que los ácidos rosmarínico y carnósico presentaron una alta capacidad antirradicalaria con valores de IC_{50} de 27 y 32 μM respectivamente. La influencia de las condiciones ambientales del sur de Inglaterra en el contenido de los ácidos rosmarínico y carnósico en plantas de romero fue estudiada durante un periodo de un año. Los resultados mostraron que el ácido carnósico alcanzó concentraciones máximas durante el mes de diciembre, disminuyendo su concentración en un 50% durante los meses de verano. El ácido rosmarínico mostró, sin embargo, una concentración casi constante durante todo el año. La importancia de estos resultados ha sido argumentada a lo largo de este artículo.

Palabras clave adicionales: ácidos fenólicos, antioxidante, diterpenos, DPPH, romero.

Introduction

Since prehistoric times, herbs have been used as flavourings, beverages, repellents, fragrances, cosmetics and for their medicinal properties. Nowadays, the interest in herbs has considerably increased, particularly as a natural source of antioxidants for the food and pharmaceutical industries. Rosemary (*Rosmarinus officinalis* L.), for example, is an economically important herb known not only as a source of essential oils but also for its natural antioxidants (Cuvelier *et al.*, 1996; Zheng and Wang, 2001; Ibáñez *et al.*, 2003). The presence of diterpenes such as carnosic acid and carnosol, two natural compounds with antioxidant activity, has been reported (Aruoma *et al.*, 1992; Schwarz and Ternes, 1992; Frankel *et al.*, 1996) and several flavonoids and phenolic compounds such as hispidulin, cirsimaritin, apigenin, genkwanin, naringin, caffeic acid and rosmarinic acid are also present in

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rosemary extracts (Zheng and Wang, 2001; Ibáñez *et al.*, 2003).

The antioxidant activity of rosemary extracts depends on their composition. There are many reports that analysed and determined their antioxidant capacity by various methods using lipid and aqueous systems. In lipid systems, extracts with higher diterpene content were the most effective (Hopia *et al.*, 1996), while in aqueous systems rosmarinic acid exhibited the highest antioxidant activity (Frankel *et al.*, 1996; Cuvelier *et al.*, 2000). Several reports have been published analysing the distribution of rosmarinic and/or carnosic acids during growth and vegetative development of rosemary leaves (Hidalgo *et al.*, 1998; Munné-Bosch *et al.*, 1999; Munné-Bosch and Alegre, 2000; Del Baño *et al.*, 2003; Ibáñez *et al.*, 2003; Munné-Bosch and Alegre, 2003), however, to our knowledge this is the first report were the influence of south UK environmental growing conditions were studied simultaneously on both rosmarinic and carnosic aids.

Therefore, the aims of this study were: to identify and characterise the most abundant compounds (rosmarinic and carnosic acids) in rosemary plants (cultivar Sissinghurst English); to study the quantitative distribution of rosmarinic and carnosic acids in different plant organs and the seasonal variations observed during one year period using HPLC (high performance liquid chromatography) and diode array detection; and to evaluate the antiradical activity for rosemary extracts and rosmarinic and carnosic acids using the DPPH radical system and ascorbic acid as a reference.

Material and Methods

Chemicals

All solvents used in the experiments were HPLC grade and were purchased from Fisher Scientific (UK). Chemicals such as ascorbic acid, BHT (butilated hydroxytoluene) and DPPH (1,1-diphenyl-2-picrylhydrazyl) were purchased from Sigma-Aldrich Company Ltd. (UK). The standards caffeic and vanillic acids were purchased from Sigma-Aldrich Company Ltd. (UK). Rosmarinic acid was purchased from ICN Pharmaceuticals, Ltd. (UK). Carnosic acid was obtained from the National Herb Centre (Banbury, UK). Naringin, apigenin, hispidulin and cirsimaritin were obtained from the Phytochemistry laboratory, Department of Botany, University of Reading.

