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Influence of Genotype on Antioxidant Activity and Phenolic Profile of Fennel Bulbs

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Abstract: Currently, fennel bulb is becoming a highly demanded and consumed vegetable due to its licorice flavor and health benefits. Although the chemical composition of fennel essential oil has been extensively studied, the nonvolatile bioactive compounds of fennel bulbs have drawn less attention. Here, the phenolic profile and the antioxidant activity in terms of DPPH radical scavenging activity, reducing power, chelating ability of ferrous ions, and inhibition of lipid peroxidation were analyzed using four different fennel hybrid genotypes and three different extraction solvents (methanol, aqueous-methanol, and hot water). Antioxidant activity results revealed significant variation amongst fennel varieties (>3-fold difference for DPPH and reducing power and >2.7-fold for the inhibition of lipid peroxidation), with methanolic extracts exhibiting the highest antioxidant activity. Total phenol content peaked in the aqueous-methanol extracts, exhibiting a 2-fold difference across fennel genotypes. HPLC–PDA/MS analyses identified high levels of caffeic acid derivatives in hot water extracts, particularly in the commercial genotype. The therapeutic benefits associated with these compounds make it reasonable to use detailed phytochemical screening in fennel breeding programs to obtain varieties with new functionalities and thus higher added value.

Keywords: *Foeniculum vulgare*; phenolic profile; antioxidant activity; varieties; extraction solvent; caffeic acid derivatives

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1. Introduction

Nowadays, increasing health awareness has led consumers to pay more attention to their diet, which has resulted in a significant increase in the demand for health-promoting foods such as fruits and vegetables. Numerous epidemiological studies pointed out that the regular consumption of fruits and vegetables can reduce the occurrence of chronic diseases, including cardiovascular disorders, stroke, type II diabetes mellitus, some cancers [1–3], and dementia [4–6]. Current literature suggests that antioxidant phytochemicals, including carotenoids, vitamin C, tocopherol, and phenolic compounds, can be responsible for the observed protection in preventing diseases caused as a result of oxidative stress [7–10]. Phenolic compounds are widespread secondary metabolites present in plant foods, and they are commonly known as the largest phytochemical molecules with antioxidant properties from plants [11,12]. Recently, dietary phenolic compounds have been described as effective antioxidants in treating inflammatory diseases, allergies, and cancer [13–15]. The antioxidant properties of phenolics are mainly related to their chemical structures, mostly the number and position of hydroxyl groups in the molecule [16–18]. Considering the potential chemopreventive properties of these compounds, the study of the phenolic profile and antioxidant properties of plant foods is gaining increasing interest both in academia and in the agri-food and pharmaceutical industries. Moreover, although current plant breeding programs are basically focused on the development of pathogen-resistant and climate-resilient crops [19], the improvement of the nutritional quality of plants is

also an important target in breeding, seeking to increase the content of health-promoting compounds in foods [20].

Fennel (*Foeniculum vulgare* Mill.) is an umbelliferous plant native to the Mediterranean area, well-known for its aromatic and medicinal properties, and used by humans since antiquity [21]. Currently, in Europe, the bulb of sweet fennel (*F. vulgare* Mill. ssp. *vulgare* var. *azoricum* Thell.) is an important culinary element widely consumed as a vegetable in soups and salads [21]. The enchanting licorice-like flavor and aroma, which come from anethole essential oil, together with its health benefits, including antimicrobial, antiviral, gastroprotective, and antithrombotic properties [22], could explain the increasing demand for fennel bulbs. Notably, in recent years, the chemical composition of the essential oil obtained from different fennel organs (root, leaf, stem, fruit/seed, and whole aerial parts) has been the subject of intensive research [22–25]. Despite its high demand and economic value, limited information exists regarding the nonvolatile compounds, particularly phenolics, in fennel bulbs. It is well known that the extraction of phenolic compounds from plant matrices is greatly influenced by the solvent-polarity, temperature, contact time, sample-to-solvent ratio, and extraction technique used [26,27]. Furthermore, the chemical composition of the phenolic compounds to be extracted, the nature of the sample matrix, and the presence of interfering substances also substantially affect the efficiency of the extraction [27,28]. Moreover, the amount and occurrence of plant bioactive compounds are strongly influenced by environmental and edaphoclimatic conditions, as well as by the genetic background of the plant [29]. Notably, the characterization of genotypes from germplasm banks or breeding programs has become a priority for designing effective selection programs. Specifically in fennel, distinct genotypes displaying variations in drought recovery capabilities have been identified [30]. However, limited information is available regarding the variation in bioactive phenolic compounds in fennel bulbs among different genotypes. Hence, the purpose of the present work was to characterize the antioxidant activity and phenolic profile of different fennel genotypes grown under the same conditions using different extraction solvents to provide guidelines that can assist in the development of plant-breeding methods to maximize the content of health-promoting and disease-preventing phytochemicals.

2. Materials and Methods

2.1. Plant Material and Extract Preparations

In this study, three experimental breeding fennel hybrids (*Foeniculum vulgare* Mill ssp. *vulgare* var. *azoricum* Thell.) from Rijk Zwaan Ibérica S.A. (Spain) and a commercial hybrid variety were used (named thereafter varieties I to IV, respectively). Plants were grown to the commercial harvest stage following the recommended agronomic practices of the region (Torre Pacheco, Murcia, Spain). At the harvest stage, at least ten fennel bulbs with an average weight of 300–400 g (commercial weight) were randomly collected from each variety and brought to the laboratory under cool conditions (Figure 1). All the bulbs collected meet the UNECE international marketing standards applicable to fresh fennel [31]. In the laboratory, the bulbs were cut to isolate the inner young, whitish, hypertrophied leaves of the basal rosette and immediately snap frozen in liquid nitrogen and stored at −80 °C. Young leaves from three plants per variety were pooled to form a biological replicate, and three replicates were examined. Leaves were ground into a fine powder using a liquid nitrogen-cooled mill (IKA, Labortechnik, Staufen, Germany).

