Original article

Polyphenolic profiling and chemometric analysis of leaves from Italian *Ficus carica* L. varieties.

Polyphenol compounds in common fig

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Summary

Ficus carica L. is a typical crop of Mediterranean traditional agricultural landscapes. Its fruits have special historical importance in the diet of Mediterranean peoples, while fruits, roots, and leaves possess medicinal and nutraceutical activity, and are used in traditional medicines. This study explores the difference in the phenolic profile of leaves of ten Italian varieties of *Ficus carica* L. Individual polyphenols were evaluated quantitatively by means of HPLC-DAD-TOF-MS (High-Performance Liquid Chromatography-Diode-Array Detection/Mass Spectrometry) techniques. Additionally, total phenol content was measured using the Folin-Ciocalteu assay and antioxidant activity was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and oxygen radical absorbance capacity (ORAC). Chemometric analysis were used to determine fingerprinting phytochemical of the fig varieties. Eighteen phenolic compounds were determined in the fig leaves, and caffeoylmalic acid and rutin were found to be the major components. The activity of antioxidants assessed by DPPH and ORAC presented significant differences among the varieties ranging from 0.48 to 6.68 mg DW mL⁻¹, and from 49.2 to 6.3 µM Trolox equivalent (TE) per gram of dry weight, respectively. Simple linear regression analysis between the total polyphenol and antioxidant activity values gave moderate correlation, suggesting that antioxidant activity is determined by relationship between the amount of polyphenols and the various phenolic constituents. Principal Component Analysis indicated that phenolic compounds could be responsible for the differences among fig leaf samples according to variety. The results confirm that the fig leaves can be a source of phenolic compounds, and that phytochemical fingerprinting combined with chemometric analysis can be used to characterize fig germplasm.

Keywords

common fig, HPLC-DAD-TOF-MS, phenolic compounds, Folin-Ciocalteu method, DPPH, ORAC

Significance of this study

What is already known on this subject?

- The phenolic compounds and antioxidant activity of fig fruits have been studied by many research teams, while that of fig leaves has been much less and knowledge is limited to a few varieties. *Ficus carica* leaves contain a significant concentration of bioactive compounds, and leaf extracts have many benefits.

What are the new findings?

- A preliminary characterization of polyphenolic contents from leaves of Italian fig varieties.

What is the expected impact on horticulture?

- Fig leaves could be an interesting source of particular compounds with applications in the food and pharmaceutical market.

and climatic variable area, extending from Mediterranean environments, with cool winters and hot, dry summers, to tropical and subtropical regions (Veberic et al., 2015). Until recently, it was believed that fig was domesticated in the Near East during the Bronze Age, at the same time as the olive tree, vine and date palm (Zohary and Hopf, 1994). However, recently carbonized fig fruits discovered in archeological sites, dating 11,400–11,200 years ago, appear to indicate that fig domestication possibly occurred one thousand years before the cultivation of cereals and legumes (Kislev et al., 2006).

The fig is an important crop worldwide, consumed as fresh and dried fruit, or used for the production of jam (Veberic et al., 2015). Fig is a major source of calcium and fiber, but is also rich in sugars, minerals (i.e., calcium, potassium, magnesium, iron), vitamins and amino acids. Furthermore, it is a good source of bioactive compounds such as flavonoids, hydroxycinnamic acids, anthocyanins and other classes of secondary metabolites with potential benefits to human health (Mawa et al., 2013; Veberic et al., 2015). All these compounds are known to have biological properties such as antioxidant activity and potential beneficial effect on human health (Manach et al., 2004). *Ficus carica* has been incorporated in occidental Pharmacopoeias and in therapeutic guides of herbal medicines (Barolo et al., 2014).

Various plant parts (fruits, roots, and leaves) and extracts are known to possess medicinal and nutraceutical activity, and are used in traditional medicines (Gilani et al., 2008; Menichini et al., 2012; Tchombè and Louajri, 2015).

Introduction

The cultivated fig (*Ficus carica* L., family *Moraceae*), originally from southwest Asia, is distributed over a geographical

Regarding the fig leaves, they have been reported to contain a significant concentration of bioactive compounds, as well as volatile compounds (aldehydes, alcohols, monoterpenes, norisoprenoids, etc.) (Oliveira et al., 2010); coumarins (psoralen bergapten, umbelliferone, 4',5'-dihydropsoralen and marmesin) (Innocenti et al., 1982) and triterpenoids and sterols (ficusin, taraxasterol, β sitosterol, stigmasterol, lupeol, and calotropenyl acetate) (Veberic et al., 2015). The amount and composition of bioactive compounds depend greatly on the genotype, but they are also influenced by latitude, geographical position of the tree, and the time of leaf collection (Oliveira et al., 2010).

The leaf extracts have been widely used as a folk remedy for combating fever and other diseases (Mawa et al., 2013). The extracts exhibit a large spectrum of properties such as antioxidant activity, anti-inflammatory activity and hypoglycemic activity (Perez et al., 2003; Tchombè and Louajri, 2015). Recently, fig leaf extracts showed anti-proliferative effects and cytotoxicity on tumor cell line (Khodarahmi et al., 2011; Conforti et al., 2012).

Common fig has been present in Italy since ancient times, and cultivated fig trees are found in most areas of the country. The cultivation assumes economic importance primarily, in four regions (Campania, Calabria, Puglia and Sicily) of southern Italy. However, in some regions of central Italy, like Tuscany, its cultivation occupies "special niches", thanks to the production of traditional food production. The Italian fig germplasm consists of a large number of varieties (more than 300 known fig varieties) characterized by a large phenotypic variability, that is reflected also in the secondary metabolites. However, Italian fig germplasm has not been extensively studied. Recently, some authors have provided phytochemical fingerprinting restricted to a few cultivars, particularly the cultivar Dottato, the predominant fig variety cultivated in Italy (Loizzo et al., 2014; Marrelli et al., 2014; Russo et al., 2014).

