Chapter

Total Phenolic Content and Polyphenolic Profile of Tunisian Rosemary (*Rosmarinus officinalis* L.) Residues

Hcini Kheiria, Abidi Mounir, Quílez María, Jordán Maria José and Sadok Bouzid

Abstract

Plants, especially herbs and spices, have always been the major sources of numerous natural compounds with antioxidant activity and other beneficial properties and, specifically, Rosemary (*Rosmarinus officinalis* L.) has been widely accepted as one of the spices with highest antioxidant activities which appear to be related to their richness of phenolic compounds. This study was undertaken with the aim to estimate the total phenolic content, identify and quantify the polyphenolic compounds of the methanolic extracts from post-distilled rosemary, collected from two different bioclimatic areas from Tunisia. Total phenolic content (TPC) was determined by Folin–Ciocalteu method. Identification and quantification of polyphenolic compounds was performed using high-performance liquid chromatography (HPLC) analysis. TPC ranged from 85.8 to 137.3 mg GAE/g DE in rosemary extracts. HPLC analysis showed the presence of carnosic acid and carnosol, wich were found to be the most abundant compounds in all analyzed extracts (46.3 to 76.4 and 22.4 to 43.5 mg/g of plant dry weight respectively), rosmarinic acid and caffeic acid as phenolic acids, besides some flavonoids such as apigenin, luteolin, genkwanin and hesperidin. This study revealed that rosemary post-distilled residues were shown to be promising with regard to their incorporation into various foods, cosmetics and fragrances.Therefore, supplementing a balanced diet with herbs may have beneficial health effects.

Keywords: *Rosmarinus officinalis* L, total phenolic content, polyphenolic profile, HPLC analysis

1. Introduction

Herbs and plants have been used for a large range of purposes including medicine, pharmaceuticals, nutrition, food preservation, flavorings, beverages, repellents, fragrances and cosmetics. Since prehistoric times, they were the basis for medicinal therapy until synthetic drugs were developed in the nineteenth century [1, 2]. In recent decades, the use of herbs and plants has been of great interest, as

they have been the sources of natural products, commonly named as bioactive compounds, with beneficial activities, namely polyphenols, vitamins, polysaccharides and minerals [3]. Nowadays the use of natural compounds is also increasing around the world due to their mild features and low side effects [4, 5]. Cosmetic preparations from herbal origin are popular among consumers, as these agents are typically non-toxic and possess strong activities [6].

Preliminary studies demonstrated that some herbs extracts are as efficient as the synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), which have carcinogenic effects in living organisms [3, 4, 7]. Several studies have indicated that the consumption of natural antioxidant compounds protect cells against the damage of reactive oxygen species such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite (Yang et al., 2013) [8]. Recently numerous reports have described antioxidants and compounds with beneficial activities, would be beneficial for healthy life, present in fruits, vegetables, herbs and cereals extracts [3, 9]. For all the described reasons, during the last decade there has been a growing interest in the formulation of new cosmetic, food and pharmaceutical products containing natural compounds with antioxidant activity and other beneficial properties for healthy life.

The evidence for antioxidant effect of spices and herbs in food systems was initially determined by Chipault et *al.* (1952) [10] studying 32 spices, and among these rosemary and sage were considered the most effective. In general, herbs and plants are rich in compounds with antioxidant properties, such as vitamins (E and C), glutathione, enzymes and phenolic compounds [11]. Specifically, the natural compounds from the Lamiaceae family (thyme, sage and rosemary) has been reported in several studies for its antioxidant, anti-inflammatory, antimicrobial and anti-carcinogenic activities [1, 3, 12, 13]. In particular rosemary extracts possess very useful antioxidant properties, which appear to be related to their content of phenolic compounds [4, 7, 14–18].

Rosemary (*Rosmarinus officinalis* L.) is a perennial shrub belonging to the *Lamiaceae*. Native to the Mediterranean region rosemary is now widely distributed and has been cultivated around the world [19]. Its leaves have been used for thousands of years as a natural food preservative, flavorings, pharmaceuticals, alternative medicine and natural therapies [20, 21] and its applications have ranged from memory enhancement to the treatment of gastrointestinal diseases [22, 23]. Also, rosemary is the most used and economically important aromatic and medicinal plant for its essential oil and phenolic compounds [1, 24–26] and has been widely accepted as one of the spices with highest antioxidant activities of all the herbs and spices that have been investigated [3]. Antioxidant activity of rosemary extracts depends on their richness of phenolic compounds. Several investigators found that rosemary bioactive properties are connected with the presence of phenolic compounds, especially flavonoids and diterpenes, such as carnosic acid and carnosol that characterized with its antioxidant activity [1, 27–33].