2.2. Preparation of Extracts

Bulb fennel extracts were obtained using methanol (absolute, 100%), methanol:water (20:80; *v*/*v*), or hot water (90 °C). For the extractions, 500 mg of leaf powder samples were mixed with 3 mL of solvent. In both methanolic extractions, samples were incubated at room temperature with occasional shaking for 1 h in dark conditions and spun down at 10,000× *g* for 10 min at 4 °C. In hot water extractions, samples were kept in a water bath at 90 °C for 10 min in darkness, then cooled in an ice bath and centrifuged under the same conditions as above. All the clarified supernatants were stored at −80 °C until use.

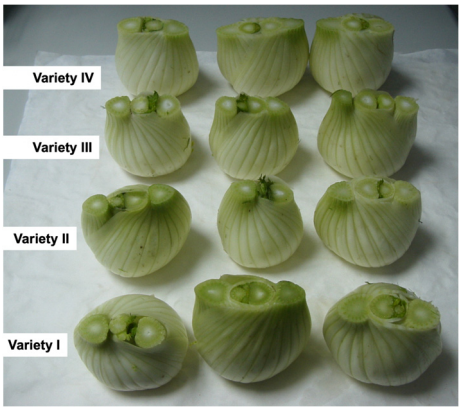

Figure 1. Representative photo of the four fennel bulb varieties used in this study. The varieties I, II, and III correspond to the experimental breeding fennel hybrids from Rijk Zwaan Ibérica S.A. (Spain), and the IV to a commercial variety.

2.3. Determination of Antioxidant Properties

2.3.1. DPPH Radical-Scavenging Assay

The free radical scavenging capacities of fennel extracts were quantified by reaction with the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical as previously described [29]. The results were expressed as micromoles of DPPH reduced per gram of fresh weight using an extinction coefficient of 12,500 M^{-1} cm^{-1} at 517 nm [32].

2.3.2. Reducing Power Assay

The antioxidant activity of the bulb fennel extracts based on the reducing power determination was determined by using the potassium ferricyanide-ferric chloride method, according to Pérez-Tortosa et al. [29]. The reducing power was expressed as micromoles of ascorbic acid equivalents (AAE) per gram of fresh weight.

2.3.3. Metal Chelating Activities

The ferrous ion chelating potential of fennel extracts was estimated according to the method of Dinis et al. [33], with some modifications. In short, 100 µL of fennel extracts were added to 300 µL of $FeSO_4 \cdot 7H_2O$ (2 mM) and vortex mixed for 30 s. Then, 600 µL ferrozine (FZ) (5 mM) (Sigma Aldrich, Madrid, Spain, Cat. No. 82950) was added, and the absorbance was read at 562 nm after a 10-min incubation period at room temperature in the dark. The amount of chelated iron ions by fennel extracts was estimated from the difference between the initial concentration of Fe(II) in the reaction media (600 µM) and the concentration of Fe(II) in the Fe(II)-FZ_3 complex, for which an extinction coefficient of 28,600 M^{-1} cm^{-1} at 562 nm was used [34].

2.3.4. Measurement of Inhibition of Lipid Peroxidation

The inhibitory ability of the extracts on lipid peroxidation was determined by following the formation of conjugated dienes at 234 nm using linoleic acid (Sigma-Aldrich, Spain) as substrate, as described by [29]. The results were expressed as the difference between the amount of conjugated diene formed in the absence (control reaction media) and in the presence of the sample extracts. The micromoles of conjugated diene formed were calculated using a molar absorption coefficient of 25,000 M^{-1} cm^{-1}.

2.3.5. Quantification of Total Soluble Phenol Content

The total soluble phenol content (TPC) was determined by the Folin–Ciocalteu procedure as previously described [29], using caffeic acid (0–1000 µM) as the standard. Total soluble phenol content was expressed as micromoles of caffeic acid equivalents (CAE) per gram of fresh weight.

2.4. HPLC-MS Analysis of Phenolic Compounds

HPLC–PDA/MS analyses were conducted on a 2695 Waters HPLC system (Waters Corporation, Milford, MA, USA) equipped with a 2996 Photodiode Array Detector and coupled to a ZQ quadrupole mass spectrometer (Micromass ZQ4000, Waters Corp.) equipped with an electrospray ionization (ESI) source. The separation was carried out following the method described by Parejo et al. [35] using a LiChroCART C-18 reversed-phase column (250 mm × 4 mm i.d., 5 µm; Merck, Darmstadt, Germany). The mobile phase was a gradient prepared from 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The composition ranged from 10% B to 26% B in 40 min. The flow rate was 1 mL min^{-1}, operated at 30 °C, and the injection volume was 20 µL. UV detection was performed at 330 and 280 nm. The ESI mass spectrometer analysis was conducted in both negative and positive ion modes. Nitrogen was used as both a cone gas and a desolvation gas. The cone voltage was set at 30 V, and the full-scan mass covered the m/z range from 100 to 1000 Da.

Identification of the phenolic compounds was carried out by comparison of the retention times and UV spectra with those of reference compounds [caffeic acid (assay ≥ 98%), chlorogenic acid (assay ≥ 95%), and rosmarinic acid (assay ≥ 98%) (Sigma-Aldrich, Madrid, Spain)]. The identified analytes were quantified by integration of the peak areas at the wavelength corresponding to their maximum absorbance using external calibration curves with seven dilutions of each standard at concentrations ranging from 10 to 100 µM (1–500 nM). The caffeic acid derivative levels were expressed as nanomoles of chlorogenic acid equivalents (CGAE) per gram of fresh weight.

2.5. Statistical Analysis

The data were expressed as the mean ± standard error (SE) of at least three biological replicates. The data were analyzed by Pearson's correlations and by one-way ANOVA followed by Tukey's HSD post hoc test ($p \leq 0.05$) using SPSS software (version 26; SPSS Inc., Chicago, IL, USA). The principal component analysis (PCA) biplot was carried out using the CANOCO software (CANOCO for Windows program v4.02).