The aim of the present study was to identify and characterize the phenolic profile in extract of fig leaves and to evaluate their antioxidant capacity. Multivariate Data Analysis (Principal Component Analysis and Hierarchical Cluster Analysis) was used to classify and compare the varieties.

Material and methods

Chemicals

All the solvents, reagents, and standards used were of analytical grade and were obtained from Sigma (St. Louis, MO, USA). The water used in sample preparation, solutions, and analysis was obtained with a Milli-Q water purification system by Millipore (Bedford, MA).

Plant material

The varieties used in this study included: 'Dottato', the predominant fig cultivar produced in Italy, and 'Brogiotto Nero', 'Dorato', 'Fiorone Bianco', 'Gigante di Carmignano', 'Portogallo', 'Saccareccio', 'San Piero', 'Sanguinello', and 'Verdino'. Fig varieties were grown in a farm located in Carmignano (Prato, Tuscany) (43°49'N, 11°01'E, 189 m a.s.l.). The trees were planted at 7.0 m spacing along the row and 7.0 m between rows and cultivated without irrigation following the traditional method. All varieties were subjected to identical cultural practices and, of course, environmental conditions. Three uniform trees were selected at random in the orchard, and leaves were sampled, in August, from multiple positions on each tree. The samples were placed in airtight

plastic bags and frozen immediately (-80°C). The samples were then freeze-dried (Freeze Dryer ALPHA 1-4 LD, Martin Christ, Germany), homogenized and frozen at -80°C prior to subsequent analysis.

Extraction of phenolics

Phenolics was extracted according to Torti et al. (1995) from 200 mg of lyophilized fig leaves. Briefly, the samples were extracted with 5 mL of 80% methanol (methanol:water 80:20 % v/v), sonicated at ambient temperature for 30 min and centrifuged (10,000 rpm at 10°C) for 10 min. The supernatant was transferred in a flask and extraction was repeated. Both supernatants were combined and then adjusted to 10 mL with extraction solvent. The obtained extracts were then added with an equal volume of petroleum to remove the chlorophyll, agitated and centrifuged at maximum speed for 5 min. Petroleum was subsequently discarded and remaining extracts were used for all subsequent analyses. Before HPLC analysis, all samples were centrifuged (4 min at 14,000 rpm) to remove traces of suspended materials.

HPLC-DAD-TOF-MS

The analyses were carried out using an HP-1100 liquid chromatograph equipped with a DAD detector (Agilent Technologies, Palo Alto, USA). The column was a Poroshell, 150×3 mm, 2.7 μ m from Agilent Technologies. The mobile phase was (A) water at pH 3.2 acidified by formic acid and (B) acetonitrile. The following multi-step linear gradient was applied: from 95% of A to 70% in 25 min, to 45% A in 10 min, to 0% A in 6 min, which was maintained for 4 min. Total time of analysis was 38 min, flow rate 0.4 mL min⁻¹ and oven temperature 28±0.5°C. UV/Vis spectra were recorded in the 190–600 nm range and the chromatograms were acquired at 280, 330 and 350 nm. The above HPLC system was interfaced with an Agilent 6210 Time Of Flight Mass Spectrometer (TOF-MS) equipped with an ESI source (Agilent Corp, Santa Clara, CA, USA). Experiments were performed in positive and negative ion mode using a Dual Spray ESI source; the mass range was set at m/z 100–1500; the capillary voltage was 4 kV; the nebulizer gas was at 20 psi and the drying gas flow and temperature were 6 L min⁻¹ and 350°C, respectively. The fragmentor voltage was set at 200 V to obtain an in-source fragmentation of the ions eluting from the ESI spray. Spectra were recorded at 1.03 spectra sec⁻¹ and internally calibrated using the reference mass at 922.009798 m/z. Data analysis was controlled using Agilent LC-MS TOF Software (Agilent, USA). Identification of phenolic compounds was carried out by comparing their UV-Vis spectra (280–350 nm) and mass spectra with available literature and retention times relative to available external standards. Quantification of individual polyphenolic compounds was directly performed by HPLC DAD using a five-point regression curve ($r^2 \geq 0.998$) on the basis of authentic standards. In particular, quercetin derivatives were determined at 350 nm using quercetin-3-O-rutinoside as reference compound. Kaempferol derivatives were determined at 350 nm using Kaempferol-3-O-glucoside, while flavones were determined at 330 nm using apigenin-7-O-glucoside as reference compound. Furanocoumarins were determined at 330 nm with psolaren as reference compound and *p*-coumaroyl and caffeoyl derivative were determined at 330 nm using coumaric acid and chlorogenic acid, respectively, as reference compounds.

Total phenolic content

The total phenol content was determined with the Fo-

lin-Ciocalteu reagent as described by Torti et al. (1995). The absorbance of the coloured reaction product was read at 730 nm standard using a Varian UV-Visible spectrophotometer Cary 50 Scan. Total phenolics were expressed as mg of gallic acid equivalent per gram (mg GAE g⁻¹) of lyophilized leaves powder, based on a standard curve. Each analysis was performed in triplicate.

DPPH radical scavenging assay

The DPPH radical-scavenging activity was determined using the method proposed by Brand-Williams et al. (1995). The decrease absorbance of extract (A_E) was measured at a wavelength of 517 nm after keeping samples for 20 min. at dark. The radical scavenging activity was calculated by the percentage of DPPH that was scavenged using the following formula:

$$\% \text{ Reduction} = [(A_B - A_E)/A_B] \times 100$$

where A_B was the absorbance of the blank sample, while A_E was the absorbance of the fig leaf extracts. Ascorbic acid was used as positive control. The EC₅₀ (Effective Concentration) values, defined as the amount of antioxidant required to scavenge DPPH radical by 50% were calculated from the results. Ascorbic acid was used as positive control. Each analysis was performed in triplicate.

