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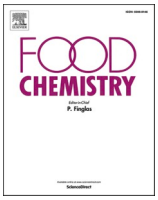

Re-discovering *Prunus* fruit varieties as antiangiogenic agents by metabolomic and bioinformatic approach

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ABSTRACT

In this work, a comparative chemical-biological study of nine plum varieties (*Prunus domestica* L. and *Prunus salicina* Lindl.) with two commercial ones was carried out to improve their cultivation and use in the agri-food chain. The chemical quali-quantitative fingerprint by HR-Orbitrap/ESI-MS showed similar profiles, being 'Rossa Casa Velasco' the richest in phenols and anthocyanins. All the extracts were investigated for their *in vitro* antioxidant as well as antiangiogenic activity by two *in vivo* models, chick chorioallantoic membrane and zebrafish embryos. Among investigated varieties 'Scarrafona', 'Rusticano', 'Marisa', 'Rossa Casa Velasco', 'Verdone', and 'Sangue di Drago' showed the best antiangiogenic activities (30–50 % inhibition). Finally, the chemical/biological datasets processed with a bioinformatic approach revealed that a large group of flavonoids, procyanidins, and anthocyanins significantly correlated with all the three antioxidant tests (DPPH, FRAP, and ABTS), while quinic acid and icariside F_2 resulted positively correlated with CAM at both 100 and 200 μg/egg.

1. Introduction

Prunus genus, belonging to the Rosaceae family, includes more than 175 species distributed worldwide (Jang et al., 2016). Some of them are fruit trees of wide gastronomic consumption, daily present in human meals such as common European plum (*P. domestica* L.), Chinese-Japanese plum (*P. salicina* Lindl.), red and white cherry plum (*P. cerasifera* Ehrh.), sour cherry (*P. cerasus* L.), sweet cherry (*P. avium* L.), peach (*P. persica* (L.) Batsch.), apricot (*P. armeniaca* L.), steppe cherry (*P. fruticosa* Pall.), and blackthorn (*P. spinosa* L.) (Popović et al., 2021). In particular, the European plum, originally from Asia, is hexaploid and it has been cultivated in the Old World since ancient times. Indeed, Pliny the Elder, in the first century AD, mentions the many varieties of plum crops having different forms and colors. The Chinese-Japanese plum, originally from China, is diploid-shaped and it is more recent (Vv.Aa., 2016). At present, the plum fruit is cultivated in all parts of the world in temperate climates (Birwal, Deshmukh, Saurabh, & Pragati, 2017).

P. domestica and *P. salicina* plums are stone fruits included in the traditional Mediterranean diet for their valuable nutritional properties. They are eaten fresh as well as used in traditional and modern popular culinary dishes and recipes; in addition they are processed in different kinds of food products such as dried prunes, canned prunes, plum juice, drinks, liquors, cakes, jams, and jelly (Birwal et al., 2017). Their high

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content in bioactive components, particularly polyphenols, is one of the remarkable features contributing to the widespread consumption. Among polyphenols, anthocyanins are responsible for the vibrant colors of many of these fruits from deep red/purple to blue hues. Anthocyanins, together with hydroxycinnamic acid derivatives such as chlorogenic acid, flavonoids in particular quercetin derivatives, proanthocyanidins, and benzoic acid derivatives were previously reported as *P. domestica* and *P. salicina* main constituents (Hong, Wang, Barrow, Dunshea, & Suleria, 2021; Jang et al., 2016; Navarro-Hoyos et al., 2021; Navarro et al., 2018). These molecules have attracted researcher and consumer attention for their health benefits, such as reducing inflammation and cancer developing risk, protecting against oxidative stress, supporting cardiovascular health, and cognitive functions (Igwe & Charlton, 2016; Fanning, Topp, Russell, Stanley, & Netzel, 2014).

In Mediterranean countries, plums, particularly the *P. domestica* varieties, have a long cultivation history and are cherished for their culinary and medicinal uses (Vv.Aa., 2014). Popular medicine recommends plum decoctions to drink for laxative purposes or boiled plums to eat for the digestive system. Another common use is to prepare infusions of dried prunes to drink as a cough suppressant, often together with other mucilaginous and aromatic fruits and plants. These infusions are also drunk as a depurative between seasons. In the past, the seeds were ground together with honey for worm-infections, especially in children (Guarrera, 2006). Nowadays, the intensification of agricultural practices with the resulting shift towards monoculture and the climate change to which some traditional plum varieties were not able to adapt, led to a loss in the genetic diversity and overall biodiversity of Mediterranean plums. The loss of biodiversity in plums has deep implications for ecosystems, food security, and cultural heritage. Plum trees not only provide delicious fruits but also support pollinators, wildlife, and the global health of the environment. Additionally, traditional plum varieties are an essential part of Mediterranean culinary traditions, local economies, and cultural identity (Ceccarelli, Antonucci, Talento, & Ciccoritti, 2021).

The aim of this work was to counteract the loss of plum biodiversity through a comparative chemical and biological study of nine Italian varieties (*P. domestica* and *P. salicina*) cultivated on the Elba Island and in Campania region with two commercially available ones. As far as we know, no previous phytochemical studies about the selected plum varieties have been reported in the literature. The chemical quali-quantitative plum extract fingerprint was carried out by means of a high-resolution Orbitrap-based electrospray ionization source mass spectrometer (HR-Orbitrap/ESI-MS).

Nowadays, the identification of dietary phytochemicals that modulate angiogenic process has become a relevant issue in health-related research as angiogenesis represents a pathway involved in the progression of many diseases (cardiovascular pathologies, tumor, diabetic retinopathy, etc.) (Diniz, Suliburska, & Ferreira, 2017). Within this framework, the eleven plum extracts were also searched for potential effect on angiogenesis using two *in vivo* models, chick chorioallantoic membrane (CAM) and zebrafish embryo. Finally, as a new contribution to the knowledge of plum fruits health potential, the chemical/biological datasets were processed with an innovative bioinformatic approach. In particular, principal component analysis (PCA), hierarchical clustering (HCL), Pearson correlation matrices, and networks were displayed in order to better find similarities, differences, and correlations among phytochemical composition and biological properties of these varieties.

2. Materials and methods

2.1. Chemicals and reagents

UHPLC grade MeOH, ACN, H_2O, and HCOOH were acquired from Merck KGaA (Darmstadt, Germany), while the analytical grade solvents from VWR (Milano, Italy). Pure standard of cyanidin 3-*O*-glucoside chloride was purchased from Extrasynthese (Genay, France), instead, quercetin 3-*O*-glucoside, catechin, icariside F_2, and chlorogenic acid standards were previously isolated and characterized by 1D- and 2D-NMR, and HR-MS techniques in authors laboratory from other plant extracts.

2.2. Fruit samples and extract preparation

The ripe fruits of eleven local and commercial varieties of European (*P. domestica* L.) and Japanese (*P. salicina* Lindl.) were collected from fruiting trees in July 2021. The samples came from two Italian regions, in particular: *P. domestica* var. 'Coscia di Frate', 'Coscia di Monaca' (Vv. Aa., 2014), and *P. salicina* var. 'Formosa', 'Goccia d'Oro', 'Marisa', 'Rossa Casa Velasco', 'Sangue di Drago' were collected on the Elba Island (Tuscany, Italy), while *P. domestica* var. 'San Francesco', 'Verdone' and *P. salicina* var. 'Rusticano' and 'Scarrafona', were gathered in Benevento territories (Campania - Italy). Prof. Fabiano Camangi performed the plums identification. The fruits morphological, phenological, and taste characteristics are shown in Table 1S-A and 1S-B. Fruit samples, without kernels, were frozen (-18 °C) and stored until extraction.

All varieties, blended after defrosting to obtain homogeneous matrices, were extracted under the same conditions. 100 g of each variety were subjected to extraction with 100 mL of a mixture EtOH/H_2O (8:2 v/v) by dynamic maceration (110 rpm; room temperature) for three consecutive days in dark bottles to prevent oxidation. All obtained residues were partitioned with *n*-BuOH/H_2O (1:1 v/v) to remove sugars. Finally, the solvent was removed under vacuum to obtain dried *n*-butanolic extracts ($R_{n\text{-BuOH}}$) that were stored in dark amber glass vials.