3. Results

3.1. Free Radical Scavenging and Antioxidant Activities of Fennel Bulbs Using Different Extraction Solvents

To detect the effect of solvent on the antioxidant properties of fennel bulb extracts, four different antioxidant methods were tested based on the DPPH radical scavenging activities, the electron donation capacity, the chelating ability of ferrous ions, and the inhibitory ability of lipid oxidation.

As shown in Figure 2, DPPH results indicate that methanolic extracts exhibited the highest antiradical capacity, followed by hot water extracts, and finally by the aqueous-methanol extracts in the four fennel varieties assayed. DPPH values were in the range of 0.31–1.20 µmol DPPH g^{-1} FW in the methanolic extracts, representing a 3.9-fold difference among the different fennel varieties tested. The fennel variety with the highest DPPH values was variety III, followed by varieties II and IV, and the lowest values were found in variety I.

Reducing power (RP) values were in the range of 2.7–8.6 µmol AAE g^{-1} FW in the methanolic extracts, representing a 3.2-fold difference among the fennel varieties tested (Figure 3). Results also reveal that the methanolic extracts from fennel varieties III and IV exhibited the highest reducing ability, followed by the aqueous-methanol and hot water extracts (4.9–5.7 µmol AAE g^{-1} FW) from these fennel varieties. The lowest RP activities were found in the methanolic extracts from fennel variety II (2.7 µmol AAE/g FW) as well as in the three extracts from fennel variety I (2.7–2.9 µmol AAE g^{-1} FW) (Figure 3).

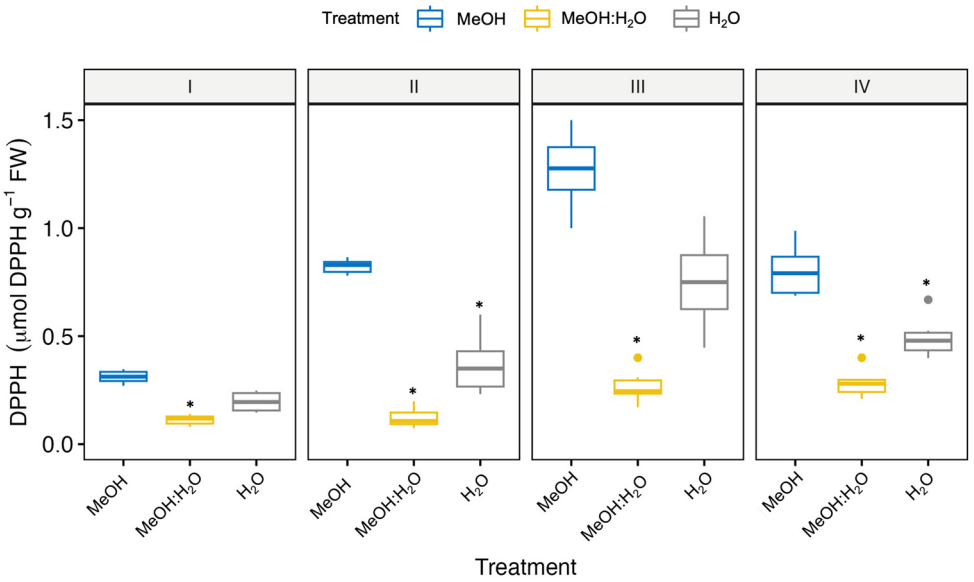

Figure 2. Antioxidant activity of methanol, aqueous-methanol, and hot water extracts of fennel bulbs from the four fennel varieties tested (**I**–**IV**) by the DPPH method. The data are means ± SE from two experiments. For each fennel variety, treatments marked with an asterisk are significantly different from the corresponding methanol extract ($p < 0.05$) by Tukey's HSD post hoc test.

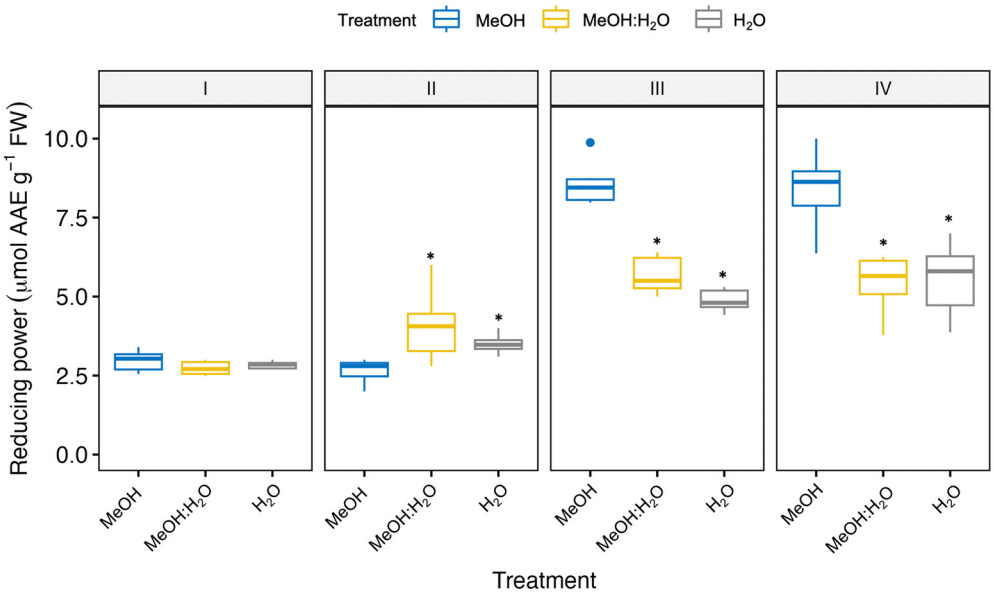

Figure 3. Antioxidant activity of methanol, aqueous-methanol, and hot water extracts of fennel bulbs from the four fennel varieties tested (**I**–**IV**) by the reducing power method. The data are means ± SE from two experiments. For each fennel variety, treatments marked with an asterisk are significantly different from the corresponding methanol extract ($p < 0.05$) by Tukey's HSD post hoc test.

