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In vitro antioxidant activities of *Rosmarinus officinalis* extracts treated with supercritical carbon dioxide

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Abstract

The leaves of *Rosmarinus officinalis* (rosemary) were subjected to supercritical CO_2 extraction (SFE). Different sources of variability, including location (Izmir, Canakkale and Mersin) and harvesting time (December, March, June and September), were considered. Among active constituents of rosemary, carnosic acid, carnosol and rosmarinic acid were analyzed by HPLC. Variability of the amounts of active constituents appears to be due to different geographical locations of growth and seasonal variations. The levels of the constituents were higher in the months of December 2003 and September 2004. In addition to this, 12 SFE extracts were screened for their radical-scavenging capacities and antioxidant activities by various in vitro assays, namely total phenol assay, DPPH radical-scavenging activity and trolox equivalent antioxidant capacity (TEAC).

The results revealed excellent correlation ($r = 0.97$) between the HPLC and total phenol assay. The results also indicated that the plants harvested in September, possessing higher levels of active constituents, had antioxidant capacities superior to those collected at other times. With respect to the location, plants harvested from the Mersin region had higher total phenol and active constituent levels resulting in superior antioxidant activity, therefore implying their potential value for the food industry.

Keywords: *Rosmarinus officinalis*; Antioxidant activity; Supercritical CO_2 extraction; Total phenol assay; DPPH; TEAC

1. Introduction

Free radicals can have a noxious effect on cell components, such as membranes, lipoproteins, proteins, carbohydrates, DNA and RNA. They are generally by-products of various endogenous processes that can be stimulated by external factors, such as air pollution, irradiation, smoking, stress and toxins present in food and/or drinking water. Antioxidants can protect against these radicals by forming an intricate network (Arts, Haenen, Voss, & Bast, 2001). Recently, various plant extracts have attracted interest as sources of natural products and rosemary is one of the most studied of these.

Several researches on the antioxidative constituents of rosemary have indicated that the most active compounds are phenolic diterpenes, such as carnosic acid, carnosol, rosmanol, epi- and, iso-rosmanol and the phenolic constituent rosmarinic acid (Senorans, Ibanez, Cavero, Tabera, & Reglero, 2000; Thorsen & Hildebrandt, 2003). On the other hand, rosmanol, epi- and iso-rosmanol are considered to be minor components resulting from degradation of carnosic acid (Schwarz & Ternes, 1992).

Several methods have been applied to extract these antioxidant compounds from rosemary, belonging to the Lamiaceae family. Steam-distillation and molecular distillation are used for concentrating the active fraction or to remove the residual aroma (Sebastian et al., 1998). Dorman, Peltoketo, Hiltunen, and Tikkanen (2003) used a two-step process, steam-distillation followed by water extraction as a cheap, simple and solvent-free method but only rosmarinic

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acid was confirmed by HPLC to be the main phenolic constituent. Ollanketo, Peltoketo, Hartonen, Hitunen, and Riekkola (2002) pointed out that diterpenes cannot be extracted from rosemary by this method. Although, high yields are reported for solvent extraction of rosemary (Chang, Ostric-Matijasevic, Hsieh, & Huang, 1977), solvent residues, which are prohibited in food by regulation, remain in the extract. In order to overcome these difficulties, there is considerable interest in replacing the traditional methods with supercritical fluid extraction technology (Reverchon, 1997).

As far as our literature survey could ascertain, antioxidant activities of supercritical CO_2 extracts of rosemary have not been compared with respect to both the location and the harvesting times. In the present study, the purpose was to screen free radical-scavenging capacity and antioxidant activities of supercritical CO_2 extracts of *Rosmarinus officinalis* (rosemary) harvested from different locations of Turkey at four different times of the year, thereby evaluating two different parameters affecting antioxidant activity.

2. Materials and methods

2.1. Chemicals

Folin-Ciocalteu's reagent, 2,2-diphenyl-1-picrylhydrazil hydrate (DPPH), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ABTS, α-tocopherol, and sodium carbonate were obtained from Sigma, gallic acid and rosmarinic acid (97%) (lot no. 456976/1) from Fluka, carnosol (98%) (lot no. A7531) and carnosic acid (93%) (A7781) from A.G. Scientific, and trolox and potassium persulfate were obtained from Acros Organics (The Netherlands). The HPLC grade organic solvents, methanol and acetonitrile, were purchased from Merck. All other chemicals were of analytical grade purity.