For the extraction of anthocyanins, fruits of each variety (1 g) and a 2 % MeOH/HCl mixture (6 mL) were used. After 15 min of stirring, the solutions were centrifuged for 5 min at 4000 rpm and the supernatants were analyzed in triplicate by UHPLC-DAD-HR-Orbitrap/ESI-MS.

2.3. UHPLC-DAD-HR-Orbitrap/ESI-MS analyses of the fruit extracts

Phenol and anthocyanin analyses were performed by Ultra High-Performance Liquid Chromatography (UHPLC; Vanquish Flex Binary pump) coupled with a diode array detector (DAD) and a high resolution (HR) Q Exactive Plus MS, based on Orbitrap technology, equipped with an electrospray ionization (ESI) source (Thermo Fischer Scientific Inc., Bremem, Germany). Chromatographic analyses were carried out using a 2.1 × 100 mm, 2.6 μm, Kinetex® Biphenyl C-18 column equipped with a Security Guard™ Ultra Cartridge (Phenomenex, Bologna, Italy) at a flow rate of 0.5 mL/min with a splitting system of 1:1 to MS and DAD/UV detector, respectively. Ionization parameters set for all analyses were: nebulization voltage of 3500 V, capillary temperature of 300 °C, sheath gas (N_2) 20 arbitrary units, auxiliary gas (N_2) 3 arbitrary units, HCD (Higher-energy C-trap dissociation) of 18 eV (D'Angiolo et al., 2022). DAD data were registered in a 200–600 nm range using three preferential channels at 254, 280, and 325 nm for phenols and one preferential channel at 515 nm for anthocyanins. Xcalibur 3.1 software was used to view the acquired chromatograms and spectra.

For each variety, *n*-butanol residues were dissolved in methanol at a concentration of 1 mg/mL. All triplicate solutions were centrifuged because of possible undissolved particles and the supernatants (5 μL) were analyzed through the LC-HR-MS system. The elution was performed using a mixture of MeOH/HCOOH 0.1 % (solvent B) and H_2O/HCOOH 0.1 % v/v (solvent A) according to a linear gradient 5 to 80 % (B) within 20 min. The HR-ESI mass spectra were acquired in the negative and positive ion modes; *m/z* scan range of 135–2000.

Anthocyanin LC was carried out with a mixture of ACN/HCOOH 0.1 % (solvent B) and H_2O/HCOOH 0.1 % v/v (solvent A) as mobile phase and a linear gradient 10 to 35 % (B) in 8 min was set. The mass data were acquired in a *m/z* scan range of 250–1200 and only in the positive ion mode.

2.4. Quantitative analyses of compounds of the fruit extracts

Five calibration curves were used to quantify the compounds found in the different varieties of *Prunus* fruits by UHPLC-DAD-HR-Orbitrap/ESI-MS. More in detail, the following pure external standards were chosen: chlorogenic acid for hydroxycinnamic acids and their derivatives, catechin for flavanol derivatives and procyanidins, quercetin 3-*O*-glucoside for flavonol and flavanone derivatives, and icariside F_2. All triplicate solutions were prepared in a concentration range of 1.95–15.63 μg/mL from a stock solution of 1 mg/mL. These concentrations, related to the respective peak areas, showed a good linearity over the entire range and a correlation coefficient (R^2) equal to 0.982 for chlorogenic acid, 0.994 for catechin, 0.990 for quercetin 3-*O*-glucoside, and 0.984 for icariside F_2. Instead, the quantitative analysis of anthocyanins was performed using cyanidin 3-*O*-glucoside as an external standard. Four different concentrations (1–50 μg/mL) were prepared in triplicate and related to the integration of the peaks. Good linearity was displayed over the entire range and a calibration curve was obtained showing R^2 equal to 0.987. Microsoft® Office Excel program was used to calculate each compound amount (mg/100 g of fresh weight (FW) ± standard deviation (SD) for phenols and anthocyanins).

2.5. Determination of total phenolic content

A modified Folin-Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventós 1999) was used to determine the total phenolic content (TPC) in *Prunus* fruits extracts. Each extract (100 μL) was mixed with Folin-Ciocalteu reagent (1000 μL) and distilled water (900 μL). Then, Na_2CO_3 (15 %, 1000 μL) was added to the mixture prior incubation at room temperature in the dark for 1 h. The absorbance was read at 765 nm. A calibration curve of gallic acid was used to express TPC content as mg gallic acid equivalents (mg GAE/g extract).

2.6. Determination of DPPH radical scavenging activity

The scavenging activity was measured using the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical following the method of Yoshida (Yoshida et al., 1989) with minor modifications (De Leo et al., 2021). Briefly, 0.5 mL of tested sample was added to 3.0 mL of DPPH methanolic solution (0.1 mM). After shaking, the mixture was stored in the dark for 30 min at room temperature and finally the absorbance was measured at 517 nm. The results were reported as mg ascorbic acid (reference standard) equivalents (mg AAE/g extract).

2.7. Fe^{3+} reducing antioxidant power (FRAP)

The FRAP test was evaluated following the method reported by Uysal et al. (2017). The results were reported as mg Trolox equivalents (mg TE/g extract), used as a reference compound.

2.8. ABTS radical scavenging assay

The free radical scavenging activity of plum extracts was also determined by the ABTS test following the method reported by Wang, Wang, Pu, & Li, (2013). Results were expressed in terms of mg Trolox equivalents (mg TE/g extract), used as a reference compound.

2.9. Chick chorioallantoic membrane (CAM) assay

The evaluation of antiangiogenic activity was determined by the chick chorioallantoic membrane (CAM) assay according to the method reported before (De Leo et al., 2021). Firstly, stock solutions of extracts (10 mg/mL) were prepared in methanol and then diluted with Tris buffer (0.2 %, pH 7.4) to obtain final concentrations for the test (100–200 μg/egg). Control CAM were treated with the buffer while retinoic acid (1.0 μg/egg) was used as reference standard. After treatment, the eggs were incubated for 48 h at 37 °C. At the end of the process, the CAM was visualized by a stereomicroscope equipped with a digital camera. The number of blood vessel branch points in a standardized area of the CAM was counted to evaluate the antiangiogenic activity and results were expressed as % of inhibition respect to the control. Results were expressed as mean ± SD. Statistical analysis was carried out using Student's *t*-test.

2.10. Zebrafish embryo: generation and staging, treatment protocol

Adult wild type zebrafishes (*Danio rerio*), maintained in flowthrough aquaria at 28.5 °C on a 14/10 h (light/dark) photoperiod according to the standard protocol, were used (Westerfield, 2007). The stage of embryo development was monitored by microscopic observation. Embryos at 24 h after fertilization (24 hpf) were selected to be used in the experiment, manually dechorionated, and distributed in 96-well microplates (one embryo per well). Subsequently, the embryos were treated with 100 μL of sample extracts (10–20 μg/embryo) and incubated for 48 h. The vehicle used for those treatments was DMSO (0.2 % v/v). 2-Methoxyestradiol (2 μM) was used as a reference standard due to its proven antiangiogenic activity. All experiments were conducted in compliance with the European Directive 2010/63/EU and the ethical guidelines described in the "National Institutes of Health Guide for Care and Use of Laboratory Animals". Results were expressed as mean ± SD. Statistical analysis was carried out using Student's *t*-test.

2.11. Quantitative determination of endogenous alkaline phosphatase activity

After incubation with the extracts, the embryos were dehydrated using increasing concentrations of ethanol (50, 75, 95 % for 10 min and then 100 % for 30 min), washed three times with 1 M diethanolamine buffer (0.5 mg/mL, pH 9.8) and finally incubated for 30 min at room temperature with disodium salt of *p*-nitrophenyl phosphate (*p*NPP, chromogenic substrate). NaOH (2 M, 50 μL/well) was added after incubation to stop the reaction. The absorbance of the supernatant solution was measured spectrophotometrically at 405 nm using a microplate reader (Multiskan GO; Thermo Scientific, Waltham, MA, USA). The results were expressed as the percentage of inhibition (%) of the treated groups compared to the control group (100 % of activity) (De Leo et al., 2021).

