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Correlation Between Polyphenolic Compounds and Antioxidant Activity of Tunisian Wild Rosemary (*Rosmarinus Officinalis* L.) Post-distilled Residues

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Abstract: Rosemary (*Rosmarinus officinalis* L.) constitutes a reliable and inexhaustible source of biologically active molecules with antioxidant activity which confers beneficial effects for human health. This study was undertaken with the following aims: to evaluate the TPC (total polyphenolic content), to assess the antioxidant potential and to identify and quantify the phytochemical compounds of Tunisian wild rosemary postdistilled residues. Furthermore, the correlation between polyphenolic compounds and the antioxidant activity was studied. The TPC, estimated by the Folin-Ciocalteu assay, reached the value of 113.28 mg gallic acid equivalent/g dry extract (mg GAE/g DE). The antioxidant potential, assessed by DPPH (2, 2-diphenyl1-picrylhydrazyl) and FRAP (Ferric-Reducing Antioxidant Power) assays, was 19.96 µg/mL and 48.13 mMFe2+/mg DE, respectively. The polyphenolic profile, determined by HPLC (high-performance liquid chromatography) analysis, showed the presence of eighteen polyphenolic compounds of which carnosic acid and carnosol were the major abundant components (76.36 and 43.53 mg/g DE, respectively), followed by rosmarinic acid and hesperidin (26.02 and 10.60 mg/g DE, respectively) The correlation between many identified polyphenolic compounds and the antioxidant activity was found highly significant ($p < 0.05$). These results proved that the post-distilled rosemary residue extracts constitute an effective natural antioxidant due to their high content of bioactive molecules, and seemed to be incorporate in the soft pharmaceutical products, safety foods, and biocosmetics industries, with beneficial effects for human well-being.

Key words: *Rosmarinus officinalis* L., post-distilled residues, polyphenolic compounds, antioxidant activity, correlation.

1. Introduction

Aromatic and medicinal plants have always been the main sources of natural products, commonly named as bioactive compounds, with beneficial activities, namely polyphenols, vitamins, polysaccharides and minerals [1-4]. Specifically, the natural compounds from the Lamiaceae family (thyme, sage and rosemary) have been reported in several studies for its antioxidant, anti-inflammatory, antimicrobial and anti-carcinogenic activities [5-7]. In particular Rosemary (*Rosmarinus officinalis* L.) extracts possess very useful antioxidant properties, which appear to be related to their content of essential oil and phenolic compounds [5, 8-10].

Rosemary (*Rosmarinus officinalis* L.), currently named *Salvia rosmarinus* Spenn., belongs to the Lamiaceae family, and is the most used and economically important aromatic and medicinal plant for its essential oil and phenolic compounds [9, 10]. Leaves of rosemary have been used for a long time in

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Mediterranean cuisine, not only to improve or modify the flavor of foods, but also to avoid its deterioration. Dried leaves, or their extracts, are characterized by their richness in phenolic compounds with known anti-inflammatory, antioxidant, antiaging, antibacterial and anticancer properties [5, 8]. On the other hand, the search for natural antioxidants in wastes of plant origin is also being explored as an alternative to the synthetic antioxidants used in food and pharmaceutical industries [5, 9, 11, 12]. Rosemary is among the most promising sources used for the recovery of essential oils through hydrodistillation. Yet, the exploitation for polyphenols recovery from the post-distillation wastes, which may be used as antioxidants in foods, food supplements or cosmetics, is really limited. Nevertheless, some previous studies have shown that the post-distillation waste materials from rosemary still possess antioxidant activity. Furthermore, the residues remaining after essential oil recovery have been studied for their content of a diversity of bioactive compounds such as phenolic acids and flavonoids, which is useful in increasing the shelf life of food [5, 9, 12].

In light of the above, the aim of this study was to determine the total polyphenolic content, to evaluate the antioxidant activity and to identify and quantify the polyphenolic compounds of post-distilled residues of rosemary aerial parts extracts. Furthermore, the correlation between polyphenolic compounds and the antioxidant activity was studied. This phytochemical characterization was carried out to revalorize this wild plant by recovered phenolic compounds from their by-product as a source of bioactive molecules.