Regarding the chelating ability of ferrous ions, it was noted that methanolic extracts weakly interfered with the formation of the ferrous complex with the heterocyclic amine ferrozine (Figure 4). Moreover, the chelating activity of the methanolic extracts was similar in the four fennel varieties (about 4.9 µmol chelated Fe(II) g^{-1} FW), while the highest chelating activities were found in the aqueous-methanol extracts, whose values ranged from 7.7 to 10.5 µmol chelated Fe(II) g^{-1} FW, closely followed by the hot water extracts (6.8–8.5 µmol chelated Fe(II) g^{-1} FW) (Figure 4).

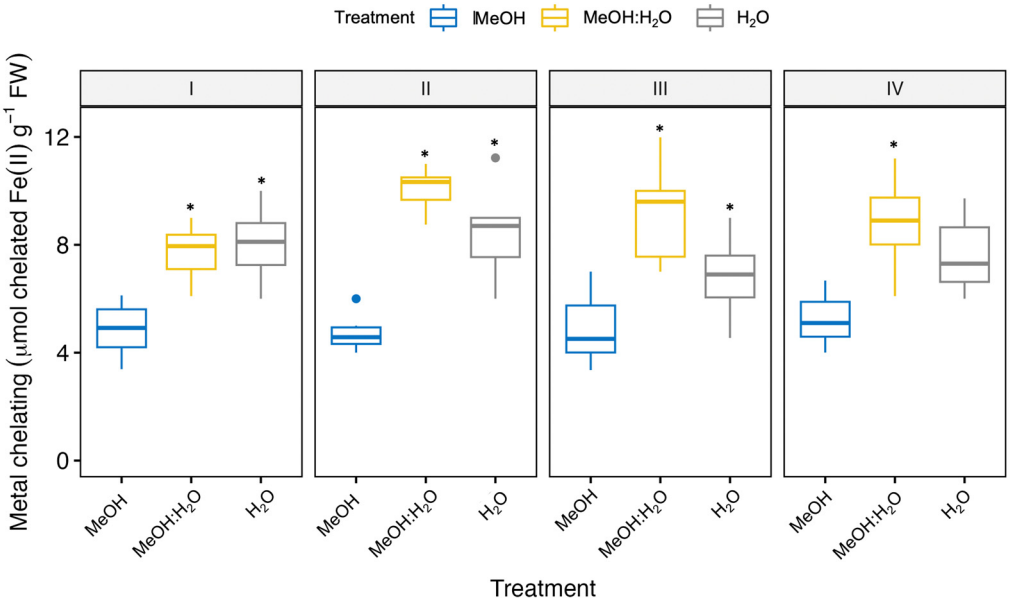

Figure 4. Antioxidant activity of methanol, aqueous methanol, and hot water extracts of fennel bulbs from the four fennel varieties tested (**I–IV**) by the metal chelating method. The data are means ± SE from two experiments. For each fennel variety, treatments marked with an asterisk are significantly different from the corresponding methanol extract ($p < 0.05$) by Tukey's HSD post hoc test.

The ability of the fennel extracts to inhibit the lipid peroxidation of linoleic acid under autooxidation conditions is shown in Figure 5. In general, the ability of extracts to prevent the formation of conjugated dienes was inversely correlated with the polarity of the solvent used in all fennel varieties analyzed. The highest antioxidant capacity was found in the methanol extracts, in which values were in the range of 1.8–4.9 μmol g^{-1} FW, representing a 2.7-fold difference among the four fennel varieties. In contrast to the above methods, fennel varieties I and II exhibited the highest lipid peroxidation inhibitory activity.

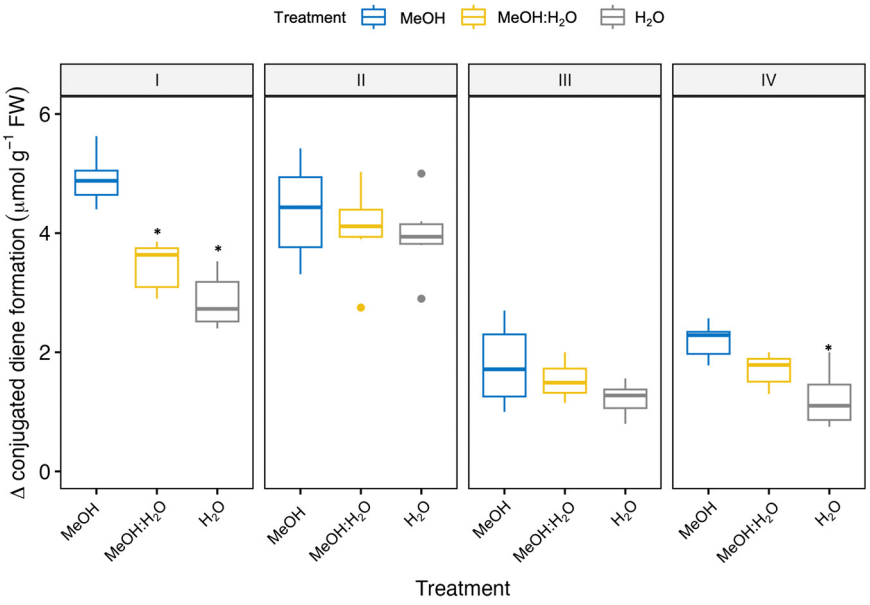

Figure 5. Inhibition of lipid peroxidation of methanol, aqueous-methanol, and hot water extracts of fennel bulbs from the four fennel varieties tested (**I–IV**). The data are means ± SE from two experiments. For each fennel variety, treatments marked with an asterisk are significantly different from the corresponding methanol extract ($p < 0.05$) by Tukey's HSD post hoc test.