2.12. Bioinformatics analyses of chemical and bioassay data

All phytochemical and biological data have been examined using an ANOVA + Tukey's pairwise *t*-test as reported in Parisi et al. (2022). On the contrary, PCA and HCL have been generated as previously shown (Di Meo et al., 2019; Grosso et al., 2018, respectively). Similarly, symmetric metabolite-metabolite correlation matrix has been produced as described in (Ahrazem et al., 2018). Finally, correlation networks have been accomplished as reported in De Leo et al. (2021), with slight differences: in addition to cumulative networks for antioxidant and antiangiogenic activities, singular network for each test/concentration were also performed, in which the trait of interest was placed as central hub, and distance by the center of each additional node was inversely proportional to the Pearson correlation coefficient (ρ).

3. Results and discussion

3.1. Chemical profiles of Prunus fruit extracts

The metabolomic fingerprint and quantitative determination of all *Prunus* variety extracts (Fig. 1A and B) were performed using UHPLC-DAD-HR-Orbitrap/ESI-MS instrument. Comparing retention times, UV absorption, HR full mass spectra, fragmentation patterns with data reported in previous works and considering a mass error < 5 ppm on the

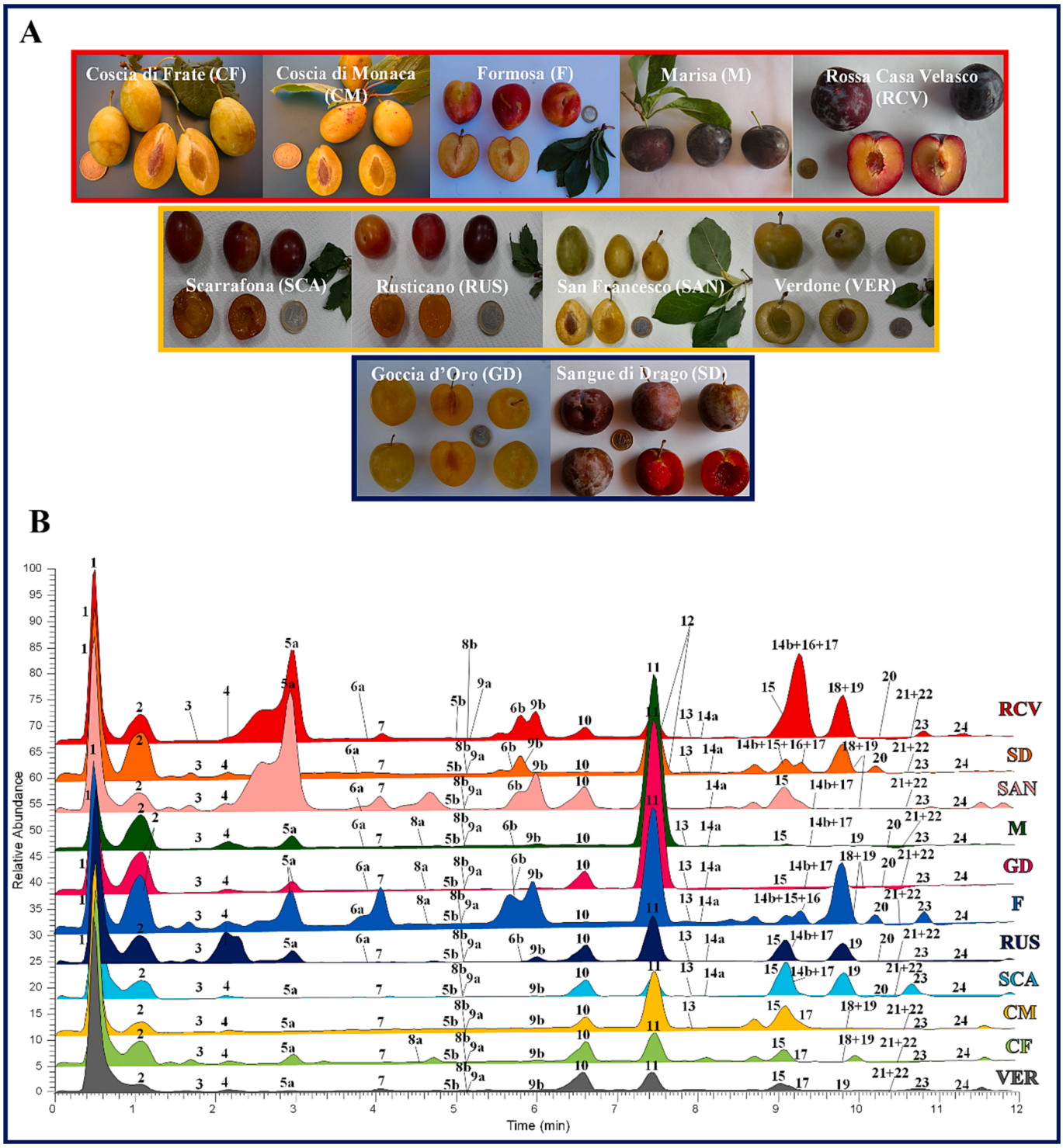

Fig. 1. Fruits of *Prunus* varieties and LC-MS chromatograms. **A.** CF, CM, F, M, RCV fruits were collected in the Elba Island (Italy); SCA, RUS, SAN, VER fruits were collected in Benevento, Campania (Italy); GD and SD fruits were taken as references. CF = 'Coscia di Frate'; CM = 'Coscia di Monaca'; F = 'Formosa'; GD = 'Goccia d'Oro'; M = 'Marisa'; RCV = 'Rossa Casa Velasco'; SCA = 'Scarrafona'; SD = 'Sangue di Drago'; RUS = 'Rusticano'; SAN = 'San Francesco'; VER = 'Verdone'. **B.** Comparison of qualitative profiles of all studied plums, obtained in negative ion mode. Each number corresponds to a molecule elencated in Table 2S.

experimental molecular formula, a total of 32 compounds (hydroxycinnamic acids and their derivatives, flavonoids, and anthocyanins) were tentatively identified (Table 2S).

Among hydroxycinnamic acids (**1**, **5a**, **5b**, **7**, **8a**, **8b**, **9a**, and **9b**), compound **1** (t_R = 0.52 min) was annotated as quinic acid due to the deprotonated ion $[M-H]^-$ at *m/z* 191.0557 and the product ion $[M-H-18]^-$ at *m/z* 173.04 corresponding to the loss of a water molecule (Navarro-Hoyos et al., 2021). Compounds **5a** (t_R = 2.93 min) and **5b** (t_R = 4.80 min) showed the same deprotonated ion $[M-H]^-$ at *m/z* 353.0878 in the full MS, while in the MS/MS experiment a base ion peak at *m/z* 191.05 (quinic acid) and a caffeoyl ion product at *m/z* 179.03 were displayed, thus the two compounds were tentatively attributed to caffeoylquinic acid isomers (Jaiswal et al., 2013; Navarro et al., 2018). Compound **7** (t_R = 4.12 min; $[M-H]^-$ at *m/z* 337.0931) was annotated