2. Material and Methods

2.1 Plant Material

Rosemary plants were randomly collected from wild population of Gafsa, Tunisia (lower arid, Longitude (N): 34°22’49.8’’, Latitude (E): 9°3’23.4’’, Altitude (m): 1165) at the full bloom phenological stage. Fresh aerial parts of plants were firstly dried at room temperature for ten days and afterwards dried in oven at 35 °C for 48 hours, until they reached a constant weight.

2.2 Preparation of the Plant Extracts

After hydrodistillation, the plant material was dried in oven at 35 °C for 48 h and then ground. Dried samples (0.5 g) were extracted using 150 mL of methanol in a Soxhlet extractor (B-811) (Buchi, Flawil, Switzerland), for 2 h. RE (rosemary extracts) were taken to dryness at 35 °C under vacuum conditions in an evaporator system (Syncore Polyvap R-96) (Buchi, Flawil, Switzerland). The residue was re-dissolved in methanol and made up to 5 mL [13]. The yield of the extracts was expressed in terms of milligrams of dry methanolic extract per gram of dry plant weight (mg DE/g DPW). Final extracts were kept in vials at -80 °C until their corresponding analyses.

2.3 Determination of Total Polyphenolic Content

TPC (total polyphenolic content) was determined by the Folin-Ciocalteu reagent method [14]. Briefly, 15 µL of extracts were added to 1,185 µL of distilled water and 75 µL of 10% Folin-Ciocalteu reagent. A vigorous stirring was performed and 225 µL of a sodium carbonate (20%) were added. After 30 min of incubation, the absorbance of the resulting blue-colored solution 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 0.1 to 1 mg/mL of gallic acid. TPC was expressed as mg gallic acid equivalents per gram of dry extract (mg GAE/g DE). Analyses were done in triplicate.

2.4 Evaluation of Antioxidant Activity

2.4.1 DPPH (2, 2-diphenyl1-picrylhydrazyl) • Radical-Scavenging Activity

The study of the DPPH• free radical scavenging activity of the rosemary methanolic extracts was performed according to the method described by

Brand-Williams et al. [15] with some modifications. Briefly, 50 µL and 100 µL of sample were added to eppendorf tubes containing respectively 850 µL and 800 µL of methanol, and then 100 µL of 1 mM DPPH• were added. The scavenging activity was evaluated by measuring the absorbance at 515 nm after 30 min reaction at 25 °C, in a Synergy MX UV-vis spectrometer (BioTek Instruments Inc; Winooski, VT, USA). The absorbance of the control consisting of 900 µL of methanol and 100 µL of DPPH• solution was measured daily. Measurements were performed in triplicate. The inhibition percentage (%I) of DPPH• in the steady state was determined following the equation:

$$\%I = [(Abs_{control} - Abs_{sample}) / Abs_{control}] \times 100.$$

The results were expressed as the inhibitory concentration of the extract needed to decrease DPPH• absorbance by 50% (IC_{50}). Concentrations are expressed in micrograms of dry extract per milliliter of methanol (IC_{50}, µg/mL).

2.4.2 FRAP (Ferric-Reducing Antioxidant Power)

The ferric-reducing ability of extracts was measured according to the method developed by Benzie and Strain [16]. Antioxidant compounds are able to reduce ferric iron, in the ferricyanide complex, Fe^{3+} to ferrous iron Fe^{2+}, which develops a blue color. To prepare the FRAP reagent, a mixture of 0.1 mM acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl and 20 mM ferric chloride (10:1:1) was made. An aliquot of 40 µL of each sample was added to 1.2 mL of the FRAP reagent. The absorption 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 ($FeSO_4.7H_2O$) of 0.05 to 1 mM were used to obtain the calibration curve and results were expressed as $mMFe^{2+}$ equivalent per milligram of dry extract ($mMFe^{2+}$/ mg DE).