3.2. Effect of Different Extraction Solvents on the Phenolic Content in Fennel Bulbs

Figure 6 depicts the effect of the extraction solvents on the level of TPC. In general, the highest extraction of total phenolic compounds was recorded for the aqueous-methanol extracts, followed by hot water extracts and methanolic extracts. TPC values ranged from 0.40 to 0.85 µmol CAE g^{-1} FW in the aqueous methanol extracts, representing a 2-fold difference among the different fennel varieties tested. The fennel variety with the highest TPC values was variety III with the three solvents tested, whereas the lowest TPC was found in the methanolic extracts from variety IV (0.29 ± 0.03 µmol CAE g^{-1} FW).

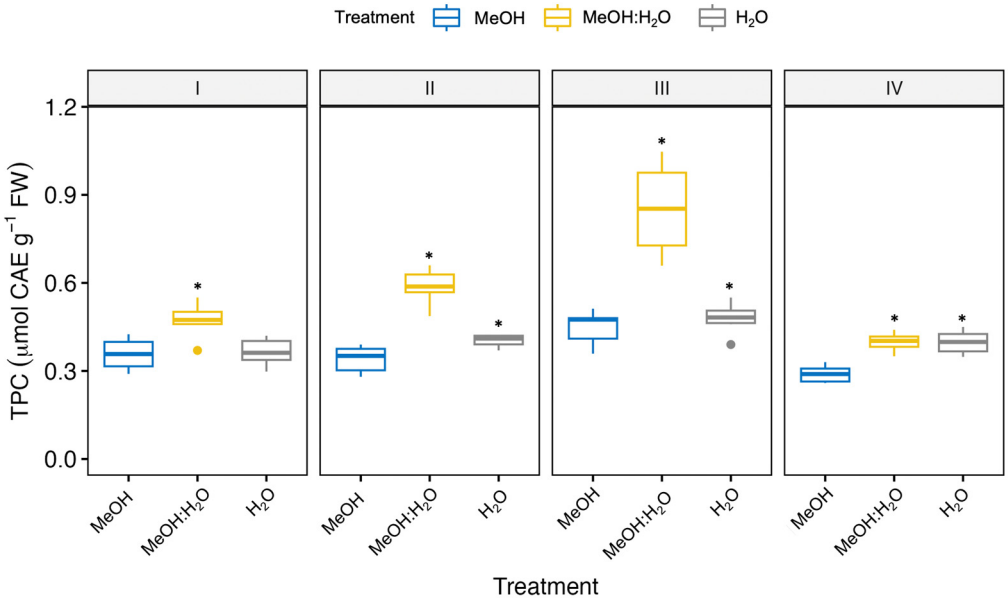

Figure 6. Total phenol content (TPC) of methanol, aqueous-methanol, and hot water extracts of fennel bulbs from the four fennel varieties tested (**I**–**IV**). The data are means ± SE from two experiments. For each fennel variety, treatments marked with an asterisk are significantly different from the corresponding methanol extract ($p < 0.05$) by Tukey's HSD post hoc test.

HPLC analyses were carried out to verify to what extent the extraction solvent influences the phenolic patterns of the bulbs from the four fennel varieties tested. Significant qualitative and quantitative differences in phenolic composition were found in the different extracts analyzed (Supplementary Figure S1). These differences were observed not only across distinct extraction methods employed for a particular fennel variety, as expected, but also among different fennel varieties utilizing the same extractant. In general, the most complex peak patterns were obtained when the methanol-water mixture was used as the extraction solvent, followed by hot water. From a qualitative point of view, the simplest chromatograms were obtained with the fennel varieties II and IV, whereas the analysis of the fennel variety III, and particularly the variety I, exhibited a high number of peaks.

The analysis of the UV-Vis spectra of the resolved peaks revealed that most of the resolved analytes showed the spectral characteristics of caffeic acid derivatives, with an absorption maximum in the region between 320 and 330 nm and a shoulder around 300 nm (Supplementary Figure S2). Also striking was the finding that these compounds with the caffeic acid spectral signature were distributed throughout the entire chromatogram. The verification that most of the compounds separated by HPLC were derivatives of caffeic acid allowed us to obtain an estimate of their accumulation in the different fennel bulb extracts. As shown in Figure 7, the levels of caffeic acid derivatives were in the range of 372–553 nmol CGAE g^{-1} FW in the hot water extracts, representing a 1.5-fold difference among the different fennel varieties tested. The fennel variety with the highest levels of caffeic acid derivatives was variety III with the three solvents tested. The results also revealed a marked reduction in the concentration of this family of compounds in the two

methanolic extracts, which ranged between 6 and 38% of their respective hot water ones, in the four fennel varieties tested (Figure 7).