as coumaroylquinic acid, displaying a base ion peak at *m/z* 163.04 corresponding to the loss of a quinoyl moiety and a product ion at *m/z* 173.04 due to the loss of a *p*-coumaroyl unit (Navarro-Hoyos et al., 2021). Compounds **8a** and **8b** (t_R = 4.69 and t_R = 5.17 min) were identified as *p*-coumaroyl hexoside isomers, having the same deprotonated molecular ion $[M - H]^-$ at *m/z* 325.0930 and a *p*-coumaroyl moiety as a product ion at *m/z* 163.04 ($[M - H - 162]^-$) due to the loss of a hexose residue (Navarro-Hoyos et al., 2021). Feruloylquinic acid isomers were attributed to peaks **9a** and **9b** (t_R = 5.36 and t_R = 6.00 min) by considering the same deprotonated ion $[M - H]^-$ at *m/z* 367.1035 and the product ion at *m/z* 193.05 (feruloyl residue) (Navarro-Hoyos et al., 2021). In addition to hydroxycinnamic acids, other organic acids were identified in the *Prunus* fruits. Hydroxy-glutaric acid (**2**, t_R = 0.98 min, $[M - H]^-$ at *m/z* 147.0293) showed the loss of a water molecule (-18 u) and a diagnostic fragment ion at *m/z* 85.03 due to the loss of the carboxylic unit as CO_2 ($[M - H - 44 - 18]^-$). Compound **3** (t_R = 1.87 min) showed a deprotonated molecular ion $[M - H]^-$ at *m/z* 153.0188 and a product ion $[M - H - 44]^-$ at *m/z* 109.03 generated by the loss of a carboxylic moiety, thus it was identified as protocatechuic acid (Navarro et al., 2018). Compound **4** (t_R = 2.02 min; $[M - H]^-$ at *m/z* 161.0451) differed from compound **2** only for a methyl group, thus annotated as 3-hydroxy-3-methylglutaric acid. All *Prunus* fruits showed the presence of flavonoids. Catechin and epicatechin were attributed to peaks **6a** and **6b** (t_R = 3.93 and t_R = 5.81 min), showing the deprotonated molecular ion $[M - H]^-$ at *m/z* 289.0719 and a product ion at *m/z* 245.08 ($[M - H - 42]^-$) due to the *retro*-Diels-Alder as reported by Navarro-Hoyos et al. (2021). In addition, compounds **12**, **14a**, **14b**, and **16** were identified as procyanidin oligomers in accordance with Jaiswal et al. (2013) and Navarro-Hoyos et al. (2021). In particular, procyanidin B-type trimer (**12**, t_R = 7.40 min; $[M - H]^-$ at *m/z* 865.1995) showed a typical fragmentation pattern with product ions at *m/z* 577.14, 575.12, 289.07, and 287.06 due to the cleavage of the interflavonoids bonds. Procyanidin B-type dimer (**16**, t_R = 9.16 min; $[M - H]^-$ at *m/z* 577.1340) displayed a fragment ion at *m/z* 559.12 due to the loss of a water molecule and diagnostic ions at *m/z* 451.10 (loss of phloroglucinol molecule), 425.09 (*retro*-Diels-Alder), and 287.05 (quinone-methide pathway). **14a** and **14b** (t_R = 8.13 and t_R = 9.14 min; $[M - H]^-$ at *m/z* 575.1201) were identified as two procyanidin A-type dimer isomers, showing fragment ions at *m/z* 449.09 (loss of phloroglucinol molecule), 423.07 (*retro*-Diels-Alder), 289.07 and 285.04 (quinone-methide pathway). Flavonols are also well represented in the *Prunus* fruits. Compound **13** (t_R = 7.80 min; $[M - H]^-$ at *m/z* 303.0512) showing product ions at *m/z* 285.04 ($[M - H - H_2O]^-$) and 275.06 ($[M - H - CO]^-$) was annotated as dihydroquercetin (Hong et al., 2021). Compounds **15**, **17**, **19**, **20,** and **23** were all attributed to quercetin derivatives, as deduced by the presence in the MS/MS of the same base ion peak at *m/z* 300.03 (aglycon portion). Based on the loss of saccharide residues these compounds were identified as rutin (**15**, $[M - H]^-$ at *m/z* 609.1465) (Khallouki, Haubner, Erben, Ulrich, & Owen, 2012), quercetin hexoside (**17**, $[M - H]^-$ at *m/z* 463.0886) (Jaiswal et al., 2013; Navarro-Hoyos et al., 2021), quercetin pentoside (**19**, $[M - H]^-$ at *m/z* 433.0779) (Hong et al., 2021; Jaiswal et al., 2013), quercetin deoxyhexoside (**20**, $[M - H]^-$ at *m/z* 447.0935), and quercetin acetylhexoside (**23**, $[M - H]^-$ at *m/z* 505.0992) (Navarro-Hoyos et al., 2021). Compounds **21** (t_R = 10.67 min; $[M - H]^-$ at *m/z* 623.1620) and **22** (t_R = 10.78 min; $[M - H]^-$ at *m/z* 477.1040) were identified as isorhamnetin derivatives, exhibiting both the product ion at *m/z* 315.05 and the loss of a disaccharide unit (-308 u) and a monosaccharide portion (-162 u), respectively, leading to identify **21** as isorhamnetin rutinoside and **22** as isorhamnetin hexoside. Furthermore, the metabolomic analysis revealed the presence of two flavanones, identified as eriodictyol hexoside (**18**, t_R = 9.77 min; $[M - H]^-$ at *m/z* 449.1093) and naringenin hexoside (**24**, t_R = 11.38 min; $[M - H]^-$ at *m/z* 433.1143), both displaying the loss of a hexose unit (-162 u) in the MS/MS experiments (Navarro et al., 2018). Among other constituents, compound **10** (t_R = 6.66 min), showing a deprotonated molecular ion $[M - H]^-$ at *m/z* 401.1455 and a formate adduct $[M + HCOO]^-$ at *m/z* 447.1509, was identified as icariside F_2. Its retention time and fragmentation pattern were compared with those of a pure standard. Compound **11** (t_R = 7.38 min) was annotated as hydroxyoctanedioic acid due to the presence of a deprotonated molecular ion $[M - H]^-$ at *m/z* 189.0765 and fragment ions at *m/z* 171.06 ($[M - H - H_2O]^-$) and 127.07 ($[M - H - H_2O - CO_2]^-$).

According to the literature, anthocyanins were found as typical components of *Prunus* fruits. Compounds **25** (t_R = 1.33 min; $[M]^+$ at *m/z* 449.1075) and **26** (t_R = 1.43 min; $[M]^+$ at *m/z* 595.1658) were annotated as cyanidin glucoside ($[M - 162]^+$ at *m/z* 287.05) and cyanidin rutinoside ($[M - 308]^+$ at *m/z* 287.05), respectively, showing the same base ion peak given by the cyanidin fragment (Tomić, Štampar, Glišić, & Jakopič, 2019). Compounds **27**, **28**, **29a**, and **29b** were all annotated as delphinidin glycosides, as deduced by the presence in the fragmentation pathway of the base ion peak at *m/z* 303.05 attributed to the aglycon portion of delphinidin, thus tentatively identified as delphinidin rutinoside (**27**, $[M]^+$ at *m/z* 611.1605), delphinidin hexoside (**28**, $[M]^+$ at *m/z* 465.1023), and delphinidin pentoside isomers (**29a** and **29b**, $[M]^+$ at *m/z* 435.0918). A petunidine derivative was also detected. Compound **30** displayed the molecular ion $[M]^+$ at *m/z* 625.1752 and a diagnostic base ion peak ($[M - 308]^+$) at *m/z* 317.07 due to petunidin portion, leading to tentatively identify the molecule as petunidin rutinoside. Compounds **31** and **32** were not completely identified, but based on some characteristic fragments, only the aglycon portion was assigned. Compound **31** ($[M]^+$ at *m/z* 507.1128) was supposed to be a delphinidin derivative, exhibiting a base ion peak at *m/z* 303.05, while **32** ($[M]^+$ at *m/z* 577.1337) displaying a base ion peak at *m/z* 287.05, was attributed to a cyanidin derivative.