2.5 Identification and Quantification of Polyphenolic Compounds by HPLC (high-performance liquid chromatography)

Phenolic compounds were identified and quantified by HPLC. Chromatographic analyses were 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, based on the method adapted from Zheng and Wang [1]. 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. The results were expressed as mg of compound per gram of dry plant weight (mg/g DPW).

Statistical analyses: All experiments were performed in triplicate (n = 3) and data were reported as means ± standard deviation (SD). A one-way ANOVA, followed by Duncan's multiple range tests, was carried out to assess for significant differences between various experiments (a significant model was accepted for a p-value < 0.05) using Excel and STATISTICA software version 5.1. Pearson's correlation coefficients were calculated. A p value less than 0.05 was considered to be statistically significant.

3. Results and Discussion

3.1 Total Polyphenolic Content

The TPC (total polyphenolic content) in the rosemary post-distilled extract, estimated by the Folin-Ciocalteu assay, reached the value of 112.55 mg gallic acid equivalent/g dry extract (mg GAE/g DE) (Table 1). These values prove that rosemary hydro distillation wastes are rich in polyphenolic compounds. Our result showed higher TPC in comparison with that obtained by Jordan et al. [5] in the case of Spain rosemary. A recent investigation including several rosemary species revealed lower amounts of total phenolics of non-distilled plant material [17, 18]. In certain cases, cell wall phenolics or bound phenolics could be released consequently to heat exposure, thus

generating more phenolics to be extracted.

It seems that the increase in temperature, in the range of 50-70 °C, produces a higher solubility of the compounds and increases their rate of transfer [19, 20]. Residues from the hydro-distillation process of essential oils from aromatic plants have been studied for their contents of a diversity of biologically active compounds, including antioxidants such as phenolic acids and flavonoids, which could be used to increase food shelf life in food industries [5, 7, 11, 21-23]. In fact, the biological activities of these compounds are directly related with the concentration of the principal components present in these polyphenolic extracts. Furthermore, the variability found in the total polyphenolic content produced by aromatic species depends on several factors, such as the species, the part of the plant, the harvest season, the geographical origin, as well as the extraction methods, and consequently their bioactive properties [24-27].

3.2 Antioxidant Activity

The antioxidant potential of rosemary post-distilled residues extracts, assessed by DPPH and FRAP assays, reached the value of 19.96 µg/mL and 48.13 $mMFe^{2+}$/mg DE, respectively (Table 1). These results show that the plants with high antioxidant capacity are characterized by high levels of total polyphenolic content. Antioxidants present in plant-based waste are also being studied as an alternative to synthetic antioxidants used in the food and pharmaceutical industries. Alternatively, by-products from essential oil production, i.e., distilled residues of aromatic plants, constitute a potential reservoir of compounds with high antioxidant activity and may contribute to protective effects on human health. Thus, residues remaining after distillation of aromatic plant oils are considered a natural source of antioxidants [5, 7, 21].

Indeed, several studies have confirmed the influence of the distillation process on the polyphenol content of several Lamiaceae species, notably *Salvia officinalis* L., *Rosmarinus officinalis* L. and *Thymus zygis* subsp. gracilis (Boiss) [6, 28]. Results reported by Parejo et al. [11] 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. In certain cases, cell wall phenolics or bound phenolics could be released consequently to heat exposure, thus generating more phenolics to be extracted. 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 in food industries [12].

3.3 Polyphenolic Profile

The HPLC chromatogram of rosemary post-distilled residues extract is shown in Figure 1. The qualitative and quantitative analysis revealed the presence of eighteen polyphenolic compounds (Table 2). Among the mentioned phenolic compounds, carnosic acid and carnosol were the major diterpenic components quantified in rosemary extract (76.36 and 43.53 mg/g DPW, respectively) followed by rosmarinic acid, 12-CH_3-carnosic acid and hesperidin (26.02, 16.70 and 10.60 mg/g DPW, respectively). Chemical structures of the identified compounds main are presented in Figure 2.