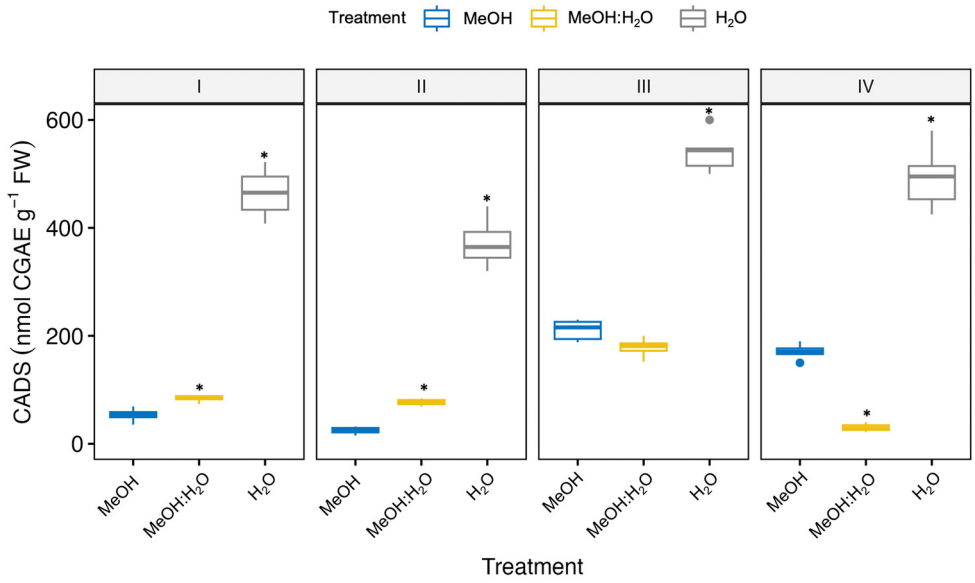

Figure 7. Levels of caffeic acid derivatives determined by HPLC found in methanol, aqueous-methanol, and hot water extracts of fennel bulbs from the four fennel varieties tested (**I**–**IV**). The data are means ± SE from two experiments. For each fennel variety, treatments marked with an asterisk are significantly different from the corresponding methanol extract ($p < 0.05$) by Tukey's HSD post hoc test.

Moreover, the combination of UV-Vis spectral data, retention times, and mass spectra led to the identification of the presence of chlorogenic acid and caffeic acid, although they were unevenly distributed in the samples (Supplementary Figures S2 and S3). While chlorogenic acid appeared in all extracts, except for the hydroalcoholic extracts from the fennel varieties II and III, caffeic acid could only be determined in the aqueous extracts of the fennel varieties II and IV and in the methanolic extracts of the latter. The differences in the accumulation of chlorogenic acid and caffeic acid were not only qualitative but also quantitative, with the former being more abundant by several orders of magnitude (Table 1).

Table 1. Levels of caffeic acid (CA), chlorogenic acid (CGA), 3-caffeoylquinic acid (3-CQA), 1,3-dicaffeoylquinic acid (1,3-diCQA), 1,5-dicaffeoylquinic acid (1,5-diCQA), and 1,4-dicaffeoylquinic acid (1,4-diCQA).

Fennel Genotype/Solvent	CA (nmol g^{-1} FW)	CGA (nmol g^{-1} FW)	3-CQA (nmol CGAE g^{-1} FW)	1,3-diCQA (nmol CGAE g^{-1} FW)	1,5-diCQA (nmol CGAE g^{-1} FW)	1,4-diCQA (nmol CGAE g^{-1} FW)
I						
MeOH	nd	6.27 ± 0.60 ^{h}	nd	nd	nd	nd
MeOH:H_2O	nd	1.17 ± 0.13 ^{i}	nd	nd	nd	nd
H_2O	nd	66.58 ± 0.88 ^{d}	9.01 ± 0.43 ^{d}	4.27 ± 0.12 ^{b}	nd	17.38 ± 1.07 ^{d}
II						
MeOH	nd	13.16 ± 1.27 ^{g}	nd	nd	nd	nd
MeOH:H_2O	nd	nd	nd	nd	nd	nd
H_2O	8.15 ± 0.15 ^{a}	232.42 ± 3.99 ^{b}	23.76 ± 0.15 ^{b}	5.02 ± 0.30 ^{b}	4.87 ± 0.74 ^{b}	23.31 ± 0.88 ^{c}
III						
MeOH	nd	47.43 ± 4.48 ^{f}	nd	nd	nd	4.54 ± 0.52 ^{e}
MeOH:H_2O	nd	nd	nd	nd	nd	nd
H_2O	nd	117.07 ± 2.08 ^{c}	13.75 ± 0.88 ^{c}	nd	3.66 ± 0.63 ^{b}	21.57 ± 1.26 ^{c}
IV						
MeOH	4.06 ± 0.15 ^{b}	96.58 ± 2.32 ^{c}	nd	nd	nd	32.72 ± 1.19 ^{b}
MeOH:H_2O	nd	5.00 ± 0.15 ^{h}	nd	nd	nd	nd
H_2O	8.10 ± 0.30 ^{a}	315.36 ± 5.17 ^{a}	30.89 ± 0.30 ^{a}	19.74 ± 0.22 ^{a}	15.58 ± 0.19 ^{a}	78.68 ± 1.38 ^{a}

Different letters in the same column indicate significant differences according to Tukey's HSD test ($p < 0.05$). nd: non-detected.

Although no other compound present in the analyzed extracts could be unequivocally identified, the chromatograms obtained with the MS detector and the consequent fragmentation patterns of some of the resolved peaks, together with the information available in the bibliography, allowed us to identify, with a reasonable degree of certainty, four caffeoylquinic acid derivatives, in particular, 3-caffeoylquinic acid and 1,3-, 1,5-, and 1,4-dicaffeoylquinic acids according to their order of elution in the chromatography (Supplementary Figure S4). The highest levels of these caffeoylquinic acid derivatives were found in the hot water extracts of fennel variety IV, and, in general, the lowest levels were determined in variety I (Table 1).

3.3. Principal Component Analysis and Pearson's Correlations

A principal component analysis (PCA) was carried out to visualize data trends and detect possible clusters within samples. The first two PCA components (PC1 and PC2) account for 68% of the variance in the overall data sets (Figure 8a). PC1 was associated positively with DPPH radical scavenging activity, RP, and caffeic acid derivatives (CADS). PC2, which accounted for 22.9% of the total variance, was best explained by TPC and the chelating ability of ferrous ions on the positive side of the Y-axis and by the inhibitory ability of lipid oxidation (Diene) on the negative Y-axis.