Qualitative investigation confirmed the presence of many compounds, including phenol derivatives and anthocyanins, previously reported in the literature. However, as far as we know, icariside F_2, delphinidin, and petunidin derivatives were never reported before as plums constituents. Overall, all the varieties showed similar chromatographic profiles, differing from a qualitative prospective, especially catechin, *p*-coumaroyl hexoside, procyanidin B-type trimer and dimer, delphinidin pentoside, cyanidin glucoside, and cyanidin rutinoside were the point of difference (Table 2S). In particular, 'Rossa Casa Velasco' variety exhibited the most diversified profile resulting very similar in term of composition to 'Sangue di Drago', widely spread in the market. On the contrary, 'Coscia di Monaca' and 'Coscia di Frate' varieties showed an overlapping chemical fingerprint, resulting free of catechin and procyanidin derivatives.

3.2. Quantitative analysis of Prunus fruit extracts

Quantitative analysis highlighted similarities and differences among varieties (Table 1). Particularly, all red varieties showed a larger amount of flavonoids such as catechin/epicatechin, procyanidin derivatives, and anthocyanins than the yellowish varieties. 'Rossa Casa Velasco' (red) variety distinguished from all the other ones in term of phenol content, having a hydroxycinnamic acid derivative/flavonoid total content of 76.9 ± 1.0 mg/100 g of fresh weight (FW) and an anthocyanin total content of 61.1 ± 1.3 mg/100 g FW. 'Coscia di Monaca' (yellowish) and 'Goccia d'Oro' (yellow) varieties resulted similar in the poor total content both of hydroxycinnamic acid derivatives/flavonoids (19.2 ± 0.34 and 15.8 ± 0.61 mg/100 g FW, respectively) and anthocyanins (2.82 ± 0.26 and 2.74 ± 0.16 mg/100 g FW, respectively). 'Verdone' variety showed a quinic acid content much higher than all the other varieties (17.4 ± 0.51 mg/100 g FW), while 'Rusticano' resulted the richest variety in rutin (7.74 ± 0.023 mg/100 g FW), icariside F_2 (8.70 ± 0.021 mg/100 g FW) and delphinidin rutinoside (6.01 ± 0.24 mg/100 g FW). Taken together results evidenced a trend of higher phenol and anthocyanin amounts in the ancient varieties (e.g. 'Rossa Casa Velasco, 'Formosa, 'Rusticano', and 'San Francesco') compared to the commercial ones ('Sangue di Drago' and 'Goccia d'Oro').

Table 1
Quantitative results of phenols and anthocyanins (mg/100 g fresh weight ± standard deviation) found in *Prunus* fruit extracts.

Variety (mg/100 g fresh weight ± standard deviation)											
Compounds	**'Goccia d'Oro'**	**'Sangue di Drago'**	**'Rossa Casa Velasco'**	**'Coscia di Monaca'**	**'Coscia di Frate'**	**'Formosa'**	**'Marisa'**	**'Scarrafona'**	**'San Francesco'**	**'Rusticano'**	**'Verdone'**
Quinic acid	2.79 ± 0.20	4.03 ± 0.80	6.01 ± 0.23	4.94 ± 0.10	11.2 ± 0.29	2.72 ± 0.14	2.69 ± 0.39	7.26 ± 0.61	5.93 ± 0.17	10.1 ± 0.043	17.4 ± 0.51
Caffeoylquinic acid I (chlorogenic acid)	1.73 ± 0.056	0.321 ± 0.059	12.6 ± 0.099	0.811 ± 0.011	2.08 ± 0.012	3.49 ± 0.060	3.12 ± 0.048	0.248 ± 0.0013	13.9 ± 0.13	3.82 ± 0.051	0.822 ± 0.049
Caffeoylquinic acid II	0.491 ± 0.013	0.0575 ± 0.0095	1.39 ± 0.018	0.117 ± 0.00024	0.444 ± 0.0050	1.30 ± 0.030	0.602 ± 0.010	0.117 ± 0.016	2.92 ± 0.029	1.87 ± 0.035	0.126 ± 0.0023
Coumaroylquinic acid	0.279 ± 0.010	0.0927 ± 0.012	1.53 ± 0.015	0.352 ± 0.0064	0.562 ± 0.0069	2.99 ± 0.037	0.888 ± 0.019	0.118 ± 0.0032	1.87 ± 0.049	0.388 ± 0.0033	1.15 ± 0.10
p-Coumaroyl hexoside I	0.0658 ± 0.0030	nd	nd	nd	1.19 ± 0.012	0.169 ± 0.0059	0.141 ± 0.0028	nd	nd	nd	nd
p-Coumaroyl hexoside II	0.0779 ± 0.0025	0.0613 ± 0.0090	0.0950 ± 0.0021	0.0627 ± 0.0010	0.337 ± 0.016	0.101 ± 0.00063	0.0703 ± 0.0020	0.0741 ± 0.0031	0.112 ± 0.0020	0.183 ± 0.0083	0.0733 ± 0.0049
Feruloylquinic acid I	0.209 ± 0.011	0.0273 ± 0.0034	0.463 ± 0.009	0.214 ± 0.0014	0.474 ± 0.0062	0.414 ± 0.0056	0.432 ± 0.0090	0.148 ± 0.0034	0.503 ± 0.036	0.290 ± 0.013	0.814 ± 0.067
Feruloylquinic acid II	0.657 ± 0.017	0.114 ± 0.011	3.61 ± 0.10	0.120 ± 0.0012	0.782 ± 0.018	1.26 ± 0.020	1.09 ± 0.026	0.0558 ± 0.00091	3.72 ± 0.014	1.81 ± 0.032	0.139 ± 0.0051
Rutin	0.938 ± 0.027	3.00 ± 0.11	5.26 ± 0.024	4.43 ± 0.040	3.69 ± 0.041	2.09 ± 0.040	1.99 ± 0.067	6.81 ± 0.12	4.19 ± 0.16	7.74 ± 0.023	2.99 ± 0.13
Quercetin hexoside	0.399 ± 0.0084	2.70 ± 0.077	16.6 ± 0.14	2.68 ± 0.013	0.954 ± 0.024	2.42 ± 0.026	0.953 ± 0.014	1.49 ± 0.013	2.39 ± 0.013	1.65 ± 0.026	2.01 ± 0.053
Eriodictyol hexoside	0.0115 ± 0.00071	0.100 ± 0.0044	0.764 ± 0.0075	trace	trace	trace	nd	nd	0.0146 ± 0.00013	nd	nd
Quercetin pentoside	2.11 ± 0.062	4.75 ± 0.13	8.55 ± 0.079	0.0145 ± 0.00085	trace	7.19 ± 0.15	1.75 ± 0.013	5.50 ± 0.094	trace	7.46 ± 0.033	trace
Quercetin deoxyhexoside	0.616 ± 0.023	1.90 ± 0.12	0.670 ± 0.010	nd	nd	1.92 ± 0.059	0.539 ± 0.0045	0.986 ± 0.026	nd	1.14 ± 0.010	nd
Isorhamnetin rutinoside	trace	0.0370 ± 0.00028	0.193 ± 0.0052	0.233 ± 0.0079	0.155 ± 0.0028	trace	0.0132 ± 0.0020	2.59 ± 0.053	0.168 ± 0.0077	1.14 ± 0.014	0.302 ± 0.014
Isorhamnetin hexoside	trace	0.0274 ± 0.0028	0.461 ± 0.013	0.0609 ± 0.00034	0.0108 ± 0.00013	0.0212 ± 0.0019	trace	0.230 ± 0.0029	0.0499 ± 0.0032	0.0497 ± 0.0020	0.0533 ± 0.0052
Quercetin acetylhexoside	0.187 ± 0.0082	0.297 ± 0.020	2.32 ± 0.028	0.793 ± 0.018	0.388 ± 0.0081	2.06 ± 0.053	0.167 ± 0.0020	1.12 ± 0.025	0.823 ± 0.017	0.981 ± 0.027	0.166 ± 0.0053
Naringenin hexoside	0.0531 ± 0.0025	0.268 ± 0.0080	1.63 ± 0.026	0.445 ± 0.0016	0.0532 ± 0.0004	0.0331 ± 0.0020	0.0110 ± 0.00093	trace	0.0841 ± 0.0063	0.0143 ± 0.0021	0.0348 ± 0.0018
Icariside F_2	4.81 ± 0.13	1.72 ± 0.058	4.75 ± 0.069	3.93 ± 0.14	6.69 ± 0.12	0.790 ± 0.023	1.16 ± 0.024	5.35 ± 0.039	5.59 ± 0.12	8.70 ± 0.021	7.45 ± 0.11
Catechin	0.0787 ± 0.0044	0.672 ± 0.081	0.624 ± 0.010	nd	nd	2.24 ± 0.021	0.176 ± 0.0051	nd	0.0125 ± 0.00041	0.545 ± 0.013	nd
Epicatechin	0.0663 ± 0.0020	3.88 ± 0.37	6.22 ± 0.061	nd	nd	0.336 ± 0.0068	0.0449 ± 0.0020	nd	trace	0.259 ± 0.0050	nd
Procyanidin B-type trimer	nd	0.171 ± 0.0052	0.485 ± 0.0056	nd	nd	nd	nd	nd	nd	nd	nd
Procyanidin A-type dimer I	0.143 ± 0.0065	0.301 ± 0.011	0.333 ± 0.0062	nd	nd	0.367 ± 0.0043	0.656 ± 0.0022	0.0358 ± 0.00069	0.0305 ± 0.0020	1.17 ± 0.029	nd
Procyanidin A-type dimer II	0.122 ± 0.011	1.65 ± 0.057	2.09 ± 0.092	nd	nd	0.189 ± 0.011	0.539 ± 0.012	trace	trace	0.341 ± 0.0076	nd
Procyanidin B-type dimer	nd	0.0702 ± 0.0089	0.220 ± 0.032	nd	nd	0.0146 ± 0.0038	nd	nd	nd	nd	nd
TOTAL	15.8 ± 0.61	26.2 ± 2.1	76.9 ± 1.0	19.2 ± 0.34	29.0 ± 0.61	32.1 ± 0.74	17.0 ± 0.66	32.1 ± 0.93	42.4 ± 0.76	49.7 ± 0.40	33.5 ± 1.1
Variety (mg/100 g fresh weight ± standard deviation)											
Anthocyanins	**'Goccia d'Oro'**	**'Sangue di Drago'**	**'Rossa Casa Velasco'**	**'Coscia di Monaca'**	**'Coscia di Frate'**	**'Formosa'**	**'Marisa'**	**'Scarrafona'**	**'San Francesco'**	**'Rusticano'**	**'Verdone'**
Cyanidin glucoside	nd	1.75 ± 0.066	27.7 ± 0.66	nd	nd	0.145 ± 0.030	4.32 ± 0.15	0.288 ± 0.017	nd	0.0520 ± 0.0073	0.0665 ± 0.00054
Cyanidin rutinoside	nd	0.927 ± 0.11	2.84 ± 0.091	nd	nd	0.106 ± 0.0073	2.55 ± 0.061	0.0218 ± 0.0019	nd	nd	nd
Delphinidin rutinoside	1.69 ± 0.093	0.755 ± 0.0060	7.57 ± 0.12	1.58 ± 0.14	2.47 ± 0.18	3.12 ± 0.31	1.23 ± 0.023	2.73 ± 0.21	2.24 ± 0.041	6.01 ± 0.24	0.840 ± 0.0093
Delphinidin hexoside	0.761 ± 0.063	0.936 ± 0.019	21.9 ± 0.43	1.05 ± 0.10	0.719 ± 0.074	2.33 ± 0.22	0.577 ± 0.028	0.428 ± 0.024	1.19 ± 0.045	1.28 ± 0.076	0.456 ± 0.019