2.2. Plant material

R. officinalis specimens were collected from three different locations, namely, Canakkale (southern Marmara region, the coolest climate), Izmir (Aegean region, moderately hot) and Mersin (eastern Mediterranean region, the hottest) at four different time intervals, December 2003 (represented by C-SC1, I-SC1, M-SC1, respectively), March (C-SC2, I-SC2, M-SC2), June (C-SC3, I-SC3, M-SC3) and September, 2004 (C-SC4, I-SC4, M-SC4). The specimens were dried at 30°C in a conventional oven in order to avoid activity losses reported to be caused by thermal treatment (Ibanez et al., 1999) and stored in the cold room of Ege University Science and Technology Center.

2.3. Preparation of SFE extracts

One hundred grammes of plant material were distilled by means of a Clevenger apparatus (4 h) in order to remove volatile oils. After that, the distillate was filtered, air-dried and then extracted by using supercritical CO_2 extraction. An Isco (Isco Inc., Lincoln, Nebraska) supercritical fluid extractor was used to perform all of the experiments. 0.49 g of sample was placed in a 2.5 ml stainless steel cartridge. CO_2 flow rate was controlled using a valve; flow rates were from 2 to 3 ml/min, with an average of 2.5 ml/min. Quite a number of experiments were conducted to optimize the conditions for the extraction (data not shown). Finally, the sample was extracted at 350 bar and 100 °C. Extraction time was 5 min of static extraction, followed by 35 min of dynamic extraction. In regard to the co-solvents, both 2% and 5% of methanol, ethanol and propanol were tested. Best compositions were achieved with 5% of methanol as co-solvent. At the conditions of 350 bar, 100 °C, 40 min, 5% methanol, the amounts of carnosol and carnosic acid in the extracts were 15.8% and 143% higher than the amounts obtained under the same conditions without a co-solvent. Supercritical fluid extracts were collected in vials. Afterwards, the extracts were stored at −20 °C.

2.4. HPLC analysis of the extracts

HPLC analyses were performed with a Waters 2695 instrument equipped with an autosampler (injection volume 20 μl). The column was a Zorbax C_{18} type, 5 μm, 250 × 4.6 mm. The mobile phase was a mixture of solvent A (methanol) and solvent B (10 mM acetic acid in acetonitrile) according to a linear gradient, lasting 35 min, changing from 90% B to 0% B in 30 min, at a flow rate of 1.5 ml/min. The detection was attained by using a Waters 2487 Dual absorbance UV detector. The signals at a wavelength of 285 nm were stored and collected by Chromperfect data management software (Justice Laboratory Software, UK).

2.5. Antioxidant assays

2.5.1. Total phenol assay

The total phenols in the plant extracts were determined by the Folin-Ciocalteu method described by Dorman et al. (2003) with some modifications. Briefly, a 10 μl aliquot of rosemary extract was added to a tube containing Milli-Q water (final volume 10 ml). Then 500 μl of Folin-Ciocalteu reagent were added and the solution was stirred vigorously by vortex and left to stand for 5 min. Finally, 1.5 ml of a saturated sodium carbonate solution were added, stirred vigorously for the last time and left to stand at room temperature for 1 h. Absorbance was determined spectrophotometrically at 760 nm using a Unicam Helios-alfa spectrophotometer. Gallic acid was used as a standard and determination of total phenols was carried out in duplicate; the results are mean values and given as gallic acid equivalent (GAE) per gramme of extract.

2.5.2. DPPH radical-scavenging activity (RSA) assay

DPPH assay was carried out as described by Amarowicz, Pegg, Moghaddam, Barl, and Weil (2004) with

minor modifications. This radical serves as the oxidizing radical to be reduced by the antioxidant (AH) and as the indicator for the reaction $DPPH^{\cdot} + AH \rightarrow DPPH\text{–}H + A^{\cdot}$ (Frankel & Meyer, 2000). Antioxidant extracts were dissolved in 4 ml of methanol and then added to a 1 mM methanolic solution of $DPPH^{\cdot}$ (final volume 4.5 ml). The contents were stirred vigorously for 15 s and then left to stand at room temperature for 30 min. Decrease in colorization was measured spectrophotometrically at 517 nm, using a Unicam Helios-alfa spectrophotometer. The radical-scavenging activity (RSA) was calculated using the equation below:

$$\%\mathrm{RSA} = 100 \times (1 - A_E/A_D)$$

where A_E is the absorbance of the solution containing antioxidant extract whereas A_D is the absorbance of the $DPPH^{\cdot}$ solution.