Oxygen radical absorbance capacity (ORAC) assay

The method was adapted from that described by Cao and Prior (1998) and the instrument was a fluorescence spectrophotometer (Varian Cary Eclipse) (Palo Alto, CA, USA). The sample was added to a free-radical generator (AAPH, 2, 2'-azobis (2-aminopropane) dihydrochloride) and the inhibition of the free radicals was measured. Fluorescein was used as a target for free-radical attack. The exciting λ was 490 nm and the emission λ was 512 nm. Total antioxidant capacity or ORAC unit (μM) was obtained by the following formula:

$$\text{ORAC unit } (\mu\text{M}) = 20 k (S_{\text{sample}} - S_{\text{blank}}) / (S_{\text{Trolox}} - S_{\text{blank}})$$

where k = dilution factor, S_{sample} = the under curve area of the sample, S_{blank} the under curve area of the blank and S_{Trolox} the under curve area of the standard. ORAC values were expressed as μM Trolox equivalents g⁻¹ of dry leaf using the

standard curve established previously. Each analysis was performed in triplicate.

Statistical analysis

The data of the chemical analysis were tested for differences between the varieties using the one-way analysis of variance (ANOVA). The differences were tested with Tukey's high significance difference (HSD) test at the 0.05 significant level (Statgraphics Plus, version 5.1 for Windows). The data are presented as mean $\pm$ standard deviation (SD).

Multivariate analysis (Principal Component Analysis PCA, and Cluster analysis) were carried out on phenolic composition data obtained by HPLC. To study differences between fig cultivars, data from HPLC were collected in a 10 $\times$ 18 matrix, which was analyzed by means of Principal Component Analysis (PCA).

To study the relationship between every single HPLC determination, the total phenolic content (TPC) and the estimators of anti-oxidant activity (DPPH, ORAC), Multiple Linear Regression (MLR) associated to a variable selection algorithm was used.

Data were processed using the NIPALS algorithm and Unscrambler X1 software (CAMO, Oslo, Norway).

Hierarchical agglomerative cluster analysis (HCA) was applied to the standardized data to investigate the similarity and dissimilarity between the fig varieties. Cluster analysis was applied to obtain hierarchical associations using Ward's method applying squared and Euclidean distance as the distance or similarity measure. Cluster analysis was currently performed using Statgraphics Plus, version 5.1 for Windows.

Results and discussion

Analysis of phenolic compounds

The identity of the phenolic compounds was ascertained using data from HPLC-DAD-TOF-MS analyses through comparison and combination of their retention times, UV/Vis and mass spectra with authentic standards and according to the literature. Polyphenol compounds found in the investigated fig leaves can be classified as flavonoids and non-flavonoids (phenolic acids-derivates of hydroxycinnamic acids and furanocoumarins). Obtained results are reported in Table 1 and representative chromatogram is presented in Figure 1.

Hydroxycinnamic acids were represented by chlorogen-

FIGURE 1. Chromatogram profile of fig leaves recorded at 330 nm. For the peak detection see Table 1.

TABLE 1. Phenolic compounds characterized using the negative ionization mode and quantitative data in the fig leaf extracts.

Peak	Rt (min)	Compound	[M+H] ⁺ measured m/z	[M-H] ⁻ measured m/z	Diagnostic m/z ions (%)	λ max	Mean* (mg g ⁻¹ DW)	SD
1	10.5	Chlorogenic acid (5- <i>O</i> -caffeoylquinic acid)		353.0890	353 (100), 191 (40), 179 (2), 135 (2)	218, 242, 300, 326	0.79	0.48
2	13.1	Caffeoylmalic acid		295.0477	295 (100), 179 (25), 133 (30)	218, 242, 300, 326	5.35	2.35
3	15.4	<i>p</i> -Coumaroyl derivative		-	-163 (20)	210, 228, 312	0.24	0.27
4	16.1	<i>p</i> -Coumaroylquinic acid		337.0947	337 (100), 191 (30), 163 (5)	210, 228, 312	0.92	0.78
5	16.7	<i>p</i> -Coumaroylmalic acid		279.0523	279 (100), 163 (30)	210, 228, 312	0.59	0.36
6	16.8	Caffeic acid derivative		-	133 (30)-	218, 242, 300, 326	0.47	0.18
7	16.9	Isoschaftoside		563.1419	-563 (100)	270, 338	0.51	0.25
8	17.0	Schaftoside		563.1431	-563 (100)	270, 342	0.28	0.13
9	17.3	Quercetin derivative		-	301 (15)-	256, 344	0.17	0.17
10	18.9	Quercetin derivative		-	301 (15)-	256, 354	0.09	0.07
11	19.2	Quercetin-3- <i>O</i> -rutinoside (Rutin)		609.1480	609 (100), 301 (20)	256, 354	4.20	2.46
12	19.8	Psolaric acid glucoside		365.0964	365 (35)	244, 288, 330	0.28	0.20
13	19.9	Quercetin 3- <i>O</i> glucoside		463.0889	463 (40), 301 (10)	256, 354	0.74	0.47
14	21.1	Quercetin 3- <i>O</i> -malonyl- glucoside		549.0860	549 (40), 505 (10), 301 (5)	256, 354	0.80	0.80
15	21.3	Kaempferol 3- <i>O</i> glucoside		447.0902	447 (100), 285 (5)	266, 348	0.17	0.13
16	22.2	Kaempferol derivative		-	-	266, 348	0.01	0.02
17	30.6	Psoralen	187.0382		187 (100)	210, 244, 294, 330	0.81	0.41
18	34.2	Bergapten (5-methoxypsoralen)	214.0499		217 (100)	222, 250, 268, 312	0.39	0.22
Total							16.53	5.35

* Values are the means of all of the genotypes analyzed. Rt: retention time; SD: Standard Deviation.