Plant Material

Rosmarinus officinalis L. plants (cultivar Sissinghurst English) were selected at the National Herb Centre (Banbury, UK). Rosemary plants were grown in pots of 2 L capacity with a mixture of soil: peat: sand (1:1:1 v v^{-1}) for 12 months before being used in any experiment. The plants were maintained in a glasshouse with ambient day temperatures of 17-25ºC during sunless days and 28-35ºC during sunny days, and they were watered daily with tap water twice a week with Hoagland solution. In order to analyse the seasonal variations of rosmarinic and carnosic acids, 40 plants grown as described above were transplanted into the experimental fields of The University of Reading on July 2001 and were watered with 10 mm d^{-1} during this month. Thereafter, plants grew under normal environmental conditions for the southern UK, receiving water exclusively from rainfall.

Sampling

Rosemary plants were sampled at the beginning and at the end of each month before 9 am between winter 2001 and winter 2002. Seasonal variations of rosmarinic and carnosic acids were analysed by using only the leaves of rosemary plants. The plant distribution of rosmarinic and carnosic acids was analysed by collecting petals, sepals, leaves, stems and roots. The plants' water content was monitored by measuring the relative water content (RWC) as: RWC (%) = (fresh weight)-(dry weight)/(turgid weight)-(dry weight) × 100. Leaf dry weight was calculated after 24 hours at 85ºC while the turgid weight was calculated after equilibration in distilled water for 24 hours.

Extraction method

Fresh plant material (1 g) was ground in liquid nitrogen and extracted three times with 15 ml of methanol for 15, 10 and 5 min at room temperature (RT), in a sonic bath. The combined extracts were evaporated to dryness under reduced pressure at 30ºC. The residues were dissolved in 1 ml of methanol and kept at -20ºC for no more than 24 h before the analysis.

HPLC analysis

Before the HPLC analysis all the samples were filtered through a 0.45 μm filter. Aliquots of 20 μl were injected into a reverse phase Hypersil H5 ODS column (250 × 4.6 mm i.d.). A Waters 600 System controller coupled with a photodiode array detector Waters 994 series or a Waters 490E programmable multiwavelength detector were used. Separation and quantification were achieved at 25ºC by using the gradient acetonitrile (solvent A) and acidified water containing 2.5% of acetic acid (solvent B). The gradient was as follows: 0 min, 10% A; 10 min, 20% A; 30 min, 30% A; 35 min; 50% A; 50 min, 60% A; 55 min, 90% A; 57 min, 100% A; 67 min, 100% A; 68 min; 10% A.

After 68 min the gradient was recycled to initial conditions and held for 10 min before a new injection. The flow rate was 1 ml min^{-1} and the detection was set at 280 nm, a wavelength at which all compounds could be detected and quantified. Identification of individual compounds was based on the comparison of the actual retention time to those of reference authentic standards. Carnosol was quantified as carnosic acid and all other compounds as themselves. The values obtained for carnosol using carnosic acid were recalculated using a relative response factor of 1.36 at 280 nm to get an accurate estimate of carnosol content (Thorsen and Hildebrandt, 2003).

DPPH assay conditions

The total DPPH radical scavenging activity of rosmarinic and carnosic acids was estimated using the stable DPPH radical (Lu and Foo, 2001). Freshly made DPPH radical (200 μM) (Sigma-Aldrich) was mixed with methanolic extracts of rosemary main secondary metabolites to start the reaction. Rosemary extracts were also tested using fresh plant material ground in liquid nitrogen and extracted with methanol at room temperature (RT) in a sonic bath as described before. The total μg ml^{-1} of antioxidants used was calculated from the total phenolic content of the extracts (the sum of rosmarinic acid, caffeic acid, vanillic acid, naringin, apigenin, hispidulin, cirsimaritin, carnosol, and carnosic acid concentrations), quantified by the HPLC.