Rosemary is among the most promising sources for the recovery of essential oils through hydrodistillation and polyphenols. After extracting the essential oils, the residues remaining postdistillation (wastes of hydro-distillation) is considered a natural source of antioxidants with several biological activites [9, 30]. Up to today, their exploitation for polyphenol recovery has been limited. The residues remaining after essential oil recovery currently disposed as waste, could be extracted to obtain natural extracts rich in phenolic compounds and with a high antioxidant activity [34].

This current study was undertaken with the aim to identify and quantify the polyphenolic compounds (polyphenolic profile) and to determine the total phenolic content of the residues (waste of hydro-distillation) of rosemary aerial parts in

order to revalorize this plant and to increase the economic values of this valuable products as a source of bioactive molecules with beneficial properties that might confer benefits to human health.

2. Materials and methods

2.1 Collection of plant materials

A total of 40 individual samples of wild rosemary shrubs (4 locations with 10 plants/area) from two different bioclimatic areas (Semi-arid superior and lower arid) were randomly collected at the full bloom phenological stage (spring 2017). Voucher specimens of rosemary from every location are deposited at the Herbarium of Laboratory of Aromatic and Medicinal Plants of the Research Group on Rainfed Crops for the Rural Development, Murcia Institute of Agri-Food Research and Development (IMIDA), Murcia, Spain.

Details of collection sites are given in **Table 1**. Fresh aerial parts of plants were firstly dried at room temperature for ten days and afterwards dried in a forced-air drier at 35°C for 48 h, until they reached a constant weight.

2.2 Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH•), ascorbic acid, potassium persulfate, the Folin–Ciocalteu reagent, gallic acid and high purity standards were purchased from Sigma-Aldrich (Madrid, Spain). Methanol, acetonitrile, formic acid, anhydrous sodium carbonate and sodium acetate were supplied from Scharlau Chemie S.A. (Sentmenat, Spain). Methanol was of HPLC grade and other reagents were of analytical grade.

2.3 Extraction of polyphenolic coupounds

After extracting the essential oils, the residues remaining postdistillation (wastes of hydro-distillation) is considered a natural source of antioxidants [35, 36]. In order to avoid interferences from the essential oil components, individual plants were firstly distilled in a Clevenger system for 3 h, after this, the oil free distilled plant material was dried in a forced-air drier 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 extracted using 150 mL of methanol in a Soxhlet extractor (B-811) (Buchi, Flawil, Switzerland), for 2 h under a nitrogen atmosphere. Methanolic extracts (ME) were taken to dryness at 40°C under vacuum conditions

Collection site	Bioclimatic stage	Rainfall mm/year	Temp °C/year	Geographical location		
				Longitude (N)	Latitude (E)	Altitude (m)
ElKef-Menzel Salem (KMS)	Semi-arid superior	446	16.2	35°51′24.0”	8°28′34.0”	995
Elkef-Sers (KS)	Semi-arid superior	441	16.9	36°4′36.2”	9°1′21.8”	887
Gafsa-Orbata (GO)	Arid lower	223	19.6	34°22′49.8”	9°3′23.4”	1165
Gafsa-Sened (GS)	Semi-arid superior	222	17.3	34°28′1.2”	9°16′1.2”	392

Table 1.
Samples collection sites and their eco-geographic characteristics.