Considering our results, the highest values of free radical scavenging activity of rosemary extract post-distilled residues could be due to their higher contents of phenolic components. It was the greater presence of these components that was responsible for the increased antioxidant capacity of the corresponding extracts. Consequently, it can be concluded that the concentration of the chemical compounds of polyphenolic extracts has an indispensable role in their antioxidative power [7]. Several authors have published the important role of carnosic acid, carnosol, and rosmarinic acid on the antioxidant power of the rosemary extract [29-31].

Table 1 Total polyphenolic content (TPC), DPPH radical scavenging activity, and Ferric-Reducing Antioxidant Power (FRAP) of rosemary polyphenolic extract.

	Total phenolic content (TPC, mg GAE/g DE)	DPPH IC_{50} (µg/mL)	FRAP ($mMFe^{2+}$/mg DE)
Gafsa	113.28 ± 3.60	19.96 ±1.02	48.13 ± 2.09

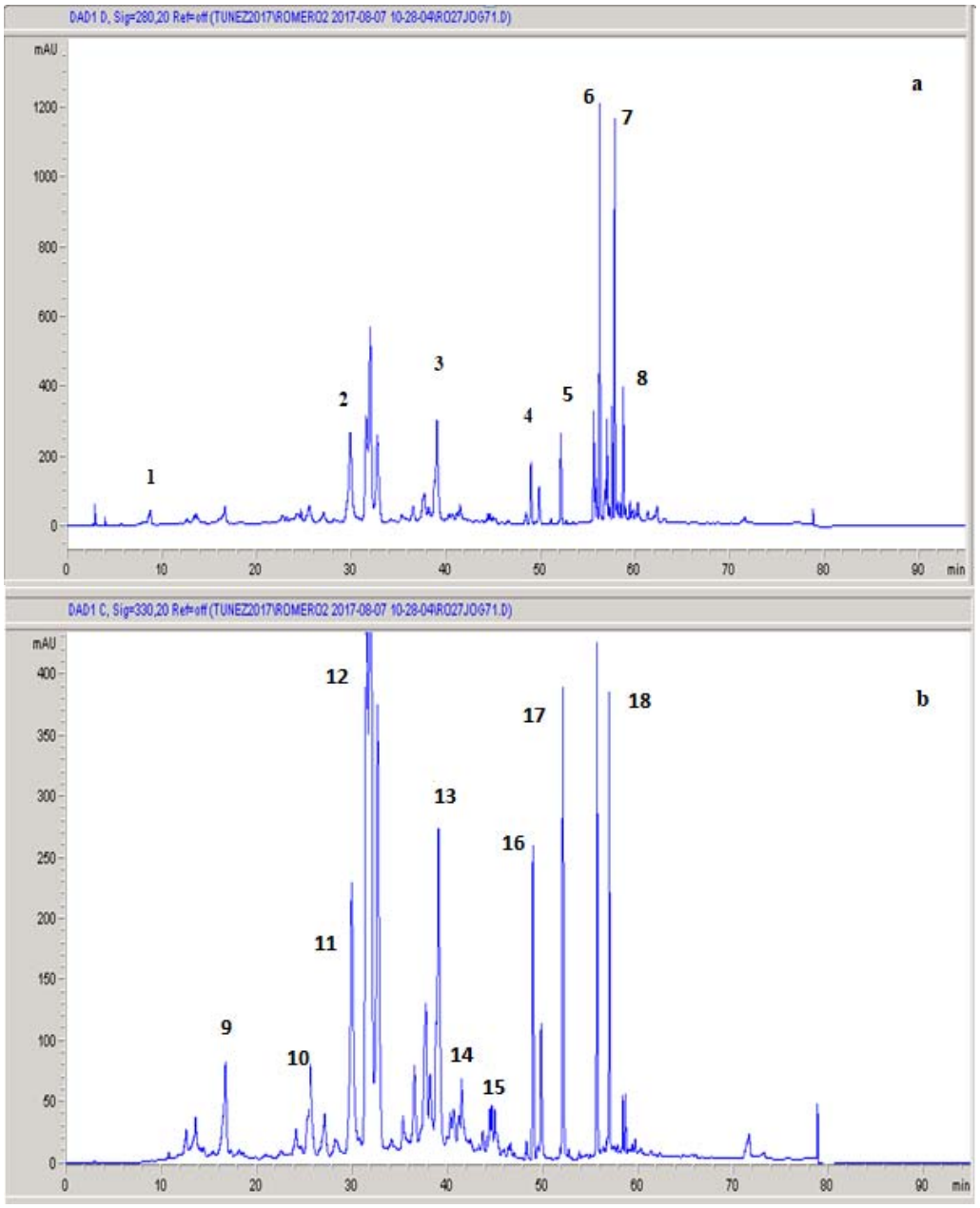
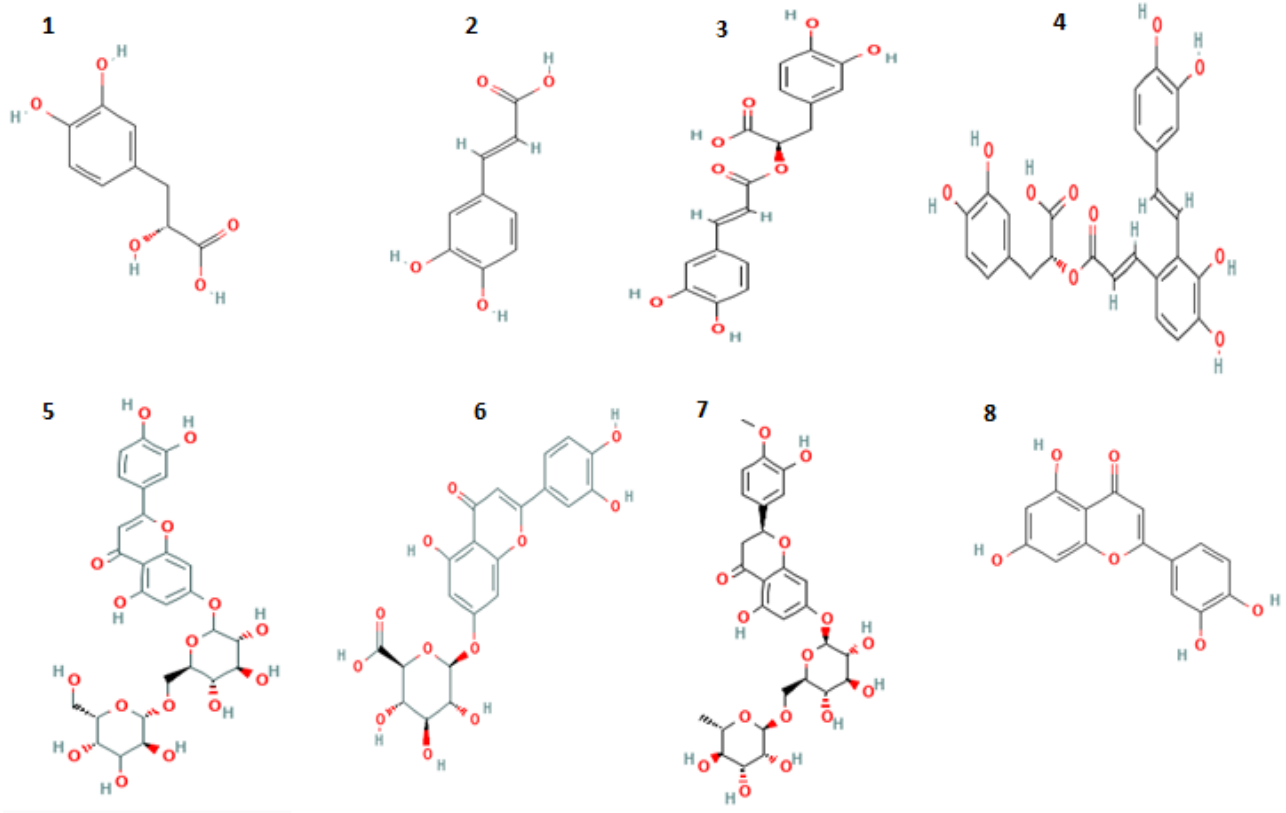