ic acid (trans-5-*O*-caffeoyl-quinic acid; compound 1) and caffeoylmalic acid (compound 2). The main fragment ion for compound 2 was at m/z 295 (quasi molecular ion) while the minor fragment ions were detected at m/z 179 (caffeic acid moiety), at m/z 133 (malic acid moiety) and at m/z 591 (dimer ion). Compounds 4 with [M-H]⁻ 337.0947 and with fragment ions at m/z 191 (quinic acid moiety) and at m/z 163, (coumaroyl moiety) was identified as *p*-coumaroylquinic acid. Compounds 5 was identified as *p*-coumaroyl malic acid, showing the principal ion at m/z 279 corresponding to the [M-H]⁻ and the ion at m/z 163 related to the coumaroyl group (Ammar et al., 2015). Other hydroxycinnamic derivatives were identified on the basis of the UV/Vis spectra and diagnostic ions as *p*-coumaroyl and caffeoyl derivatives, compound 3 and 6 respectively. Caffeoylmalic acid showed the higher mean value (5.35 mg g⁻¹ DW) while the amounts of 5-*O*-caffeoylquinic acid, *p*-coumaroylquinic acid and *p*-coumaroyl malic acid were considerably lower (Table 1).

Flavonols were characterized by quercetin 3-*O*-rutinoside (compounds 11), quercetin 3-*O*-glucoside (compost 13), quercetin 3-*O*-malonyl-glucoside (compost 14), and kaemp-

ferol 3-*O*-glucoside (compost 15) (Table 1). The main fragment ion for compound 14 was at m/z 549 (quasi molecular ion) while the minor fragment ions were detected at m/z 301 (quercetin moiety) and at m/z 505 [M-CO₂-H]⁻. Compound 15 showed a retention time (RT) of 21.3 min, a molecular ion with m/z and the fragment at m/z 285. These compounds have been previously reported in fig leaves (Takahashi et al., 2014; Ammar et al., 2015). Through UV and MS spectra, other quercetin derivatives (peaks 9–10) and kaempferol derivatives (peaks 16) were detected. Flavones were characterized by as schaftoside, C-glycoside of apigenin, and the structural isomer isoschaftoside (compounds 7 and 8). Their fragmentation pattern agreed with the Takahashi analysis (Takahashi et al., 2015). A previous study reported the presence in fig leaves and fruit of schaftoside and isoschaftoside (Siewek and Herrmann, 1985).

Two furanocoumarins (peaks 17 and 18) were detected in the fig leaves. Compound 17 presented a molecular ion with m/z 187 [M+H]⁺ and was identified as psoralen. Compound 18 was recognized as bergapten (5-methoxypsoralen). This showed a molecular ion with m/z 217 [M+H]⁺ and the

formula of the molecular ion was calculated to be $C_{12}H_9O_4$. A phenylpropanoid acid related to psoralen was assigned as psoralic acid glucoside according to Takahashi et al. (2015). Compound 12 showed a molecular ion with m/z 365 [M-H], confirmed by the presence of a dimer ion with m/z 731 [2M-H]. The formula of the molecular ion was calculated to be $C_{17}H_{17}O_9$. The phenolic profiles of leaves of the fig varieties were in general agreement with previous reports, revealing rutin as the principal flavonol, and caffeoylmalic acid as the main hydroxycinnamic acid (Oliveira et al., 2009; Ammar et al., 2015; Takahashi et al., 2015). However, there were some important differences in the presence or absence of certain compounds and in their relative concentration.

Polyphenol variations in fig varieties

Fig varieties significantly differed in the contents of individual polyphenols and their amounts were specific for each variety (Table 2). 'Dorato' variety showed the highest level of total phenol content. This is mainly due to its high amounts of some flavonoid glycosides. All the varieties examined, with the exception of 'Gigante di Carmignano' and 'Sanguinello', showed a total phenol content higher than the commercial cultivar Dottato (Table 2).

Hydroxycinnamic acids were the most represented class of the phenolic compounds, with an average of 53% to total phenolic compounds, although they varied from 39% ('Brogiotto Nero') to 67% ('Dorato'). Caffeoylmalic acid showed a concentration between 9.20 mg g⁻¹ DW and 1.39 mg g⁻¹ DW, followed by 5-*O*-caffeoylquinic acid and *p*-coumaroylquinic acid (Table 2). Chlorogenic acid concentrations fluctuate from 0.40 mg g⁻¹ DW ('Gigante di Carmignano') to 2.06 mg g⁻¹ DW ('Dorato'), and *p*-coumaroylquinic acid ranged from 2.64 mg g⁻¹ DW to 0.35 mg g⁻¹ DW.

Total flavonol contents were found in the range of 14.7–4.6 mg g⁻¹ FW with an average value of 9.6 mg g⁻¹ DW. The flavonol rutin was the predominant compound. The highest amount of rutin was detected in the fig leaves of the 'Dorato' and 'Brogiotto Nero' varieties, whereas 'Dottato' and 'Sanguinello' varieties had the lowest amount (Table 2). Quercetin glycosides were predominantly present in 'Dorato' variety. These compounds showed potential beneficial effects for human health, reducing the incidence of cardiovascular diseases, contributing to the antioxidant defenses of blood, and prevented chronic inflammation (Bentz, 2009). The 'Gigante di Carmignano' variety was characterized by the absence of kaempferol 3-*O*-glucoside (Table 2).

Several authors have reported the qualitative and quantitative differences in fig varieties. Oliveira et al. (2009) documented that rutin content in fig leaves of two Portuguese varieties ranged from 14.6 mg g⁻¹ to 17.4 mg g⁻¹ of lyophilized extract, while the amount of 5-*O*-caffeoylquinic acid ranged from 1.16 to 0.47 mg g⁻¹. Recently, experiments with five fig cultivars in Japan showed significant differences in polyphenol content between the genotypes (Takahashi et al., 2015). The authors reported CMA amounts between 20.6 and 14.4 mg g⁻¹ DW and rutin content between 11.4 and 7.2 mg g⁻¹ DW. These concentrations are higher than that obtained in our research. These variations may be due to genetic control and physiological factors. However many other factors, such as differing growth periods, geographic location and horticultural practices could affect the biosynthesis of polyphenols (Bruni and Sacchetti, 2009).