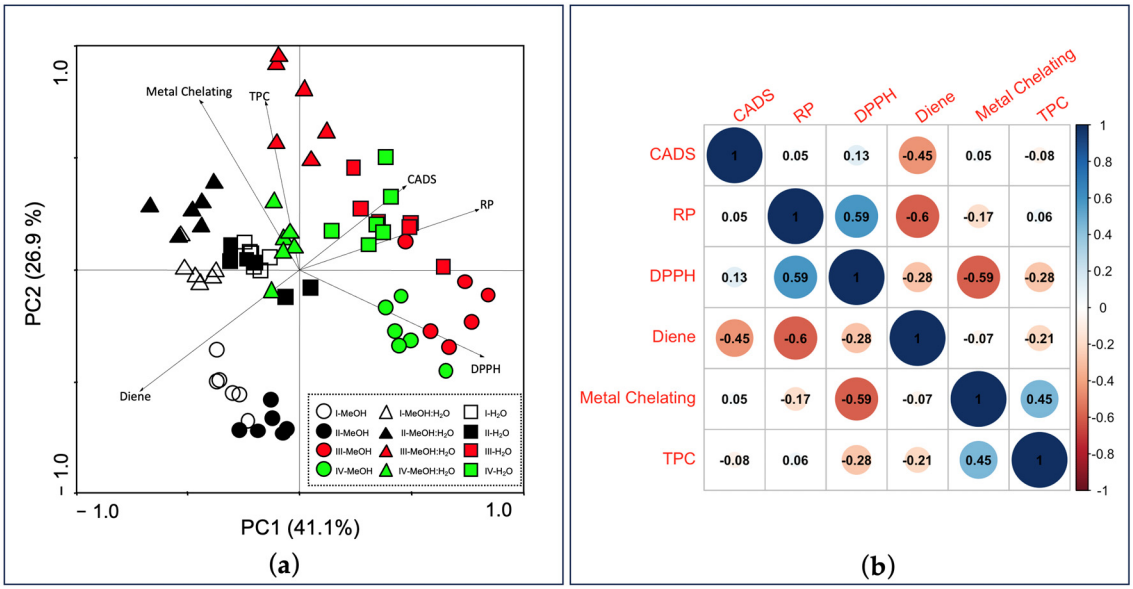

Figure 8. (**a**) Principal component analysis based on the correlation matrix of antioxidant activity parameters [DPPH, reducing power (RP), metal chelating, and conjugated diene formation (Diene)] and phenolics [total phenolic content (TPC) and levels of caffeic acid derivatives (CADS)] in methanol (○), aqueous-methanol (Δ), and hot water (□) extracts from the four fennel varieties tested (I, white; II, black; III, red; IV, green); (**b**) Pearson's correlation coefficients among fennel bulb antioxidant activities and phenolics (TPC and CADS).

PCA also revealed evident clustering differences between fennel varieties I and II, which were mostly located on the left side of PC1, whereas fennel varieties III and IV were located on the right side of PC1 (Figure 8a). The main differences between fennel varieties I and II and fennel varieties III and IV were associated with the inhibitory abilities of lipid oxidation, CADS, and RP. Moreover, methanolic extracts were mainly associated with DPPH, whereas the hydroalcoholic extracts were associated with metal chelating activity and TPC.

Pearson's correlation analysis revealed a small correlation between TPC and free radical scavenging activities against DPPH ($r = -0.28$; $p < 0.05$), while a weak to moderate correlation between TPC and metal chelating activity was found ($r = 0.45$; $p < 0.001$) (Figure 8b).

4. Discussion

The current paper aims at evaluating the antioxidant activity and phenolic profile of bulbs from different fennel varieties using different extraction solvents. Since natural products are multifunctional antioxidants, a reliable antioxidant evaluation requires the use of different assays covering different mechanisms of antioxidant action [36,37]. Here, four different in vitro assays testing free radical scavenging activity, electron donation capacity, chelating ability, and inhibition of lipid peroxidation were chosen to evaluate the antioxidant potential of fennel bulbs. The results underscore notable differences in the antioxidant activities among the examined fennel varieties, with the varieties III and IV showing the highest potential in terms of DPPH and RP, whereas the varieties I and II exhibited the highest inhibition of lipid peroxidation. Diverse antioxidant capacities among different cultivars/varieties in a wide range of plant-derived foods have been frequently reported in the literature, such as apple, pear, mango [38,39], grape [40], legumes [41], and wild, edible, and medicinal fennels [42], confirming the relevance of genotype in determining the antioxidant capacity in plant foods. Moreover, the significant differences in the antioxidant capacity values found in the bulbs of fennel varieties studied (up to 4-fold) could be exploited by plant breeders for improving fennel quality by enhancing antioxidant content.

Due to the uneven distribution of antioxidants in plant tissues and their different structures and polarities, the type of extraction solvent used is considered a critical factor that influences the extraction efficiency and the resulting antioxidant activity of the obtained extracts [27]. Among the tested solvents, in general, absolute methanol increased the scavenging ability of DPPH radicals, inhibition of lipid oxidation, and RP. The highest antioxidant capacity detected in fennel methanolic extracts could be related to the lower polar character of this solvent. Methanol is known to favor the extraction of highly hydroxylated aglycone forms of phenolics, whereas the most polar phytochemicals can be extracted using water [43]. In the literature, higher values of antioxidant activity using methanolic extracts have previously been reported for different plant materials and plant by-products [29,44–46].

The data of this study revealed that absolute methanolic extracts yielded the lowest TPC yield and the simplest HPLC chromatograms, which contrast with the results obtained using aqueous methanol solvent. This implies that the phenolic compounds in the tested fennel bulbs might be readily soluble in aqueous methanol and more sparingly soluble in absolute methanol. Moreover, it is worth mentioning that, apart from metal chelating activity, TPC showed no correlation with the rest of the antioxidant assays analyzed. Studies on the relationship between TPC and antioxidant activities show contradictory results; whereas some authors have reported high correlation values [29,47,48], others found no correlation between these parameters [39,49]. These results suggest that phenolic compounds are not the only contributors to the antioxidant activities in some plant extracts, and other phytochemicals could also contribute to the total antioxidant activity of these extracts. Apart from phenolics, ascorbic acid, carotenoids, tocopherols, and sterols, to cite just a few, are well-known contributors to the antioxidant potential of plants [50]. Among them, ascorbic acid is the most likely contributor to the antioxidant activity found in the methanolic fennel extracts by using DPPH and inhibition of lipid oxidation methods as regards its solubility in this solvent [51] and its levels in fennel bulbs (22 mg/100 gFW [52]). The antioxidant properties of ascorbic acid are related to the C2 and C3 hydroxyl groups of the enediol-lactone group. This enediol-lactone resonant structure plays an effective role in the reduction of $DPPH^{\bullet}$, exhibiting fast-kinetic reactions [53], and in the inhibition of lipid peroxidation [54].