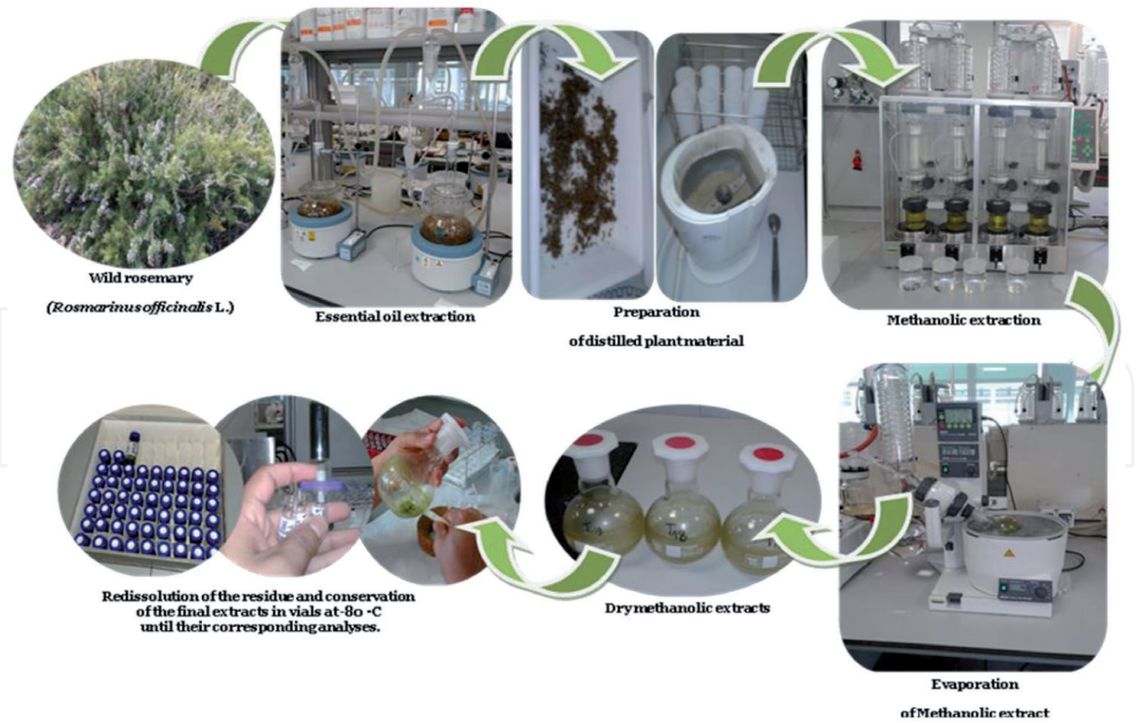

Figure 1.
Preparation of methanolic extracts of post-distilled rosemary plant.

in an evaporator system (Syncore Polyvap R-96) (Buchi, Flawil, Switzerland) (**Figure 1**). The residue was re-dissolved in methanol and made up to 5 mL [37]. 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 analyses.

2.4 Determination of the total phenolic content

Total phenolic contents in the extracts were determined by the Folin–Ciocalteu method [38]. A reaction mixture of 15 μL of methanolic extracts, 1185 μL of distilled water and 75 μL of Folin–Ciocalteu reagent were prepared. A vigorous stirring was performed and 225 μL of a sodium carbonate (20%) were added. The tubes were incubated in the dark for 30 min and then the absorbance was measured at 765 nm and 25°C with a Shimadzu (UV-2401PC, Japan) spectrophotometer. Standard curve was prepared by using different concentrations ranging from 100 to 1000 mg/L of gallic acid. Total phenolic content was expressed as mg gallic acid/g dry extract (mg GAE/g DE). Analyses were done in triplicate.

2.5 HPLC analysis

For the HPLC analysis, a method adapted from Zheng and Wang [1] was performed on a reverse phase ZORBAX SB-C18 column (4.6 x 250 mm, 5 μm pore size, Hewlett Packard, USA) using a guard column (ZORBAX SB-C18 4.6 x 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 were injected in 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 0.05% 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 and then 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. 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 mg per g of dry plant weight.

2.6 Statistical analyses

All data were reported as means ± standard deviation (SD). A one-way ANOVA was carried out to assess for significant differences (significant model was accepted for a p-value <0.05) using the IBM SPSS Statistic Program (v. 25). Next, Fisher's LSD pairwise comparison was performed on the data.

3. Results and discussion

3.1 Determination of total phenolic content (TPC)

Residues of the hydro-distillation process of aromatic plants oils had been studied for their contents of a diversity of biologically active compounds including antioxidants such as phenolic acids and flavonoids, that could be employed to increase the shelf life of food [13, 35]. The total phenolic content (TPC) in the post-distilled rosemary extracts ranged from 85.8 to 137.3 mg GAE/g DE (**Table 2**). Among the studied collection sites, post-distilled plants of GO population had the highest phenolic content (137.3 mg GAE/g DE). GS population had the lowest phenolic content (85.8 mg GAE/g DE). Our result showed higher TPC in comparison with that obtained by Jordan et *al.* (2013) in the case of Spain rosemary [16]. A recent investigation including several rosemary species revealed lower amounts of total phenolics of non-distilled plant material [18, 39].