In relation to furanocoumarins the 'Portogallo' variety had the highest level of psoralen (1.49 mg g⁻¹ DW) and bergapten (0.62 mg g⁻¹ DW). Notably, psoralen was not found in 'Gi-

gante di Carmignano' and bergapten was absent in 'Gigante di Carmignano' and 'San Piero' (Table 2). The concentration of these two compounds in the studied varieties was lower than that reported by Takahashi et al. (2015) in fig leaves of Japanese cultivars and by Oliveira et al. (2009) in 'Pingo de Mel' and 'Branca Tradicional'. Psoralen is used alongside UV radiation (PUVA, psoralen photo chemotherapy) in the treatment of skin diseases such as psoriasis, and aqueous decoction of *F. carica* leaves used in diabetes control, in insulin-dependent diabetes mellitus (IDDM) patients (Bruni and Sacchetti, 2009). The toxic effects of furanocoumarins are also well known. The photo-toxicity threshold of psoralen is equivalent to about 15 mg person⁻¹ day⁻¹ (Dolan et al., 2010). Thus, considerable attention should be directed to oral administration of these preparations. The content of psoralen in the leaves should by necessity be lower than the threshold of toxicity. Our results show that some fig varieties possess, generally, a low content in furanocoumarins. This feature could be useful in their potential use in the cosmetic market, or may make the fig leaves suitable in the production of beverages rich in antioxidants for human consumption. The results of the HPLC analysis showed in general, that fig leaves are a valuable, natural source of caffeoylmalic acid and rutin. In particular, the results have highlighted both quantitative (*how much*) and qualitative (*what is it*) information on the polyphenol composition of fig leaves. Because all the leaf samples were obtained from the accessions grown in the same place and under the same cultivation conditions, such variations may result from the inherent variability of the accessions.

Chemometric classification

Although the differences between phenolic profiles of varieties are already evident in the ANOVA analysis, we have analyzed the data in a more comprehensive way, using Multivariate Analysis. Principal component analysis (PCA) and Hierarchical agglomerative cluster analysis (HCA) are the most widely used statistical methods to reduce the number of data set, identify the most significant variables in the data set and classify different genotypes (Hossain et al., 2011). Preliminary results showed that PCA analysis could determine the separation among fig varieties. The eigenvalues obtained by PCA indicate that the first three components provide a good summary of the data. They explained 96% of the total variability. The first principal component explained 48% of the total variance and showed high loading for rutin (0.94) and quercetin 3-*O*-malonyl-glucoside (0.70). In particular, the contribute of 3-*O*-malonyl-glucoside to the PC1, is due not only to its variance, but also to its correlation with rutin. The contribution of other compounds was weaker. The second PC explained the 41% of the total variance, and showed high loading for caffeoylmalic acid (-0.98). Principal Component 3 explained 7% of the total variance and was dominated by *p*-coumaroylquinic acid (loading -0.72) and *p*-coumaroyl malic acid (loading -0.33). *P*-coumaroylquinic acid and *p*-coumaroyl malic acid showed the strongest and more stable correlation of the whole dataset. The score plots of PC1 versus PC2 and PC1 versus PC3 describe the differences between 10 fig varieties (Figures 2A and 2B). The score plot of PC1 versus PC2 gathered fig varieties in three groups. 'Portogallo', 'Dottato' and 'Verdino' were confined in the upper left portions of the score plot, characterized by high content of caffeoylmalic acid, whereas 'Sanguinello' and 'Gigante di Carmignano' appeared on the lower left side with lower contents of caffeoylmalic acid and rutin (Figure 2A). 'Saccareccio', 'San Piero',

TABLE 2. Quantification of individual polyphenols, total polyphenols content (TPC) and antioxidant activity (DPPH and ORAC) in the leaves of ten *Ficus carica* varieties.