2.5.3. TEAC assay

TEAC assay was carried out as described in a protocol by Re et al. (1999) with slight modifications. This method is based on the reaction between ABTS and potassium persulfate giving blue/green ABTS radical ($ABTS^{\cdot}$). With the addition of the antioxidants, decolorization is attained and measured spectrophotometrically at 734 nm. The results were expressed as mmol trolox per fresh weight (kg) of rosemary plants. ABTS is dissolved in water to a concentration of 7 mM and reacted with 2.45 mM potassium persulfate at a molar ratio of 2:1 to form the $ABTS^{\cdot}$ radical, left in the dark room overnight for 16 h. Stock solutions of rosemary extracts, BHA, BHT, α-tocopherol and trolox were prepared in ethanol. Ten μl aliquots of both the extracts, positive controls and trolox were pipetted into tubes; then the $ABTS^{\cdot}$ solution was added which had been diluted with PBS (pH 7.4) to an absorbance of 0.70 ± 0.02 AU at 734 nm, stirred vigorously, and the absorbance was measured over time by a Multiskan Spectrum Microplate Spectrophotometer (Finland).

2.6. Statistics

Statistical analyses of the data were performed by Student's *t*-test. A probability value of $P \leqslant 0.05$ was considered to denote a statistically significant difference, and $P \leqslant 0.01$ was also used to show the power of the significance. Data are presented as mean values ±SEM. (standard error of the mean).

3. Results and discussion

3.1. Seasonal and regional variation of phenolic diterpenes: carnosol and carnosic acid

The compositions of each sample are presented in Table 1. Fig. 1 shows a typical HPLC chromatogram of one of the samples (M-SC4) and the standard compounds.

With respect to cultivation geography, the Mersin samples had high levels of carnosol, while Canakkale samples had slightly higher amounts than did Izmir samples. The major component in all of the samples was carnosic acid. The highest content of carnosic acid was also found in the samples harvested from Mersin. The highest carnosol value was attained for Mersin samples and it varied from 18.4 to 11.1 mg/g. In all the samples, the carnosol contents ranging from 3.3 to 18.4 mg/g, were lower than the carnosic acid contents, which ranged from 5.0 to 115.5 mg/g.

As can be seen in Fig. 1, no rosmarinic acid could be extracted under the conditions used. The seasonal profiles of quantified compounds showed significant differences among the samples harvested in December, March, June and September. Carnosol contents in the Izmir and Canakkale samples peaked in June (10.1; 11.1 mg/g), also showed a high profile in September (9.9; 10.9 mg/g) and had a declining trend between December (7.7; 9.3 mg/g) and March (3.3; 8.1 mg/g), particularly reaching the lowest values in March. These results were in accordance with the findings of Del Bano et al. (2003) where the highest values for carnosol (for leaves) were observed between June and September, followed by a declining trend until February. However they observed an increase in March. In the Mersin samples, the carnosol levels peaked in September (18.4 mg/g), decreased gradually between September and December (15.2 mg/g) and reached the lowest values in March (12.3 mg/g) and June (11.1 mg/g).

In the case of carnosic acid, Canakkale and Mersin samples had similar trends, possessing lowest values in March

Table 1
Phenolic diterpenes quantified by HPLC

	Carnosol (mg/g) ± SEM	Carnosic Acid (mg/g) ± SEM
I-SC1	7.7 ± 0.01	23.4 ± 0.10
I-SC2	3.3 ± 0.00	5.0 ± 0.14
I-SC3	10.1 ± 0.08	31.7 ± 0.21
I-SC4	9.9 ± 0.08	50.0 ± 0.46
C-SC1	9.3 ± 0.01	41.2 ± 0.17
C-SC2	8.1 ± 0.01	12.7 ± 0.01
C-SC3	11.1 ± 0.00	20.8 ± 0.04
C-SC4	10.9 ± 0.00	29.4 ± 0.07
M-SC1	15.2 ± 0.01	115.5 ± 0.47
M-SC2	12.3 ± 0.01	60.9 ± 0.45
M-SC3	11.1 ± 0.02	65.6 ± 0.04
M-SC4	18.4 ± 0.00	111.1 ± 0.06