A control containing no tested compounds or extracts was included. The absorbance at 517 nm of DPPH was measured in a spectrophotometer (Ciba-Corning UK, 2800 Spectroscan) against a blank of pure methanol after 30 min at RT. DPPH radical scavenging capacity was estimated from the difference in absorbance with or without tested compounds or extracts and expressed as a percentage of DPPH scavenged in solution. The IC_{50} value represents the concentration of an individual compound required to quench 50% DPPH under experimental conditions. All the tests were done in triplicate.

Results

HPLC analysis and rosmarinic and carnosic acids variations in rosemary plants

Although numerous phenolics, flavonoids, and diterpenes have been reported in rosemary extracts, only vanillic acid, caffeic acid, rosmarinic acid, naringin, hispiduling, cirsimaritin, carnosol, and carnosic acid were present in sufficient amount to be identified and quantified in this study. For quantification purposes and to guarantee full extraction and reproducibility of the method, one sample was subjected to a set of extraction conditions using different amounts of material, solvent, and extraction times (data not shown). The best results were obtained using a three-fold extraction (see Material and Methods). In addition, the quantification of rosemary compounds at 280 nm, a wavelength at which all compounds were detected, made routine analysis more feasible allowing the quantification of all compounds in only one HPLC run, even when the photodiode array detector was not available.

Concentrations of the eight compounds from rosemary extracts were studied by using the optimum extraction and HPLC methodology. Rosmarinic and carnosic acids were the most abundant compounds followed by naringin and carnosol (Table 1). Additionally, when the plant distribution of these two main components was studied in rosemary extracts, rosmarinic acid was found in leaves, stems, and roots, while sepals and petals hardly contained the phenylpropanoid (Fig. 1a). However, carnosic acid was only found in leaves, sepals and petals of rosemary plants while the diterpene was not detected in stems and roots (Fig. 1b).

The foliar content of rosmarinic and carnosic acids in rosemary plants growing in the experimental fields of The University of Reading was studied over a year. Carnosic acid concentration levels decreased by 50% during the summer months (Fig. 2a), corresponding with the highest temperature and lowest precipitation rates, showing a recovery during September, October and

Table 1. Identified compounds and concentration levels in rosemary plants. Retention times are expressed in minutes, and concentrations in mg g^{-1} fresh weight biomass. The data represent the mean ± standard deviation for n = 6 different determinations. ND indicates not detected

Compound	Retention time	UV, λ max	Concentration
Apigenin	37	267, 340	ND
Caffeic acid	9	296, 324	0.012 ± 0.0006
Carnosic acid	57	284	12.18 ± 0.609
Carnosol	52	284	0.53 ± 0.0219
Cirsimaritin	43	274, 334	0.080 ± 0.0040
Hispulin	38	270, 336	0.020 ± 0.0010
Naringin	20	284, 334	0.57 ± 0.028
Rosmarinic acid	23	290, 330	2.15 ± 0.104
Vanillic acid	8	260, 292	0.004 ± 0.0002
Total phenolics			15.54 ± 0.769

November and reaching maximum levels in December (Fig. 3). Rosmarinic acid levels showed a slight increase during the summer, reaching maximum values in September, and being almost constant during the rest of the year (Fig. 2b). However, the relative water content of rosemary leaves was not concomitant with carnosic acid variations. In fact, precipitation levels below 60 mm of rain during the summer did not affect the relative water content of rosemary leaves, keeping the relative water content values over 70% during this period (Fig. 2c).

Antiradical activity of rosemary compounds

In order to characterise the antiradical properties of rosmarinic and carnosic acids in rosemary leaves, the ability of pure rosmarinic acid, carnosic acid and related compounds was determined. Rosmarinic acid had an excellent DPPH radical scavenging activity with an IC_{50} value of 27 μM under experimental conditions. Carnosic and caffeic acids showed a similar DPPH scavenging capacity with IC_{50} values of 32 μM and 38 μM, respectively, both significantly higher than ascorbic acid, vanillic acid and naringin (47 μM, over 200 μM, and over 200 μM, respectively) (Table 2).