Alternatively, results reported by Parejo et *al.* (2002) showed that plant material submitted to hydro-distillation has been found to contain a higher amount of phenolic substances than the non-distilled plant material [35]. In certain cases, cell wall phenolics or bound phenolics could be released consequently to heat exposure, thus generating more phenolics to be extracted. In addition, many studies described several biological activities that these non-volatile fractions have, and confirmed that these properties are directly related with the concentration of the principal components present in these polyphenolic extracts [16, 40–43]. Since total phenolic content estimated by the Folin–Ciocalteu procedure does not give a full picture of the quality and quantity of the phenolic constituents, HPLC analyses for determination of individual phenolic constituents is necessary.

3.2 Identification and quantification of polyphenolic compounds by HPLC

The polyphenolic profile of rosemary has been widely described in the scientific literature [4, 5, 16, 17, 29, 31, 33, 39, 44–49]. The polyphenolic profile of these plants is characterized by the presence of carnosic acid, carnosol, rosmarinic acid and hesperidin, as major components. Based on the retention times of calibration standards, methanolic extracts of rosemary showed a phenolic profile composed of 18 identified phenolic compounds (**Table 2**). The polyphenolic profile of rosemary are composed of four phenolic acids (salvianic acid, caffeic acid, rosmarinic acid, and salvianic acid A), five phenolic diterpenes (Rosmadial, 7-CH_3-Rosmanol, carnosol, carnosic acid and12-CH_3-carnosic acid), and nine flavonoids (Luteolin

	Kef Seres	Kef Menzel Salem	Gafsa Orbata	Gafsa Sned
Total phenolic content (TPC, mg GAE/g DE)	85.8 ± 28. 4[a]	109.1 ± 23.1[b]	137.3 ± 15.6[c]	87.8 ± 15.0[a]
Phenolic acids				
Salvianic Acid	1.10 ± 0.13[ab]	1.13 ± 0.23[ab]	1.21 ± 0.39[b]	0.84 ± 0.13[a]
Caffeic Acid	1.00 ± 0.22[b]	0.90 ± 0.11b[ab]	0.74 ± 0.26b[ab]	0.69 ± 0.27[a]
Rosmarinic Acid	29.91 ± 9.33[c]	17.96 ± 3.25[ab]	26.02 ± 5.88[bc]	16.77 ± 7.59[a]
Salvianolic Acid A	1.76 ± 0.44[a]	1.20 ± 0.34[a]	2.62 ± 0.84[b]	1.32 ± 0.40[a]
Flavonoids				
Luteolin –7-O-Rutinoxide	0.98 ± 0.40[b]	0.74 ± 0.18[ab]	0.74 ± 0.37[ab]	0.57 ± 0.25[a]
Luteolin-7-Glucoronide	2.56 ± 0.80[b]	1.89 ± 0.59[ab]	1.15 ± 0.49[a]	1.28 ± 0.62[ab]
Hesperidin	10.41 ± 2.79[a]	14.0 ± 2.69[b]	10.6 ± 2.77[ab]	9.85 ± 3.47[a]
Luteolin	0.77 ± 0.10[a]	0.97 ± 0.13[b]	0.81 ± 0.11[ab]	0.78 ± 0.18[a]
Apigenin	0.23 ± 0.05[a]	0.24 ± 0.06[a]	0.24 ± 0.05[a]	0.2 ± 0.05[a]
Hispidulin	0.34 ± 0.05[a]	0.48 ± 0.09[b]	0.41 ± 0.09[ab]	0.37 ± 0.08[a]
Cirsimaritin	1.21 ± 0.58[a]	1.53 ± 0.48[a]	1.32 ± 0.41[a]	1.16 ± 0.42[a]
Genkwanin	3.30 ± 1.33[a]	2.51 ± 0.71[a]	2.07 ± 0.84[a]	2.05 ± 1.22[a]
Salvigenin	1.05 ± 0.31[a]	1.13 ± 0.3[a]	1.59 ± 0.5[b]	1.22 ± 0.37[ab]
Diterpenes				
Rosmadial	1.83 ± 0.32[a]	2.49 ± 0.41[bc]	2.68 ± 0.54[c]	1.98 ± 057[ab]
7-CH_3-Rosmanol	1.45 ± 0.35[a]	1.49 ± 0.26[a]	3.73 ± 0.75[b]	1.12 ± 0.31[a]
Carnosol	26.94 ± 4.86[ab]	29.95 ± 3.57[b]	43.53 ± 4.18[c]	22.36 ± 4.17[a]
Carnosic acid	57.33 ± 22.37[a]	54.74 ± 9.24[a]	76.36 ± 12.87[b]	46.27 ± 12.01[a]
12-CH_3- Carnosic Acid	9.60 ± 4.86[a]	10.60 ± 4.00[a]	16.70 ± 5.84[b]	10.6 ± 2.78[a]

Contents of phenolic compounds are expressed as as mg compound/g Dry plant weight. Values followed by the same letter did not share significant differences at 5% (Duncan test).