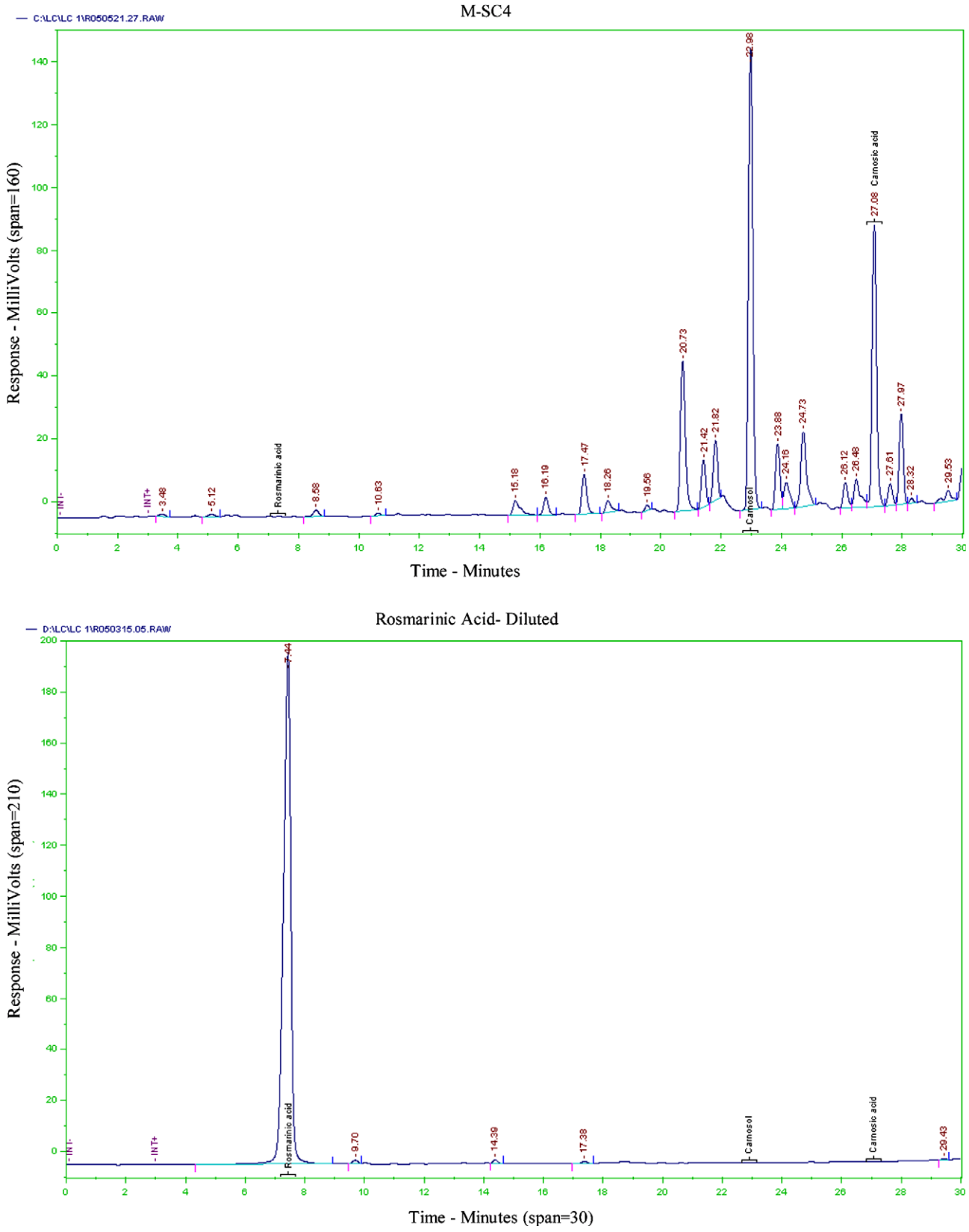