Compound (mg g ⁻¹ DW)	Varieties									
	Brogiotto Nero	Dorato	Dottato	Fiorone Bianco	Gigante di Carmig- nano	Portogallo	Sacca- reccio	San Piero	Sanginello	Verdino
Chlorogenic acid (5-O-caffeoylquinic acid)	0.793 ^{cd} ± 0.02	2.061 ^a ± 0.02	0.515 ^{ef} ± 0.05	0.84 ^c ± 0.03	0.405 ^a ± 0.04	0.766 ^d ± 0.06	0.454 ^{fg} ± 0.03	0.980 ^b ± 0.06	0.554 ^e ± 0.04	0.560 ^e ± 0.02
Caffeoylmalic acid	3.270 ^f ± 0.08	6.642 ^c ± 0.16	7.465 ^b ± 0.17	5.752 ^d ± 0.11	1.386 ^a ± 0.10	9.198 ^a ± 0.18	5.271 ^{de} ± 0.12	4.891 ^e ± 0.06	2.830 ^f ± 0.05	6.694 ^c ± 0.13
<i>p</i> -Coumaroyl derivative	-	0.713 ^a ± 0.02	-	0.442 ^b ± 0.06	0.394 ^c ± 0.02	-	-	0.450 ^b ± 0.06	0.392 ^c ± 0.03	-
<i>p</i> -Coumaroylquinic acid	1.371 ^c ± 0.06	0.350 ^f ± 0.04	0.458 ^{ef} ± 0.03	0.378 ^f ± 0.03	0.914 ^d ± 0.02	0.448 ^{ef} ± 0.05	1.208 ^b ± 0.03	0.365 ^f ± 0.04	0.499 ^e ± 0.07	2.640 ^a ± 0.03
<i>p</i> -Coumaroyl malic acid	0.774 ^c ± 0.05	0.338 ^e ± 0.02	0.372 ^e ± 0.04	0.354 ^e ± 0.04	0.593 ^d ± 0.04	0.359 ^e ± 0.02	0.988 ^b ± 0.03	0.348 ^e ± 0.02	0.384 ^e ± 0.02	1.392 ^a ± 0.03
Caffeic acid derivates	0.443 ^{ef} ± 0.03	0.517 ^{cd} ± 0.04	0.501 ^{dc} ± 0.04	0.442 ^{ef} ± 0.03	-	0.539 ^{bc} ± 0.02	0.545 ^{b-d} ± 0.02	0.592 ^{ab} ± 0.03	0.424 ^f ± 0.04	0.664 ^a ± 0.02
Isoschaftoside	0.991 ^a ± 0.04	0.648 ^b ± 0.05	0.273 ^e ± 0.04	0.522 ^d ± 0.03	0.556 ^d ± 0.02	0.273 ^e ± 0.02	0.645 ^b ± 0.02	0.399 ^e ± 0.04	0.142 ^f ± 0.03	0.616 ^c ± 0.02
Schaftoside	0.423 ^b ± 0.03	0.518 ^a ± 0.03	0.189 ^f ± 0.02	0.401 ^c ± 0.02	0.328 ^d ± 0.02	0.194 ^f ± 0.03	0.224 ^e ± 0.04	0.230 ^e ± 0.02	0.094 ^g ± 0.02	0.202 ^f ± 0.03
Quercetin derivative	0.134 ^c ± 0.02	0.417 ^a ± 0.03	0.049 ^e ± 0.02	0.294 ^b ± 0.04	0.447 ^a ± 0.03	0.064 ^e ± 0.04	-	0.099 ^d ± 0.03	0.153 ^c ± 0.05	-
Quercetin derivative	0.048 ^{cd} ± 0.03	0.211 ^a ± 0.02	0.063 ^c ± 0.03	0.189 ^{ab} ± 0.03	0.151 ^b ± 0.04	0.063 ^c ± 0.03	-	0.076 ^c ± 0.04	0.077 ^c ± 0.03	-
Rutin (quercetin-3-O-rutinoside)	7.397 ^b ± 0.06	8.218 ^a ± 0.05	1.648 ^b ± 0.07	5.745 ^c ± 0.05	2.018 ^a ± 0.02	2.509 ^f ± 0.03	4.786 ^e ± 0.06	5.662 ^d ± 0.03	1.714 ^h ± 0.05	2.384 ^f ± 0.06
Isoquercetin (quercetin 3-O-glucoside)	0.406 ^d ± 0.02	1.546 ^a ± 0.03	0.142 ^{ef} ± 0.02	0.932 ^b ± 0.02	0.126 ^{ef} ± 0.04	0.113 ^{ef} ± 0.00	0.189 ^e ± 0.04	0.631 ^c ± 0.07	0.568 ^d ± 0.05	0.076 ^f ± 0.07
Quercetin 3-O-malonyl-glucoside	0.365 ^f ± 0.02	2.621 ^a ± 0.04	0.555 ^d ± 0.05	1.781 ^b ± 0.02	0.176 ^g ± 0.05	0.542 ^{de} ± 0.02	0.340 ^f ± 0.04	0.973 ^c ± 0.04	0.491 ^e ± 0.03	0.164 ^g ± 0.04
Kaempferol 3-O-glucoside	0.213 ^b ± 0.04	0.389 ^a ± 0.03	0.061 ^e ± 0.02	0.354 ^a ± 0.02	-	0.167 ^{cd} ± 0.03	0.182 ^{bc} ± 0.02	0.135 ^d ± 0.02	0.040 ^e ± 0.03	0.144 ^d ± 0.03
Kaempferol derivative	-	0.060 ^a ± 0.04	-	0.019 ^b ± 0.05	-	-	-	-	-	-
Psolaren	1.142 ^b ± 0.04	0.960 ^c ± 0.04	0.990 ^c ± 0.05	0.714 ^e ± 0.06	-	1.492 ^a ± 0.06	0.828 ^{de} ± 0.04	0.362 ^g ± 0.02	0.918 ^{cd} ± 0.05	0.722 ^e ± 0.05
Bergapten (5 methoxypsolaren)	0.590 ^{ab} ± 0.03	0.362 ^e ± 0.02	0.504 ^{cd} ± 0.02	0.352 ^e ± 0.03	-	0.627 ^a ± 0.03	0.549 ^{bc} ± 0.05	-	0.445 ^d ± 0.03	0.481 ^d ± 0.04
Psolaric acid isobar	0.438 ^b ± 0.04	0.416 ^{bc} ± 0.03	-	0.391 ^{cd} ± 0.02	-	0.362 ^{de} ± 0.03	0.376 ^{de} ± 0.04	-	0.471 ^a ± 0.04	0.344 ^e ± 0.05
Total	18.4 ^c	26.9 ^a	13.8 ^g	19.9 ^b	7.50 ⁱ	17.8 ^e	17.1 ^e	16.1 ^f	10.1 ^h	17.1 ^d
Polyphenol tests										
TPC ^z	25.86 ^{de} ± 0.5	38.91 ^a ± 0.5	24.93 ^e ± 0.8	33.8 ^b ± 0.7	16.22 ^g ± 0.6	29.89 ^c ± 0.6	27.03 ^d ± 0.5	29.65 ^c ± 0.7	19.32 ^f ± 0.4	29.10 ^c ± 0.5
DPPH ^y EC ₅₀	1.82 ^d ± 0.08	1.05 ^f ± 0.04	6.68 ^a ± 0.06	1.35 ^{ef} ± 0.08	0.48 ^g ± 0.07	6.20 ^b ± 0.09	1.22 ^f ± 0.06	4.89 ^c ± 0.08	1.73 ^{de} ± 0.06	5.26 ^c ± 0.09
ORAC ^x	34.57 ^d ± 0.6	28.67 ^e ± 0.5	42.34 ^b ± 0.5	49.19 ^a ± 0.6	37.18 ^c ± 0.5	28.12 ^e ± 0.6	13.40 ^g ± 0.7	43.46 ^b ± 0.6	6.33 ^h ± 0.5	15.13 ^f ± 0.4

Values are the means ($n=3$) ± SD. Data were analyzed by ANOVA and different letters in each row represent significant differences ($p<0.05$) according to *post hoc* comparison (Tukey's HSD).