In addition, the scavenging capacities of pure rosmarinic acid, caffeic acid, carnosic acid and naringin were studied *in vitro* using different combinations. The results (Table 3) showed a positive relationship between the concentration used and the DPPH scavenging capacity observed when combinations of rosmarinic acid with caffeic acid, carnosic acid and naringin were used. These data reinforced the idea that the DPPH scavenging activity of rosemary extracts could be the cumulative DPPH scavenging capacities of several compounds, which contributed to the overall scavenging activity depending on their concentration in the extracts. However, studying and testing the relative concentration of other compounds in the extracts, such as carnosol, should allow the confirmation of this hypothesis (Fig. 4).

Discussion

The HPLC analysis of rosemary plants revealed eight different compounds in sufficient amounts to be quantified. These can be grouped into three classes: hydroxycinnamic acids and ester, flavonoids, and diterpenes. These three groups of compounds, as determined in this study, were similar in content and concentration to the data reported in previous studies showing rosmarinic and carnosic acids as the most

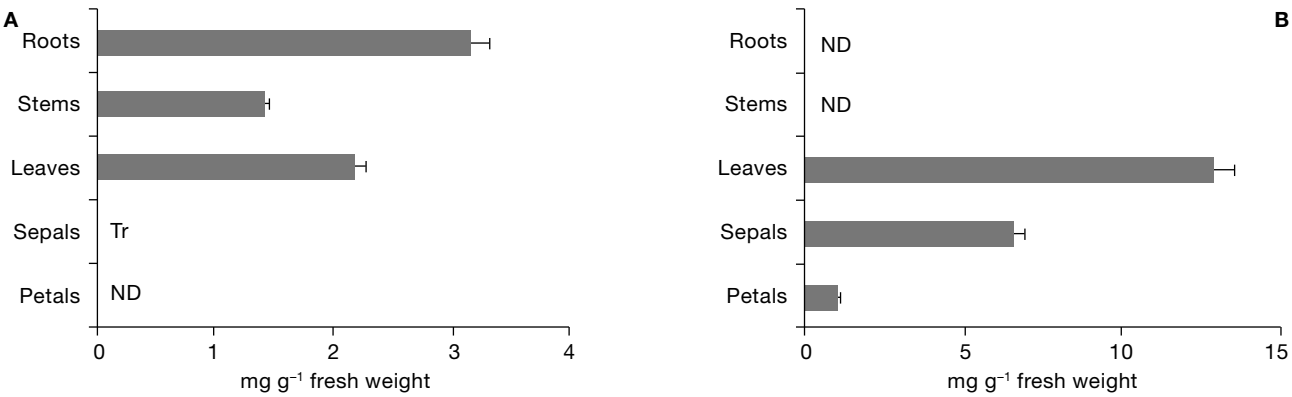

Figure 1. Tissue distribution of rosmarinic acid (a) and carnosic acid (b) in rosemary plants. The data, expressed as mg g^{-1} of fresh weight, represent the mean ± standard deviation for n = 3 different determinations.