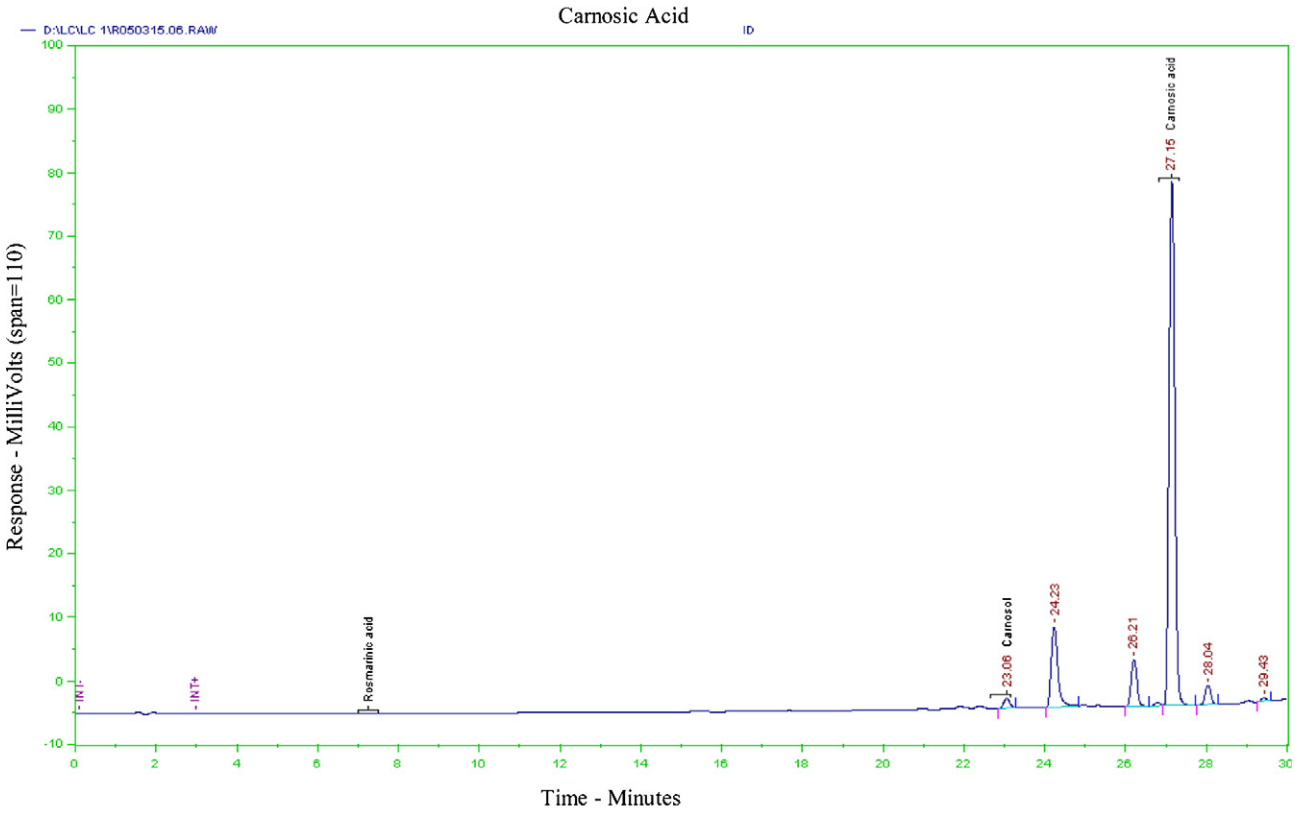