^zData expressed as mg of gallic acid equivalents per g of dry mass (mg GAE g⁻¹ DW).

^yData expressed as mg of dry material (DW) required to scavenge 50% of the initial DPPH radicals (mg DW mL⁻¹).

^xData expressed as µmol of Trolox equivalent per g of dry mass (µM TE g⁻¹ DW).

'Fiorone Bianco' were confined, close together, in the right portion of the plot. The combination of PC1 and PC2 maintained, quite distant, 'Brogiotto Nero' and 'Dorato' varieties. 'Brogiotto Nero' variety correlated well with rutin and 'Dorato' with rutin and caffeoylmalic acid (Figure 2A). PC3 separated three cultivars, 'Verdino', 'Saccareccio' and 'Brogiotto Nero', from the others. The distribution was mainly caused by relatively high contents of *p*-coumaroyl malic acid together with low contents of quercetin 3-*O*-malonyl glucoside and quercetin-3-*O*-glucoside (Figure 2B).

Cluster analysis of samples was used as an additional trial to acquire hierarchical relations between different varieties. Three major groups were obtained with cluster analysis (Figure 3). The first group included 'Dorato' and 'Fiorone Bianco' varieties, within a greater hierarchical distance, in comparison to the other samples. These varieties had similar profiles of phenolic compounds, and displayed the highest values of total polyphenols and kaempferol derivatives. The second cluster showed two sub-groups: 'Dottato', 'Portogallo', 'Sanguinello', and 'San Piero', (1st sub-cluster) and 'Brogiotto Nero', 'Saccareccio' and 'Verdino' varieties (2nd sub-cluster). All varieties showed, respectively, medium-low or high content of *p*-coumaric acid derivatives. The third cluster included 'Gigante di Carmignano' variety, characterized by the absence of psoralen derivatives (Figure 3).

FIGURE 2A AND 2B. Principal Component score of the investigated fig varieties according to PC1, PC2, and PC3 obtained by contents of polyphenols compounds, and accounting for 96% pf the total variance explained (A) PC1–PC2 and (B) PC1–PC3.

Total phenolic content (TCP) and Antioxidant activity (AA) evaluation

Polyphenols are very important plant constituents, performing a fundamental role in the strategies adopted by plants (Quideau et al., 2011). Furthermore, epidemiological studies have established that polyphenols might provide health benefits in humans due to their antioxidant activity (Manach et al., 2004).

Total phenol content (TPC) evaluated by the Folin-Ciocalteu assay and expressed as mg of acid gallic equivalent (GAE) per g of dry weight is reported in Table 2. 'Dorato' and 'Fiorone Bianco' showed the highest TPC concentration; 38.9 and 33.8 mg GAE g⁻¹ DW respectively, while 'Dottato' exhibited intermediate values (24.9 mg GAE g⁻¹ DW). The lowest concentration was determined in 'Gigante di Carmignano' (Table 2). The total phenolic content varied significantly between the studied fig varieties and increased in the following order: 'Gigante di Carmignano' < 'Sanguinello' < 'Dottato' < 'Brogiotto Nero' < 'Saccareccio' < 'Verdino' < 'Portogallo' < 'San Piero' < 'Fiorone Bianco' < 'Dorato'. Previous studies analyzed the total phenolics content of *F. carica* leaves investigating both nonspecific samples (no cultivar) and cultivars/varieties notes. Mahmoudi et al. (2016) reported, in leaves of Algerian fig varieties, values of total polyphenol content ranging from 58.7 to 42.8 mg GAE g⁻¹ DW; while the leaves of two Tunisian fig cultivars showed a significantly lower total phenolic contents (Ammar et al., 2015). Marrelli et al. (2014) reported TPC values for leaves of the 'Dottato' variety between 12.9 and 3.6 chlorogenic acid equivalents g⁻¹ DW. Our results are close to the range previously reported in the literature.

The results from the Folin-Ciocalteu assay can be, directly, compared with the sum of phenolic compounds deter-

FIGURE 3. Dendrogram obtained by cluster analyses of phenolic compounds (HPLC-DAD-TOF-MS) from ten *Ficus carica* varieties. Samples are clustered using Ward's technique with an Euclidian distance measure.

mined using HPLC. These methods (TFC and total by HPLC) presented a strong correlations ($p < 0.05$) between the two results ($r = 0.95$). The varieties with the highest ('Dorato' and 'Fiorone Bianco') and lowest ('Gigante di Carmignano' and 'Sanguinello') phenolic contents were the same for both analytical methods (Table 2). Furthermore, the TCP values obtained by the Folin-Ciocalteu method were between 1.5 and 2 times higher than those determined with the HPLC analysis. The differences between the TPC by HPLC and F-C methods can be attributed to the fact that polyphenol content is sometimes overestimated, since the presence of reducing substances (sugars and amino acids) can interfere with the F-C test. According to Alessandri et al. (2014), the F-C and HPLC-DAD estimations of total phenols content are, reliably correlated, regardless of the absolute content, and are independent of the relative composition of the phenolic fraction.

In Table 2 are compared the mean values of antioxidant activity of leaf extracts of *F. carica* varieties using two assays, DPPH and ORAC. Leaf extracts of fig varieties exhibited strong potential to act as a free radical scavenger with an EC_{50} ranging from 0.48 to 6.68 mg DW mL⁻¹ and the average value was 3.58 mg DW mL⁻¹. The DPPH values in the leaf extracts of fig varieties increased in the following order: 'Gigante di Carmignano', 'Dorato', 'Saccareccio', 'Fiorone Bianco', 'Sanguinello', 'Brogiotto Nero', 'San Piero', 'Verdino', 'Portogallo', and 'Dottato' (Table 2). The EC_{50} values of ascorbic acid was 4.9 μ g DW mL⁻¹. Total antioxidant activity measured by ORAC method, ranged from 49.2 μ M TE g⁻¹ DW to 6.3 μ M TE g⁻¹ DW (Table 2). 'Fiorone Bianco' variety showed the highest AA_{ORAC} , followed by 'San Piero' and 'Dottato' (Table 2). To the best of our knowledge, the ORAC data of fig varieties have been reported in a few studies.