(*continued on next page*)

Table 1 (*continued*)

Variety (mg/100 g fresh weight ± standard deviation)											
Compounds	**'Goccia d'Oro'**	**'Sangue di Drago'**	**'Rossa Casa Velasco'**	**'Coscia di Monaca'**	**'Coscia di Frate'**	**'Formosa'**	**'Marisa'**	**'Scarrafona'**	**'San Francesco'**	**'Rusticano'**	**'Verdone'**
Delphinidin pentoside I	0.205 ± 0.003	0.178 ± 0.013	0.386 ± 0.002	nd	nd	0.209 ± 0.015	0.0282 ± 0.0014	0.0902 ± 0.0039	nd	0.405 ± 0.013	nd
Delphinidin pentoside II	0.0664 ± 0.0033	0.0958 ± 0.0071	0.171 ± 0.0055	0.00148 ± 0.000046	0.00246 ± 0.00013	0.0331 ± 0.0043	0.0085 ± 0.00023	0.0329 ± 0.00016	0.0015 ± 0.000037	0.228 ± 0.024	0.0012 ± 0.00011
Petunidin rutinoside	0.0177 ± 0.0022	0.0250 ± 0.0011	0.524 ± 0.0062	0.184 ± 0.015	0.192 ± 0.017	0.0296 ± 0.0030	0.0176 ± 0.00051	2.05 ± 0.072	0.213 ± 0.012	2.28 ± 0.044	0.397 ± 0.0080
TOTAL	2.74 ± 0.16	4.67 ± 0.23	61.1 ± 1.3	2.82 ± 0.26	3.38 ± 0.27	5.97 ± 0.59	8.73 ± 0.26	5.64 ± 0.33	3.64 ± 0.098	10.26 ± 0.40	1.76 ± 0.037

nd = not detected.

3.3. Determination of total phenolic content

Plum fruit extracts total phenolic content is reported in Table 2. Overall, results showed a variable range from 17.31 ± 0.26 to 110.28 ± 1.94 mg GAE/g extract. However, 'Rossa Casa Velasco' variety (110.28 ± 1.94 mg GAE/g) showed the highest content, followed by 'Sangue di Drago' (85.06 ± 0.19 mg GAE/g) and 'Formosa' (73.29 ± 0.12 mg GAE/g).

3.4. Determination of antioxidant activity

In vitro test were executed to explore the plum extracts antioxidant potential (Table 2). The scavenging activity showed that, among plum extracts, 'Sangue di Drago', 'Rossa Casa Velasco', 'Formosa', and 'Rusticano' exhibited the best effects against the DPPH radical (56.98 ± 0.97, 56.82 ± 1.47, 48.29 ± 3.69, and 37.12 ± 4.56 mg AAE/g, respectively). Similarly, the antioxidant activity measured by FRAP method evidenced that 'Rossa Casa Velasco' has the strongest ability to reduce the Fe^{3+}-TPTZ complex (264.81 ± 22.76 mg TE/g) followed by 'Sangue di Drago' (194.17 ± 17.26 mg TE/g) and 'Formosa' (167.54 ± 4.27 mg TE/g). Finally, the investigated sample extracts showed also a good ABTS radical scavenging activity. However, even in this case, 'Rossa Casa Velasco' (153.43 ± 4.67 mg TE/g) exhibited the best antioxidant effect, followed by 'Marisa' (154.59 ± 1.14 mg TE/g) and 'Rusticano' (149.61 ± 20.94 mg TE/g).

3.5. Antiangiogenic effects of Prunus fruit extracts in the chick chorioallantoic membrane (CAM) assay

The antiangiogenic activity of the fruits in the CAM assay is reported in Fig. 2A. Results are expressed as inhibition % versus negative control (100 % of neovascolarization). The plum extracts from 'Scarrafona', 'Rusticano', 'Marisa', 'Rossa Casa Velasco', 'Sangue di Drago', and 'San Francesco' exhibited a significant antiangiogenic response as showed by relevant reduction of the microvasculature in the CAMs (34.44–50.55 % of inhibition at 100 and 200 μg/egg) as compared to the negative control. Retinoic acid was used as positive control (1 μg/egg, 44.55 % of inhibition). Representative images of the CAMs showing the best antiangiogenic response are outlined in Fig. 1S. As far as it can be seen, in the control CAM there is a thick vascular network with large vessels (Fig. 1S-A). Contrarily, the retinoic acid (Fig. 1S-B), 'Rusticano', 'Marisa', 'Rossa Casa Velasco', and 'Sangue di Drago' (Fig. 1S-C, D, and F) treatments produced a less dense vascular network due to the strong inhibitory effects on capillary formation.