Fig. 1. Typical chromatograms of M-SC4 and the chromatograms for the standard compounds; rosmarinic acid, carnosol and carnosic acid.

(12.7; 12.3 mg/g), then tending toward an increase during June (20.8; 65.6 mg/g), September (29.4; 111.1 mg/g) and reaching the highest values in December (41.2; 115.5 mg/g). Izmir samples showed a similar pattern as well. The increasing trend continued until September (5.0; 31.7; 50.0 mg/g) but declined in December (23.4 mg/g).



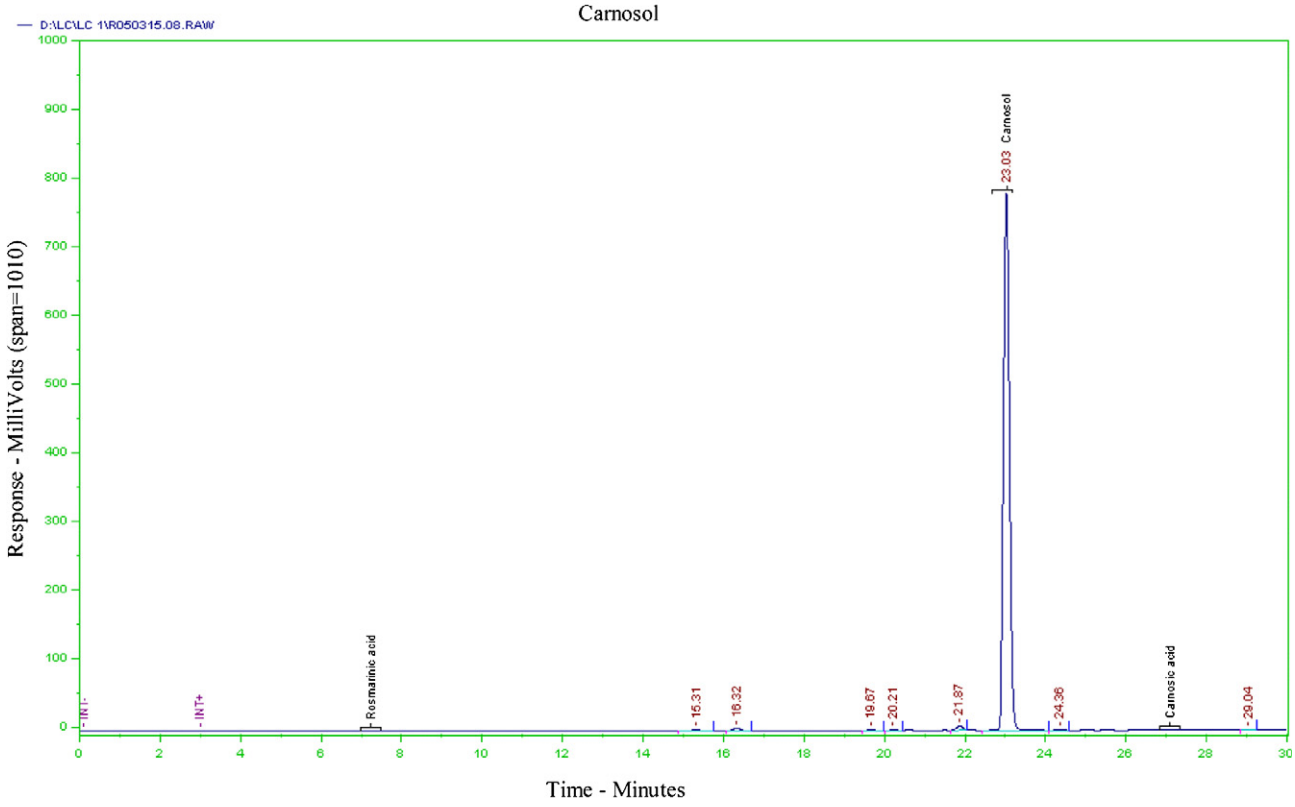


Fig. 1 (*continued*)

On the basis of the above-mentioned findings, it is to be expected that the maximum levels for the phenolic diterpenes would be attained at the same period of harvest, September. Since cooler months, especially March, do not favour the accumulation of carnosol and carnosic acid, one should avoid harvesting rosemary during the winter and spring.

The results of our study, however, are not in accordance with the observations of Hidalgo, Ubera, Tena, and Valcarcel (1998). Hidalgo and coworkers reported that carnosic acid content increased gradually during the spring, peaked in the summer months and then dropped abruptly in September. Their samples were harvested from Cordoba, Spain where the temperature was reported to be around 15 °C in September and generally lower temperatures, throughout the whole year, than in the locations reported in this study (average temperatures in September were 29, 28 and 25 °C, respectively, for Mersin, Izmir and Canakale; data obtained from Turkish State Meteorological Service). On the other hand, Munne-Bosch, Alegre, and Schwarz (2000) worked with the rosemary samples harvested from the experimental fields of the University of Barcelona, Spain and reported that carnosic acid and carnosol concentrations were relatively high from October to February and low from May until the end of August, whereas a steep increase was observed between August and October. Our results showed highest values in September samples, conflicting with their results. These differences can be explained in terms of geographical coordinates, climate and ecological conditions.

Since rosemary extract has commercial value as an antioxidant, the abundancy of phenolic diterpenes should also be considered in terms of comparing the samples in this study with the extracts discussed in the previous paragraph and commercial preparations.