By comparing AA values obtained by the individual assays, it is evident that the DPPH and ORAC methods produced different results for the varieties analyzed. For example, the 'Sanguinello' variety showed strong activity, when DPPH capacity was verified, but exhibited low antioxidant activity when the ORAC assay was used. These differences can be attributed to the specific antioxidant composition of the samples, and to different mechanisms used in the analyti-

cal methods to determine antioxidant activity (Cao and Prior, 1998). These differences in the responses of methods display the relevance of applying more than one analytical method for the determination of the antioxidant activity of an extract (Pérez-Jiménez et al., 2008). Furthermore, it is interesting to note that under our experimental conditions, varieties with the lowest total polyphenol content had significant AA values, while varieties, which showed higher values of TPC, showed AA values below average. In particular, the 'Gigante di Carmignano' variety had the lowest concentration of TP (16.2 mg GAE g⁻¹ DW) but good values of AA_{DPPH} and AA_{ORAC} .

A limited amount of literature is available regarding the analysis of the antioxidant capacity of the leaves of fig varieties, which can be compared, with our results, particularly considering the method of extraction, analysis and samples, and no specific studies have been performed with the genotypes considered in this study. Marrelli et al. (2013) investigated the antioxidant activity of leaves in 'Dottato' figs (harvested in July), found IC_{50} values of 64.00 μ g mL⁻¹ for DPPH. Oliveira et al. (2009) reported the antioxidant activities of the leaves of two cultivars, 'Pingo de Mel' and 'Branca Tradicional', and found IC_{50} values of 75.2 and 59.6 μ g mL⁻¹. Recently ORAC assay were used to determine antioxidant activity in leaves of two Tunisia fig varieties, with values of 1.32 and 1.56 μ M TE g⁻¹ sample (Ammar et al., 2016). In this study, the antioxidant activity was less than that of olive leaves. To evaluate the effective antioxidant capacity of the leaves of the varieties used in this study, can be analyzed the antioxidant activity of individual phenolic compounds. In particular, caffeoylmalic acid showed a relatively good correlation with DPPH values ($r = 0.64$) and ORAC values ($r = 0.73$). These data are in accordance with Takahashi et al. (2015). The authors have established that CMA and rutin contribute predominantly to the antioxidant activity of fig leaves. The relationship between total polyphenol content (TPC) and antioxidant activity based on the DPPH and ORAC assays are reported in Figure 4. The statistical analysis showed a moderate relationship between DPPH and TPC values ($r = 0.61$); in contrast, the results of the ORAC assay did not show any correlation with total phenol content ($r = 0.23$). A relative-

FIGURE 4. Correlation between total phenolic content and DPPH assay, total phenolic content and ORAC assay and DPPH and ORAC assay of extracts from *Ficus carica* varieties. Correlation TPC–DPPH: $r = 0.61$; correlation TPC–ORAC: $r = 0.23$; correlation DPPH–ORAC: $r = 0.63$.

ly moderate relationship was also observed between ORAC and DPPH ($r = 0.63$). The DPPH axis shows a group of 5 cultivars having higher anti-radical properties than the others (Figure 4). The fact that this group coincides with Cluster 1 individuated in the PC1-PC2 subspace, suggests that rutin is the main responsible for their high DPPH score. The exam of ORAC axis instead, shows three cultivars scoring sensibly lower than the others (Figure 4). They were the same three cultivars scoring low along PC3, which was due to their relatively high content of *p*-coumaroylquinic acid and *p*-coumaroyl malic acid, and a relatively low content of quercetin 3-*O*-malonyl-glucoside.

Previous studies conducted with fig leaves, concerning the correlation between total polyphenols content and AA, measured using several methods, reported contradicting results. Some authors (Teixeira et al., 2009; Ghazi et al., 2012) reported weak negative correlations between total phenol content and different analytical methods as well as DPPH, FRAP and ABTS; while a positive correlation was observed by Ammar et al. (2016) in Tunisian cultivars. A high number of correlation studies can be found in literature. Low and statistically insignificant correlation value between TPC and AA reported in sweet orange peel and olive leaves (Anagastopoulou et al., 2006; Mylonaki et al., 2008), while some authors showed a high correlation between the content of total phenolic compounds and antioxidant activity in various plant extracts (Gil et al., 2002; Schmitzer et al., 2010). The moderate correlation between TPC and AA in our study may be explained in numerous ways. Some authors claimed that phenolic compounds are not the only factors responsible for antioxidant activity, but the presence of other phytochemicals (e.g., carotenoids, terpenes, reducing carbohydrates) may influence total antioxidant activity. Other factors may act in the antioxidant properties of a compound such as possible synergistic and antagonistic effects among additional components, interactions between physical environment of the sample and the phenolic compounds or activity of specific phenolic compounds that were not suitably determined (Craft et al., 2012).

Conclusions

The results of this study will provide new knowledge about the composition and content of phenolic compounds in fig leaves and the antioxidant activity of their extracts. Significant differences between varieties were observed in *Ficus carica* methanolic extract with ANOVA analysis and confirmed by Multivariate Analysis, suggesting the important role played by genotypes in the determination of bioactive compounds. Chemometric analysis has confirmed the variability in the secondary metabolism of figs, despite their limited geographical distribution, by providing their phytochemical fingerprinting. Preliminary results showed that fig leaves extracts, of Tuscany varieties, possess a good antioxidant activity as measured in DPPH scavenging, and ORAC assays.

The results reported in this study require additional investigations of more fig varieties to determine a more extensive comparison between genotypes, on the chemical composition and antioxidant activity, and to confirm the possibility of using fig leaves as a raw material in medical practice, cosmetic preparations and production of dietary supplements.

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