3.6. Antiangiogenic effects of Prunus fruit extracts in zebrafish embryos

The *Prunus* fruit extracts were also tested on zebrafishes. Indeed, the embryo in the early stage of its development is considered an ideal model to estimate the effect on angiogenesis. Specifically, the endogenous alkaline phosphatase (EAP) activity released from the vascular endothelial cells of treated embryos was quantified as a marker of vessel growth. Results are reported in Fig. 2B and expressed as inhibition percentage with respect to the negative control (100 % of EAP activity).

Table 2
Total phenolic content (TPC), radical scavenging activity (DPPH), ferric reducing power (FRAP) and total antioxidant capacity (ABTS) in *Prunus* fruit extracts. Different letters within each column indicate statistically significant differences at $p < 0.05$ in an ANOVA + Tukey's pairwise *t*-test analysis.

	'Goccia d'Oro'	**'Sangue di Drago'**	**'Rossa Casa Velasco'**	**'Coscia di Monaca'**	**'Coscia di Frate'**	**'Formosa'**	**'Marisa'**	**'Scarrafona'**	**'San Francesco'**	**'Rusticano'**	**'Verdone'**
TPC (mg GAE/g)	55.84 ± 0.14 bcdefgh	85.06 ± 0.19 bcdefghil	110.28 ± 1.94 bcdefg	17.31 ± 0.26 bcd	18.27 ± 0.01 bcd	73.29 ± 0.12 bcdef	25.89 ± 0.02 bcde	38.42 ± 0.15 a	36.25 ± 0.04 bcdefghi	48.37 ± 0.05b	30.82 ± 0.07 bc
DPPH (mg AAE/g)	27.36 ± 4.02 bc	56.98 ± 0.97 bcef	56.82 ± 1.47 bcef	7.68 ± 1.49 ad	10.38 ± 4.22 ad	48.29 ± 3.69 bce	29.72 ± 1.83 bc	14.08 ± 2.86 a	34.61 ± 2.58b	37.12 ± 4.56b	6.53 ± 4.54 bcd
FRAP (mg TE/g)	101.12 ± 9.53 ah	194.17 ± 17.26 bcg	264.81 ± 22.76 bc	58.88 ± 1.78 bcde	57.69 ± 5.75 bcdf	167.54 ± 4.27b	81.09 ± 5.88 aef	84.69 ± 1.97 a	a121.54 ± 2.38 bcgh	146.56 ± 2.22b	78.6 ± 2.52 ad
ABTS (mg TE/g)	77.98 ± 0.28 af	147.91 ± 8.28 be	153.43 ± 4.67b	55.21 ± 0.79 ad	61.5 ± 1.48 ad	149.07 ± 2.28 be	154.59 ± 1.14 be	73.55 ± 0.51 a	78.26 ± 0.05 af	149.61 ± 20.94b	51.25 ± 1.22 bcd

Results were expressed as mean ± standard deviation of three independent experiments ($n = 3$).
AAE = ascorbic acid equivalents; GAE = gallic acid equivalents; TE = Trolox equivalents.

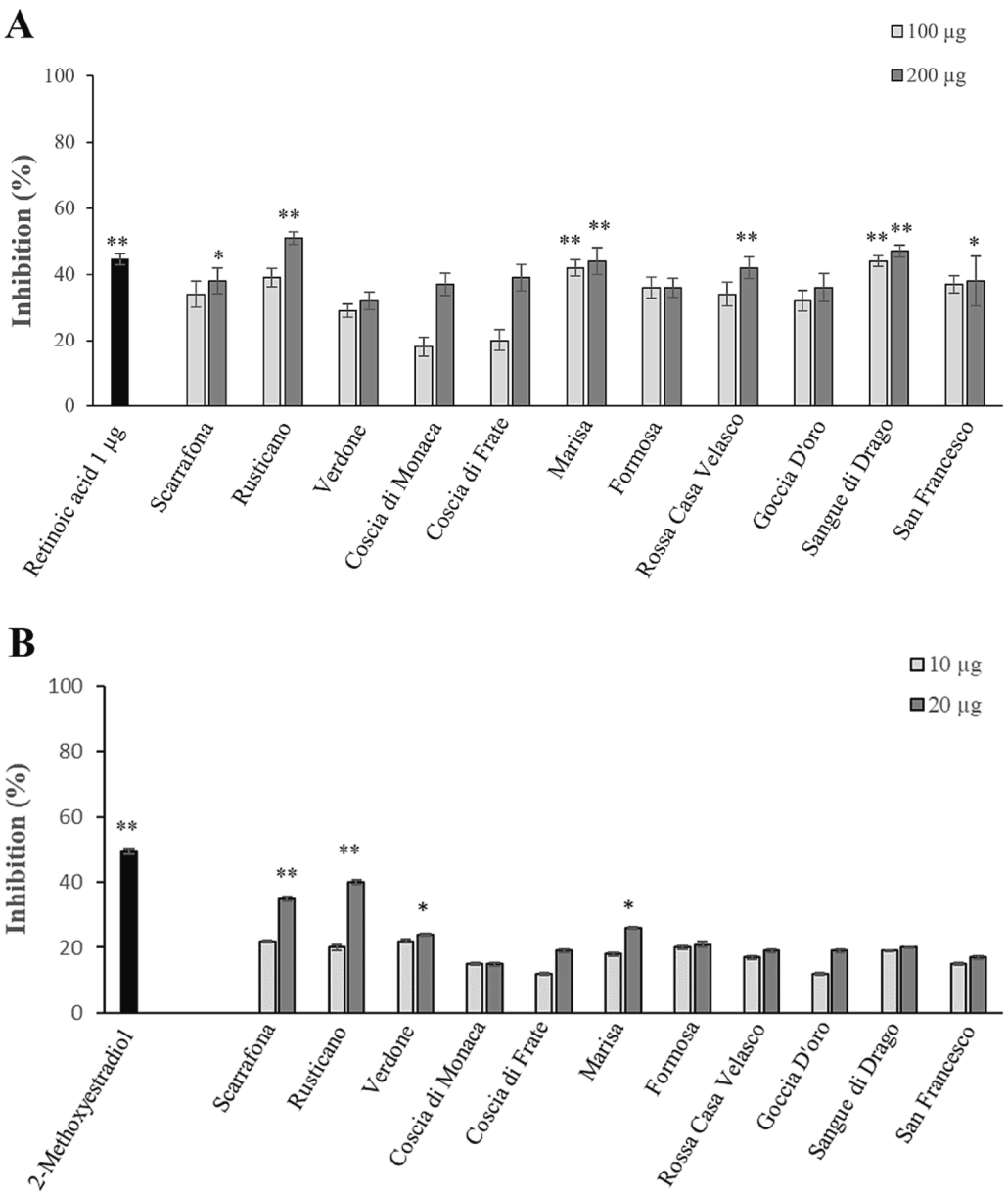

Fig. 2. Antiangiogenic activity of plum fruit extracts. **A**. Chorioallantoic membrane (CAM) assay: retinoic acid (1 µg/egg) = positive control. Vehicle = Tris buffer (pH 7.4). Mean ± SD (n = 5)**. B**. Zebrafish embryo endogenous alkaline phosphatase (EAP) assay: 2-methoxyestradiol (2 µM) = positive control. Vehicle = DMSO (0.2 % v/v). Mean ± SD (n = 10). *p < 0.05; **p < 0.01 vs control: Student's *t*-test.

In particular, treatment with 'Scarrafona' and 'Rusticano', followed by 'Verdone' and 'Marisa' extracts induced a statistically significant inhibition in terms of EAP activity (24–40 %) with respect to control embryos, at the highest concentration tested (20 µg/embryo). Conversely, the lower dose (10 µg/embryo) showed mild antiangiogenic effects. The antiangiogenic effects of plum fruit extracts were compared with the reference compound 2-methoxyestradiol (2 µM) which determined a significant reduction of EAP activity (49.5 %).