Hidalgo and coworkers (1998) reported the highest carnosic acid concentration to be 46.2 mg/g and the lowest 21.4 mg/g, while Munne-Bosch and coworkers (2000) reported 4.8 and 2.8 mg/g, respectively. In this study, the highest values for carnosic acid were observed in the Mersin region, ranging from 116 to 60.9 mg/g with an average value of 88.3 mg/g, implying the superiority of the Mersin ecotype to the Spanish counterparts. Thorsen and Hildebrandt (2003) reported that the quality as antioxidant, and the price of commercial rosemary extract are highly correlated with the content of, primarily, carnosic acid and, secondly, with the total content of phenolic diterpenes, including carnosol. Moreover, the commercial rosemary extract (P31) marketed by a Swiss company was tested by Samman et al. (2001), indicating the composition as 2.2% carnosic acid (CA), 3.5% carnosol (CL) and 2.6% rosmarinic acid (RA) whereas the compositions of Mersin samples were as follows; M-SC1 (11.5% CA, 1.5% CL), M-SC2 (6.1% CA, 1.2% CL), M-SC3 (6.6% CA, 1.1% CL) and M-SC4 (11.1% CA, 1.8% CL).

Based on these results, we suggest that rosemary samples collected from the Mersin region have high commercial value from a biological activity point of view.

3.2. Seasonal and regional variation of total phenol content

The total phenols determined by the Folin-Ciocalteu method varied from 34.1 to 119 mg GAE/g extract. Samples harvested from Mersin had the highest total phenol content, ranging from 82.3 to 119 mg/g among the locations, while Izmir and Canakkale samples showed lower values for March harvests, as shown in Fig. 2 (34.1–60.4 mg/g).

3.3. Seasonal and regional variation of antioxidant efficiency

The RSA values of 12 methanolic rosemary extracts were determined and compared. Although there was no statistically significant difference, extracts of Mersin samples harvested in September showed the highest radical-scavenging activity. With regard to variations in harvesting times, samples harvested in September exhibited higher values (Fig. 3).

TEAC values ranged from 7.4 to 3.1 mmol trolox/kg FW. Among those, the Mersin-March samples had the highest value (7.4 mmol/kg). In order to compare the calculated TEAC value with those from different fruit and vegetable extracts, some values from the work of Pellegrini et al. (2003) have been provided. For instance, the calculated TEAC value is higher than the TEAC values of blueberry, red plum, tangerine and black grape extracts (7.4, 5.1, 4.2, 3.9 mmol trolox/kg FW) and the TEAC values of all vegetable extracts, except spinach and pepper listed by Pellegrini

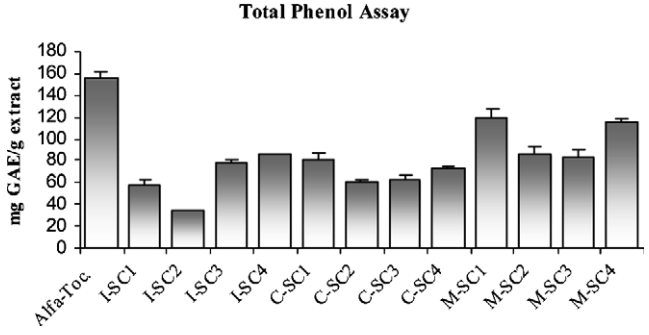


Fig. 2. Total phenol assay results for SFE extracts derived from rosemary harvested from different locations at different times.

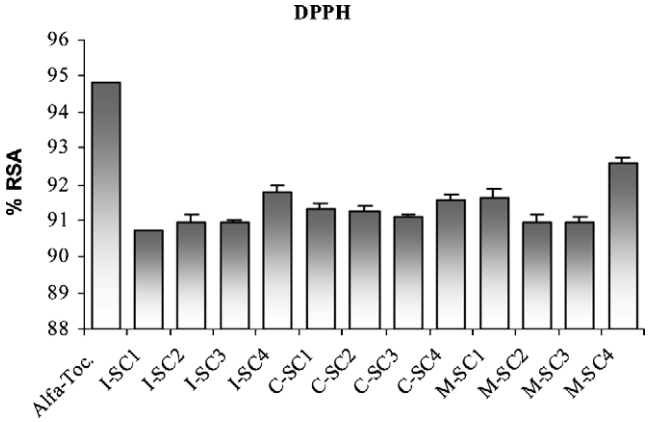


Fig. 3. DPPH radical-scavenging activity results for SFE extracts derived from rosemary harvested from different locations at different times.