Fig. 1 HPLC chromatogram of rosemary post-distilled residue extract at 280 nm (a) and 330 nm (b). 1: Salvianic acid, 2: Hesperidin, 3: Salvianolic acid A, 4: Rosmadial, 5:7-CH_3-Rosmanol, 6: Carnosol, 7: Carnosic acid, 8:12-CH_3-Carnosic acid, 9: Caffeic acid, 10: Luteolin-7-O-Rutinoside, 11: Luteolin-7-O-Glucuronide, 12: Rosmarinic acid; 13: Luteolin, 14: Apigenin, 15: Hispidulin, 16: Cirsimaritine, 17: Genkwanin, 18: Salvigenin.

Table 2 HPLC polyphenolic profile of rosemary post-distilled extracts.

No.	Identified compounds	Rt (min)	λ (nm)	Chemical formula	Gafsa
1	Phenolic acids Salvianic acid	8.6	280	$C_9H_{10}O_5$	1.21 ± 0.39
2	Caffeic acid	16.63	330	$C_9H_8O_4$	0.74 ± 0.26b
3	Rosmarinic acid	32.72	330	$C_{18}H_{16}O_8$	26.02 ± 5.88
4	Salvianolic acid A	39.09	280	$C_{26}H_{22}O_{10}$	2.62 ± 0.84
5	Flavonoids Luteolin-7-O-Rutinoxide	24.06	330	$C_{27}H_{30}O_{15}$	0.74 ± 0.37
6	Luteolin-7-Glucoronide	25.64	330	$C_{21}H_{18}O_{12}$	1.15 ± 0.49
7	Hesperidin	29.93	280	$C_{28}H_{34}O_{15}$	10.6 ± 2.77
8	Luteolin	40.54	330	$C_{15}H_{10}O_6$	0.81 ± 0.11
9	Apigenin	44.59	330	$C_{15}H_{10}O_5$	0.24 ± 0.05
10	Hispidulin	44.8	330	$C_{16}H_{12}O_6$	0.41 ± 0.09
11	Cirsimaritin	49.09	330	$C_{17}H_{14}O_6$	1.32 ±0.41
12	Genkwanin	52.19	330	$C_{16}H_{12}O_5$	2.07 ± 0.84
13	Salvigenin	55.58	330	$C_{18}H_{16}O_6$	1.59 ± 0.5^b
14	Diterpenes Rosmadial	48.56	280	$C_{20}H_{24}O_5$	2.68 ± 0.54
15	7-CH_3-Rosmanol	56.01	280	$C_{21}H_{28}O_5$	3.73 ± 0.75
16	Carnosol	56.29	280	$C_{20}H_{26}O_4$	43.53 ± 4.18
17	Carnosic acid	57.93	280	$C_{20}H_{28}O_4$	76.36 ± 12.87
18	12-CH_3-Carnosic acid	58.81	280	$C_{21}H_{30}O_4$	16.70 ± 5.84

Contents of phenolic compounds expressed as mg of compound/g of dry plant weight (mg of compound/g DPW). Results are expressed as means ± standard deviation (n = 10).