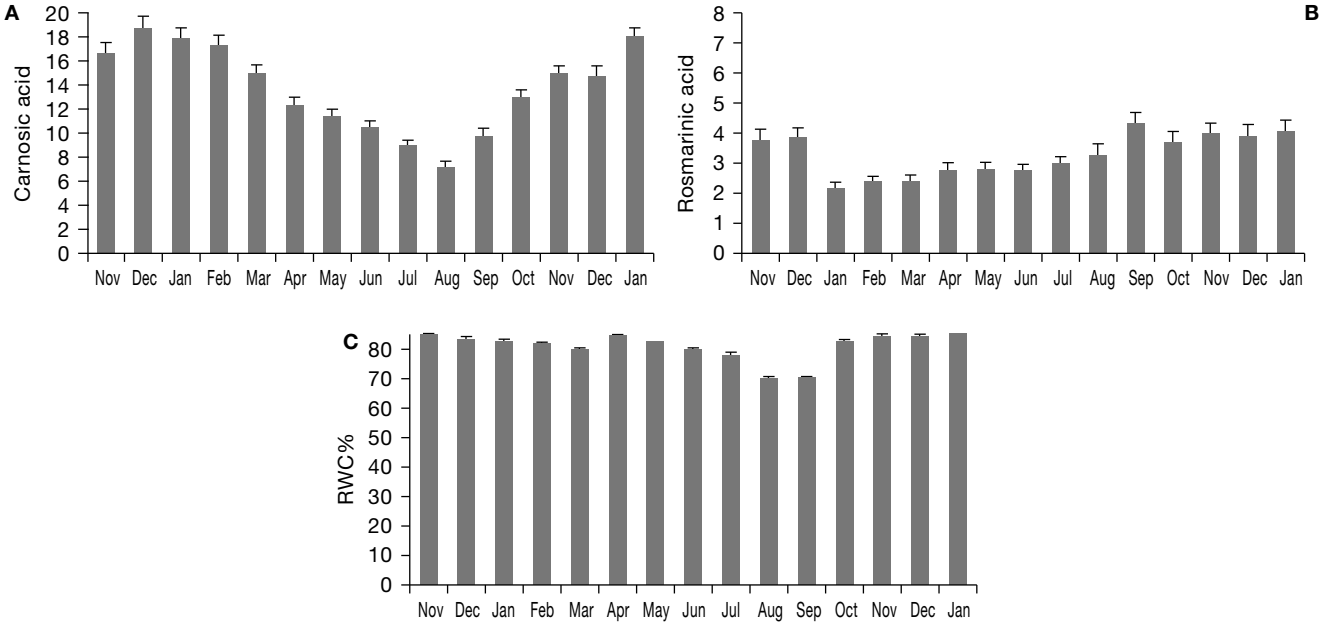

Figure 2. Foliar content values of carnosic acid (a) and rosmarinic acid (b) and relative water content (RWC%) (c) during the year 2002. Rosmarinic and carnosic acids values were expressed in mg g^{-1} of fresh weight and are the mean ± standard deviation for n = 6 measurements.

abundant compounds in rosemary extracts (Cuvelier *et al*., 1996; Zheng and Wang, 2001).

The biological activity of a compound is determined, partly, by its distribution within the plant cell. Hydroxycinnamic acids and esters, flavonoids and anthocyanins are synthesised in plant cells via the phenylpropanoid pathway localised in the cytosol, with the final stages of biosynthesis and accumulation in the vacuole. They are present in petals, sepals, leaves, stems, and roots, which is consistent with the results obtained for rosmarinic acid in this research. On the other hand, diterpenes, such as carnosic acid, are synthesised in plants via the non-mevalonate isopentenyl diphosphate pathway (McGarvey and Croteau, 1995), which has been localised, as carnosic acid, in the chloroplasts (McGarvey and Croteau, 1995; Munné-Bosch and Alegre, 2001). In fact, results from this research corroborated these results showing carnosic acid in petals, sepals and leaves while in those non-photosynthetic tissues such as stems and roots the diterpene was not detected.

In addition, rosemary plants are well adapted to withstand both the winter and summer weather conditions of southern UK. However, during summer carnosic acid concentration levels decreased, which were concomitant with low precipitation rates and high temperatures. Similar results were obtained for rosemary plants when they were exposed to Mediterranean summer conditions (Munné-Bosch and Alegre, 2000), although, the relative water content of rosemary leaves never reached values of severe water stress conditions (below 50%), which