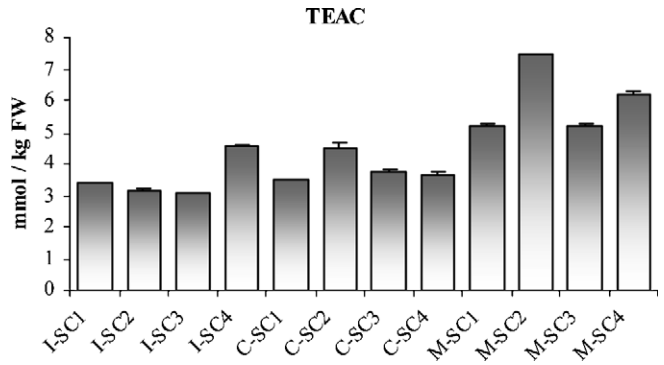


Fig. 4. TEAC values for SFE extracts derived from rosemary harvested from different locations at different times.

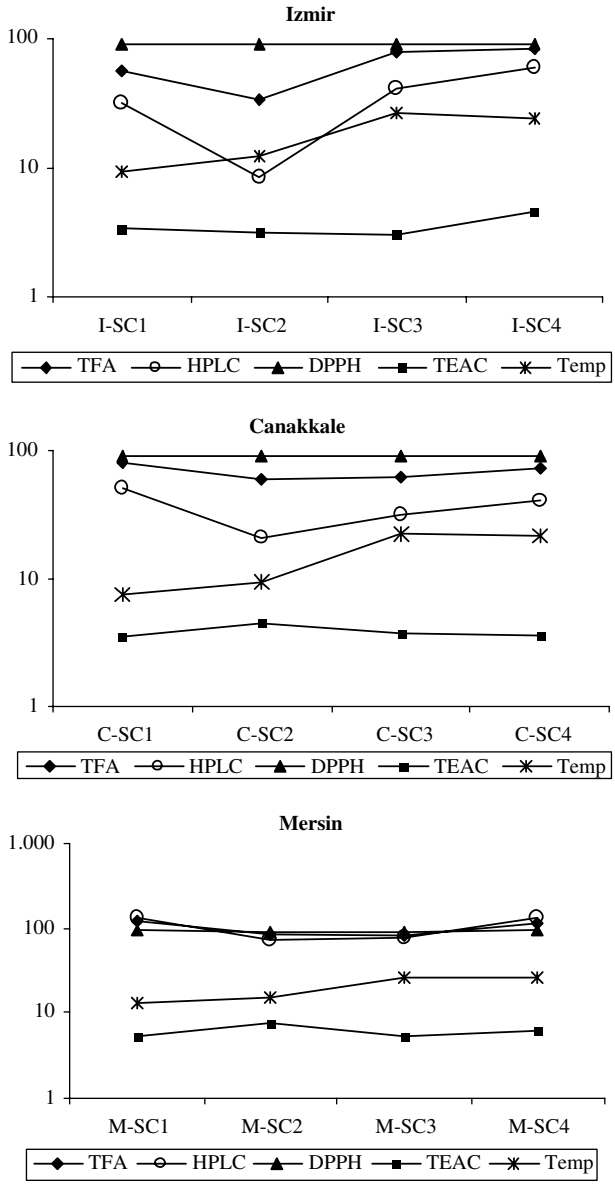


Fig. 5. Aggregated graphs for three locations (Temperatures represent the values for December 2003, March, June and September 2004, obtained from Turkish State Meteorological Service).

et al. (2003). Canakkale samples had a similar pattern where the highest values were found during March (4.5 mmol/kg). Considering the location of Izmir, SFE extracts of September harvests had higher TEAC values (4.5 mmol/kg). The differences between the values in terms of locations and different harvesting times can be seen in Fig. 4.

3.4. Relationships between different antioxidant assays

From a holistic view of the results of different assays, it can be concluded that the results of total phenol content and HPLC revealed a very good correlation ($r = 0.97$) whereas a fairly good correlation is observed for the results of total phenol content and DPPH ($r = 0.65$) and also for HPLC and DPPH ($r = 0.66$). But, on the contrary, the results of the TEAC assay did not show any correlation with total phenol content ($r = -0.17$), HPLC ($r = -0.18$) and DPPH ($r = 0.16$) which can be seen for all locations in Fig. 5.

4. Conclusions

The findings confirm that rosemary samples harvested from different locations show considerable differences in composition. Antioxidant activity of various extracts from different harvesting times also varied significantly. In general, all extracts showed high capacity in terms of neutralizing free radicals (DPPH$^{\cdot}$ and ABTS). Specifically, samples harvested from the Mersin region, possessing the highest amounts of phenolic diterpenes, could have commercial value for the food industry.

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