Table 2.
Total phenolic content and polyphenolic profiles of R. officinalis *L. methanolic extracts.*

–7-O-Rutinoxide, luteolin-7-Glucoronide, hesperidin, luteolin, apigenin, cirsimaritin, genkwanin, and salvigenin). Among the mentioned phenolic compounds, carnosic acid and carnosol were the major diterpenic components quantified in rosemary extracts, considering both provenances, (ranging from 46.3 to 76.4 mg/g and 22.4 to 43.5 mg/g respectively) followed by rosmarinic acid and hesperidin. Much lower contents were detected for luteolin, apigenin, cirsimaritin and genkwanin.

In the present study locations belonging to two different bioclimatic regions were prospected, showing, as a general pattern, that the differences in polyphenolic content should be attributed more to the genetic inheritance of the plants, than to the area of prospection. Contrary to this, studies accomplished by Yeddes et *al.* (2019) about the effect of bioclimatic area and season on phenolics and antioxidant activities of rosemary growing wild in Tunisia showed that there was a strong correlation between antioxidant activity and phenolic content depending on bioclimatic and season effects [18]. However, our results are in agreement with those published

previously by Jordán et *al.* (2013) and Luis et *al.* (2007), since as occurs at the present study, for these researchers, variation in the chemical composition of polyphenolic extracts have been attributed to many factors, including abiotic stress,, genetic heritance and the phenological stages of the plants [16, 50].

Several phenolic compounds of rosemary determined in this study were similar in content and concentration to those in previous reports [1, 24, 51]. These phenolic compounds in rosemary extracts are very potent antioxidants and are utilized in many food products. The identified compounds were previously reported in *R. officinalis* extracts: rosmarinic acid and carnosic acid [29, 52], carnosol [52], caffeic acid, ferulic acid, luteolin, apigenin [53]. Differences among phenolic compound levels, compared with our results, can be related to the distillation process, because according to Almela et *al.* [51], the drying and/or distillation treatments of *R. officinalis* strongly affected the content of the two compounds of higher antioxidant activity: rosmarinic and carnosic acid. However, our samples seem to have higher concentrations of carnosic acid compared with previous studies [1, 51].

4. Conclusion

Rosemary (*R. officinalis* L.) is a rich source of phenolic compounds and their properties are derived from its extracts. It is therefore the strong antioxidant compounds in its essential oil and extract that is making *R. officinalis* a plant of great interest in today's food and medical industries. Bioactive compounds of plant origin have been shown to have several beneficial properties. Nowadays the use of natural compounds is also increasing around the world due to their mild features and low side effects. Several studies have indicated that the consumption of natural antioxidant compounds protect cells against the damage of reactive oxygen species such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite. Detection of natural antioxidant sources would be beneficial for healthy life. Many plants, such as vegetables, fruits, and herbs, are the main sources of natural antioxidants. Phenolic compounds have attracted particular interest because these compounds demonstrate effective antioxidant potential and other beneficial properties. Unfortunately, due to their structure and nature, certain compounds such as polyphenols, is not stable and may interact easily with the matrices in which they are incorporated. Although it is crucial to benefit from the phenolic compounds, there are unsaturated bonds in the molecular structure of polyphenols and this makes them vulnerable to oxidants, light, heat, pH, water and enzymatic activities. Therefore, the stability and shelf life of phenolic compounds should be increased by being protected from chemical and physical damage prior to its application. In order to minimize aroma degradation or loss during processing and storage, it is beneficial to encapsulate volatile ingredients prior to use in foods or beverages. A bioactive compound encapsulated in a biopolymer can be efficiently protected from harmful environmental agents like light, oxygen or water. Encapsulation is becoming increasingly important in the pharmaceutical, food, cosmetics, textile, personal care, chemical, biotechnology and medicinal industries due to its potential for stabilization and delivery of delicate and precious bioactive compounds.

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Conflicts of interest

The authors declare no conflict of interest.

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