In previous works, Faudale et al. [42] compared the phenolic composition among wild, medicinal, and edible fennel varieties and reported that the latter contained the lowest amount of phenolics, with TPC values ranging from 12 to 22 GAE/mg of extract. A further study showed a total polyphenol content of 391.59 $\pm$ 0.51 mg GAE/100 g DW in fresh fennel bulbs [55]. Similarly, using a 50% aqueous-methanol-based extract of fennel

waste comprised of residual bulbs, stems, flowers, and leaf sheaths showed a TPC up to 3.9 mg/g DW [56]. The above-mentioned TPC values are comparable to those found in this study (0.40–0.85 µmol CAE g^{-1} FW, ca. 1.20–2.55 mg CAE g^{-1} DW). Nevertheless, it is necessary to be cautious when comparing data from different studies because TPC values can vary depending on phenol extraction efficiency, genotype, growing conditions, or cultivation methods [43]. Recently, differences in agronomic practices such as irrigation and soil fertilization have been reported to affect the polyphenol composition of fennel bulbs [52,57]. In this study, the four fennel varieties used were grown under the same agronomic and environmental conditions; thus, the variation noticed in the content of phenolics could be ascribed to differences in fennel genotype.

HPLC analyses clearly showed the presence of high levels of caffeic acid derivatives (372–553 nmol CGAE g^{-1} FW, ca. 2.2–3.3 mg CGAE g^{-1} DW), mainly chlorogenic acids, especially in the hot water fennel extracts. Similar concentrations of caffeic acid derivatives (CADS) have been found in edible Spanish fennel cultivars (46–97 mg/100 g DW quantified as the sum of total caffeoylquinic acids (CQA) and di-CQA), although in the Italian cultivars the CADS contents were remarkably lower (2.6–5.9 mg/100 g DW) [42]. Again, these results highlight the relevance of genotype to the phytochemical composition of fennel varieties.

Chlorogenic acids are a family of esters formed between quinic acid and certain *trans*-cinnamic acids (most commonly caffeic, *p*-coumaric, and ferulic acids) [58]. The caffeic acid group includes mono-esters of caffeic acid (CQA), di-esters (di-CQA), and other caffeic acid derivatives, including tri- and tetra-esters of caffeic acid [58]. One property of CQA is the spontaneous migration of caffeoyl residues among the hydroxyl groups of the quinic acid [59]. Isomerization and transformation of CQA are also influenced by the pH and temperature of aqueous solutions [59]. Notably, a recent metabolomic study highlights di-CQA as one of the most expressed metabolites in fennel bulb waste decoction [60]. Taken together, these results might explain, at least in part, the different CQA yield obtained in the different extraction solvents used in this work.

Recent epidemiological reports and biomedical studies in animal models have pointed out that the consumption of CQAs (including CQA, di-CQA, and other derivatives) has a range of therapeutic benefits for human health, including antiviral, anti-carcinogenic, hypoglycemic, anti-Alzheimer, and neuroprotective activity (for review, see [59]). The health benefits of CQAs are a result of their roles as direct antioxidants or as Michael acceptors and interact with the Keap1-Nrf2 pathway [59]. In fact, oxidation products of CQAs have been reported to target the Keap1-Nrf2 complex, leading to its dissociation, which increases the levels of Nrf2 (nuclear factor erythroid-2-related factor 2) in the nucleus. Nrf2 is a master regulator of cellular resistance to oxidative stress and constitutes a therapeutic target in inflammation-mediated disorders [59,61]. In such an interaction, di-CQA has been shown to be more effective than CQA [61], indicating that not only the levels of antioxidant compounds but also their profiles account for the potential therapeutic effects of plant-derived products.

5. Conclusions

In conclusion, the results of this study revealed significant variability in both the antioxidant potential and phenolic profile of fennel bulb extracts, the extent of which depends on the genotype tested. These results support the view that accurate phytochemical screening could serve as a simple tool for identifying promising genotypes in nutraceutical-oriented breeding programs. This study shows remarkable differences between genotypes in the amount and profile of CQAs present in hot water extracts of fennel bulbs. Since the therapeutic benefits associated with these compounds seem to be compound-specific, further research is needed to optimize the conditions for aqueous extraction of these compounds as well as to evaluate the biological activities of individual CQAs. All of this could lead to novel food products with enhanced functionalities and, thus, greater added value.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14030484/s1, Figure S1: HPLC chromatograms recorded at 328 nm of the methanol (left panels), aqueous-methanol (middle panels), and hot water (right panels) extracts from bulbs of the fennel variety I (A), II (B), III (C) and IV (D), Figure S2: UV-Vis spectra of representative peaks of caffeic acid derivatives resolved by HPLC in the different extract analyzed, Figure S3: Chromatograms of the aqueous extract of variety IV obtained by using HPLC-MS in the negative mode at m/z ratios 353 (A) and 179 (B), corresponding to the molecular ions of chlorogenic and caffeic acids, respectively, Figure S4: Chromatograms of the aqueous extract of variety IV obtained by using HPLC-MS in the negative mode at m/z ratios 353 (A) and 519 (B), corresponding to the molecular ions of mono- and di-caffeoylquinic acids, respectively.

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