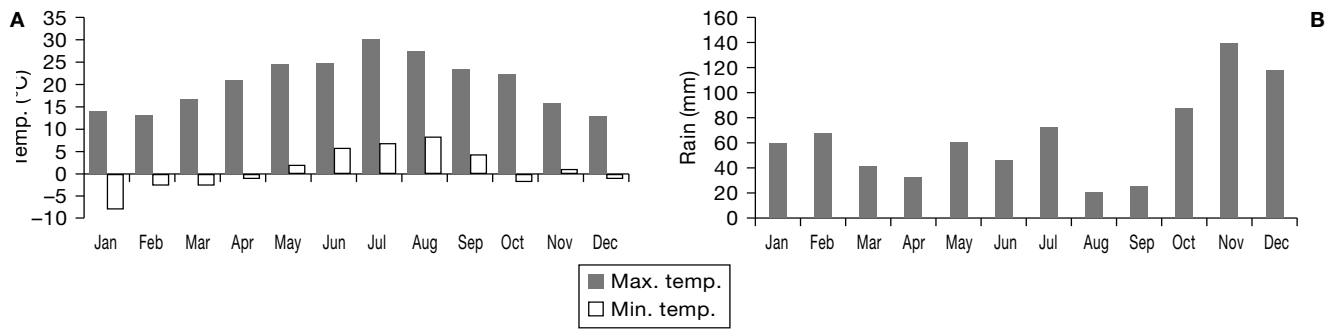

Figure 3. Maximum temperature and minimum temperature (a) and rain fall (b) during the year 2002. Courtesy of the Department of Meteorology, The University of Reading.

Table 2. DPPH radical scavenging activities of rosmarinic and carnosic acids at four selected concentrations compared with caffeic acid, vanillic acid and naringin. The data were expressed as percentage of DPPH scavenged and were the mean ± standard deviation n = 3 different determinations

Compound	Concentrations				IC_{50}
	20 µM	50 µM	100 µM	200 µM	
Rosmarinic acid	37.3 ± 1.4	82.5 ± 1.3	93.3 ± 1.4	99.8 ± 1.32	27 µM
Caffeic acid	27.5 ± 0.5	71.7 ± 1.2	94.9 ± 1.9	95.8 ± 1.98	38 µM
Vanillic acid	0.0	1.8 ± 0.2	3.8 ± 0.65	9.1 ± 0.3	> 200 µM
Naringin	0.3 ± 0.01	0.75 ± 0.01	1.7 ± 0.6	3.1 ± 0.21	> 200 µM
Carnosic acid	30.5 ± 1.2	82.7 ± 1.9	96.3 ± 1.8	99.7 ± 1.5	32 µM
Ascorbic acid	21.2 ± 0.9	75.0 ± 1.9	96.5 ± 1.4	97.8 ± 1.4	47 µM

can be seen under the Mediterranean summer. These results suggested that the reduction in carnosic acid concentration levels in rosemary plants, between winter and summer, is the result of the combination of different factors and water stress, during this study, is not among them. However, further research is needed in order to understand the evolution of carnosic acid levels under south UK weather conditions.

On the other hand, rosmarinic and carnosic acids showed powerful scavenging activity against the DPPH radical *in vitro*. The scavenging activity of rosmarinic acid was always much higher than other compounds tested, such as vanillic acid, naringin and even the «classical» antioxidant ascorbic acid. These results highlight the importance of a catechol group moiety for hydrogen-donating activity (Rice-Evans *et al*., 1997), which is present in rosmarinic, caffeic, and carnosic acids all three showing a similar DPPH scavenging capacity.

Furthermore, rosmarinic and carnosic acids have been tested in a variety of lipid systems, always giving different results depending on the assay conditions and system used (Frankel *et al.*, 1996, Cuvelier *et al.*, 2000). Results in this study showed that the free radical scavenging activity of rosemary extracts measured as DPPH scavenging activity exhibited a positive relationship with the concentration of rosmarinic and

Table 3. Cumulative DPPH scavenging activities between rosmarinic acid and caffeic acid, vanillic acid, naringin and carnosic acid. The data represent the mean ± standard deviation for n = 3 different determinations