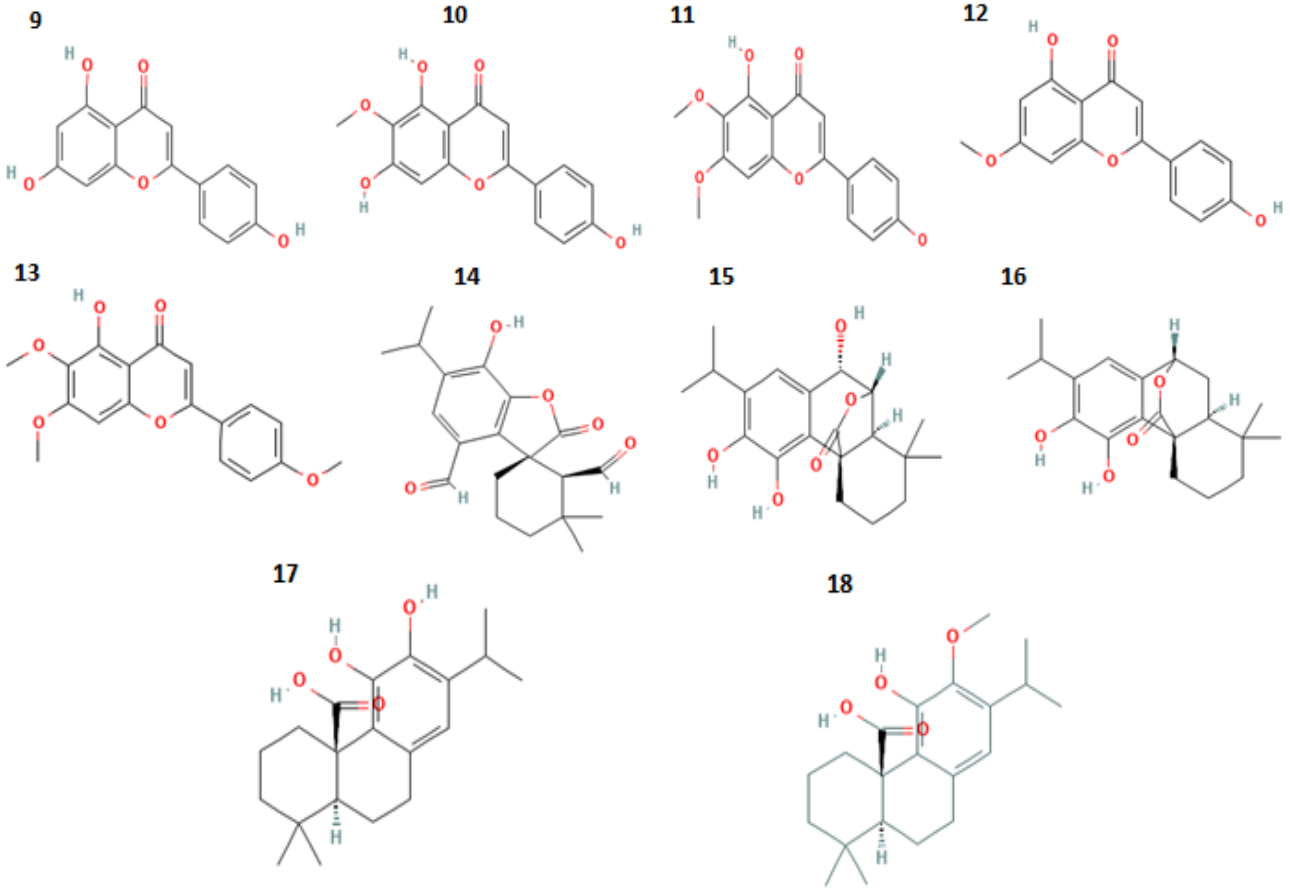


Fig. 2 Chemical structure of the main identified compounds of rosemary post-distilled residues. 1: Salvianic acid, 2: Caffeic acid, 3: Rosmarinic acid, 4: Salvianolic acid A, 5: Luteolin-7-O-Rutinoside, 6: Luteolin-7-O-Glucuronide, 7: Hesperidin, 8: Luteolin, 9: Apigenin, 10: Hispidulin, 11: Cirsimaritin, 12: Genkwanin, 13: Salvigenin, 14: Rosmadial, 15: 7-CH_3-Rosmanol; 16: Carnosol, 17: Carnosic acid, 18: 12-CH_3-Carnosic acid.

As previously reported, the antioxidant effect of rosemary is due to the polyphenol compounds (mainly rosmarinic acid, carnosol and carnosic acid), which accumulate in the fatty membranes of cells where the antioxidant effect is required [28, 32]. A significant positive correlation between the antiradical activity of Lamiaceae and the total polyphenols was reported, which demonstrates the importance of these antioxidant compounds in spices and their significant contribution to the total antioxidant activity [5-7, 33, 34]. In addition, the search for natural antioxidants in wastes of plant origin is also being explored as an alternative to the synthetic antioxidants used in the food and pharmaceutical industries. Alternatively, by-products from essential oil production, i.e., distillate residues of aromatic plants, are a potential pool of compounds with strong antioxidant activity and can contribute to protective effects on human health [9].