RA	% DPPH SC	CAF	%DPPH SC	RA + CAF	Expected	Obtained
10 µM	18.6 ± 0.93	20 µM	27.5 ± 1.3	10 +20 µM	46.1	45.0 ± 1.3
10 µM	18.9 ± 0.94	40 µM	54.8 ± 2.7	10 +40 µM	73.7	72.4 ± 0.3
10 µM	19.0 ± 0.95	60 µM	97.9 ± 0.65	10 +60 µM	100	99.1 ± 1.1
RA	**%DPPH SC**	**VAL**	**%DPPH SC**	**RA +VAL**	**Expected**	**Obtained**
10 µM	18.2 ± 0.93	20 µM	0.3 ± 0.03	10 +20 µM	18.5	18.7 ± 0.9
10 µM	18.1 ± 1.04	40 µM	0.59 ± 0.07	10 +40 µM	19.69	19.0 ± 1.1
10 µM	18.3 ± 0.95	60 µM	1.29 ± 0.09	10 +60 µM	19.59	20.3 ± 0.8
RA	**%DPPH SC**	**NAR**	**%DPPH SC**	**RA +NAR**	**Expected**	**Obtained**
10 µM	19.1 ± 0.9	20 µM	0.3 ± 0.01	10 +20 µM	19.4	18.8 ± 0.9
10 µM	18.9 ± 0.4	40 µM	0.6 ± 0.06	10 +40 µM	19.5	19.0 ± 1.1
10 µM	18.9 ± 0.5	60 µM	0.9 ± 0.09	10 +60 µM	19.8	19.3 ± 0.9
RA	**%DPPH SC**	**CAR**	**%DPPH SC**	**RA +CAR**	**Expected**	**Obtained**
10 µM	19.2 ± 0.83	20 µM	30.0 ± 1.19	10 +20 µM	49.2	48.4 ± 1.2
10 µM	18.9 ± 1.10	40 µM	59.9 ± 2.67	10 +40 µM	78.8	78.2 ± 0.9
10 µM	18.9 ± 0.65	60 µM	90.2 ± 0.60	10 +60 µM	100	98.9 ± 0.01

RA: rosmarinic acid. CAF: caffeic acid. VAL: vanillic acid. NAR: naringin. CAR: carnosic acid. %DPPC SC: percentage of DPPH scavenged.

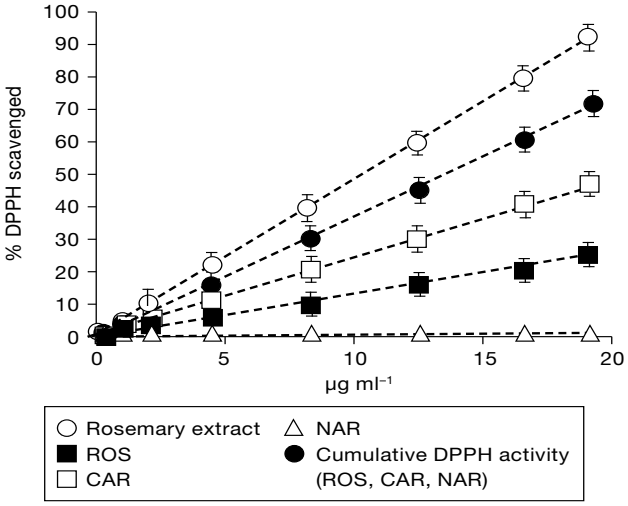

Figure 4. DPPH scavenging activity of rosemary extracts. Rosmarinic acid (ROS), carnosic acid (CAR) and naringin (NAR) DPPH scavenging activities were calculated with respect to their concentration in the extracts and the amount of extract used in the assay. The cumulative DPPH capacities of these three compounds were calculated as the sum of their DPPH scavenging capacities. The data represent the average ± standard deviation for n = 3 different determinations.

carnosic acids, both showing cumulative effects. However, the contribution of other compounds, such as carnosol, could be also important to explain the overall DPPH scavenging capacity of rosemary extracts.

In summary, the capacity of rosemary leaf extracts to scavenge DPPH radicals depends on, firstly, the concentration of rosmarinic and carnosic acids in rosemary extracts, secondly the cumulative effects of these compounds, and thirdly the presence of other important compounds such as carnosol. In addition, the influence of environmental growing conditions can modulate the contents of rosmarinic and carnosic acids and thus the antioxidant potential of rosemary plant extracts.

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