3.4 Correlation Analysis between Phenolic Compounds and the Antioxidant Activity

Table 3 shows the Pearson correlation coefficients (r) between the polyphenolic compounds of rosemary extract identified in a prior work [9], the TPC, and the antioxidant activity in an attempt to estimate the contribution of these compounds to the total antioxidant activity. The main compounds rosmarinic acid ($r = -0.72$), carnosic acid (-0.68), and carnosol (-0.65) revealed the most significant negative correlation ($p < 0.05$) with the DPPH test. While with

Table 3 Linear correlation coefficients established between polyphenolic compounds and the antioxidant activity.

Phenolic compounds	DPPH	FRAP
Salvianic acid	-0.67*	0.69*
Caffeic acid	-0.54*	0.19
Rosmarinic acid	-0.72*	0.64*
Salvianolic acid	-0.65*	0.77*
Luteolin-7-O-Rutinoxide	-0.45*	0.27
Luteolin-7-Glucoronide	-0.54*	0.19
Hesperidin	-0.35	0.03
Luteolin	-0.49*	0.12
Apigenin	-0.50*	0.51*
Hispidulin	-0.34	0.25
Cirsimaritin	-0.22	0.22
Genkwanin	-0.05	0.08
Salvigenin	-0.29	0.35
Rosmadial	-0.38	0.57*
7-CH_3-Rosmanol	-0.58*	0.88*
Carnosol	-0.65*	0.92*
Carnosic acid	-0.68*	0.79*
12-CH_3-Carnosic acid	-0.48*	0.57*
Total polyphenolic content (TPC)	-0.74*	0.84*

*Significant correlation at $p < 0.05$.

the FRAP test, these compounds revealed a significant positive correlation ($p < 0.05$). In addition, TPC had the most significant correlation with both antioxidant activity tests. As expected, these compounds are responsible for antioxidant activity in plants of the Lamiaceae family [28, 31-33]. The use of Pearson's correlation coefficients revealed significant correlations between several phenolic compounds and the antioxidant tests proving the significance of these compounds and their contribution to the antioxidant power of the plant extract [6, 7, 35]. The interaction or synergistic effect among the polyphenolic compounds contained in thyme post-distilled residues may also contribute to their antioxidant capacity. It was the greater presence of these components that was responsible for the increased antioxidant capacity of the corresponding extracts. In light of all the above, it can be concluded that the concentrations of the chemical compounds of polyphenolic extracts play an important role in their antioxidative power.

4. Conclusion

The positive correlation between the polyphenolic compounds and the antioxidant capacity confirmed that the polyphenolic compounds are responsible for the antioxidant activity of rosemary. The interaction or synergistic effect among the bioactive compounds contained in post-distilled rosemary extract may also contribute to their antioxidant capacity. Rosmary post-distilled wastes have proven to be an effective potential source of polyphenols, as natural antioxidants beneficial properties to human health, and could be useful in replacing or even decreasing synthetic antioxidants in foods, cosmetics and pharmaceutical products. This highlights the interest in extracting the phenolic compounds from the rosemary by-product in order to its antioxidant capacity is to be exploited. Therefore, supplementing a balanced diet with plants by-products may have beneficial health effects.

Authors' Contributions

Conceptualization, K. H.; Methodology, K. H., A. M. and M.Q; Formal analyses, A. M., A. A. and M. Q.; Investigation, K. H. and A. M.; Resources, K. H.; Writing-original draft preparation, K. H. and A. M.; and Writing-review and editing, Supervision, K. H. and M. J. J.

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Availability of Data and Materials

All data will be made available on request according to the journal policy

Conflicts of interest: The authors declare no conflict of interest.

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