3.7. Bioinformatic analyses of chemical and biological data

While data related to metabolic and bioactivity attitude of the *Prunus* varieties allowed for the identification of the most promising genotypes in terms of metabolite accumulation and antioxidant and antiangiogenic properties, they do not enable performing a clear and simultaneous comparison of all the varieties and the available data, as well as to assess the singular and total contribution of the detected compounds with the observed bioactivities.

In order to overcome these limits, a series of bioinformatics approaches was used spanning from multivariate PCA to Pearson correlation algorithm/coefficients (HCL, symmetric correlation matrix, and correlation networks). First of all, PCAs was applied to the chemical dataset, according either the varieties and the metabolites (Fig. 2S-A and B); whereas in the case of the former it was not possible to obtain a clear separation of the different varieties (with the notable exception of 'Rossa Casa Velasco' according component; Fig. 2S-A), an unambiguous discriminative role of the total amount for phenols and anthocyanins was found by using PCA on the metabolites (Fig. 2S-B). Interestingly, cyanidin glucoside was the only compound contributing to the total variance, placing farther from the others according both component 1 and 2 (Fig. 2S-B). PCA was also exploited to evaluate differences at variety level in terms of bioassays data (Fig. 2S-C): similarly to chemical data, no variety resulted highly discriminant, although 'Rossa Casa Velasco' and 'Marisa' were separated according component 1 and 2,

respectively, in the PCA for antioxidant activity (Fig. 2S-C); whereas 'Rusticano', 'Sangue di Drago', 'Coscia di Frate', and 'Coscia di Monaca' positioned distinctly compared to the others (Fig. 2S-D). Since PCA did not enable a satisfying separation of the different varieties, a heat-map coupled to HCL approach was used for chemical and biological data; in this way, it was possible to identify three distinct groups composed by (from the farthest to the closest): (i) 'Rossa Casa Velasco' and 'Marisa', (ii) 'Coscia di Frate', 'Scarrafona', and 'Rusticano', and (iii) the rest of the varieties, with 'San Francesco' and 'Verdone' constituting an additional sub-group (Fig. 3A). On the contrary, a partially divergent attitude at bioactivity level was observed: indeed, 'Coscia di Frate' and 'Marisa' constituted a sub-cluster for the antioxidant data, whereas 'Rusticano', 'Coscia di Monaca', and the former were the varieties clustering together and far from the others in the antiangiogenic results (Fig. 3B and C, respectively). Once assessed a hierarchical-based classification of the varieties, our interest shifted to the investigation of the relationships of the phenylpropanoid metabolism in the *Prunus* varieties at metabolite-metabolite levels. At this aim, Pearson correlation-based analyses was exploited to assess metabolite-metabolite relationships and to highlight the metabolite/s potentially most responsible for the detected bioactivities. More in depht, symmetric metabolite-metabolite correlation matrix was generated, by classifying the different compounds according to the chemical sub-classes, and ordering them either on rows and columns (Fig. 3S). Of interest, a great extent of positive

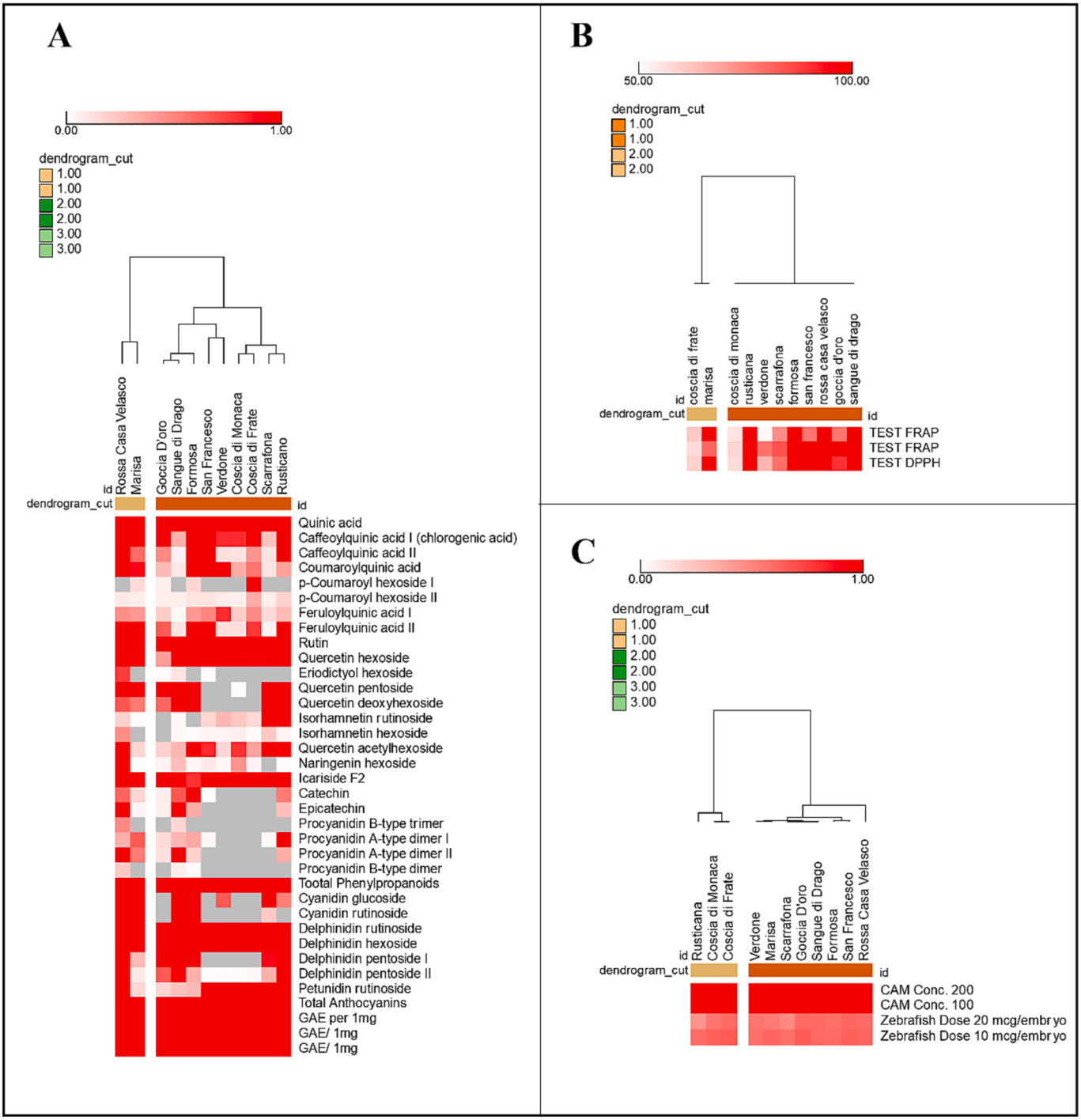

Fig. 3. Hierarchical Clustering (HCL) of phytochemical (A), antioxidant (B), and antiangiogenic (C) data of *Prunus* fruit varieties.

correlations was observed at metabolite sub-class levels (phenolic acids, flavonoids, procyanidins, and anthocyanins) as well as between compounds belonging to different sub-classes; at the opposite, and as notable exception, slight or more significant negative correlations were found between phenolic acids and all the other phenylpropanoid classes under investigation (Fig. 3S). The great level of metabolic coordination, as proved by the presence of the majority of positive correlations occurring in the dataset under study is in agreement with a series of previous studies, demostrating that metabolites in the same pathway, including phenylpropanoids, are generated according a very tight and coordinate way at different levels, ranging by gene expression to post-transcriptional and translational modifications until enzymatic controls (Chezem, & Clay, 2016; Hartline, Schmitz, Han, & Zhang, 2021). The only exception, represented by the phenolic acids and derivatives sub-class resulting negatively correlated with the other chemical classes, might find an explication with the fact that most of the compounds in

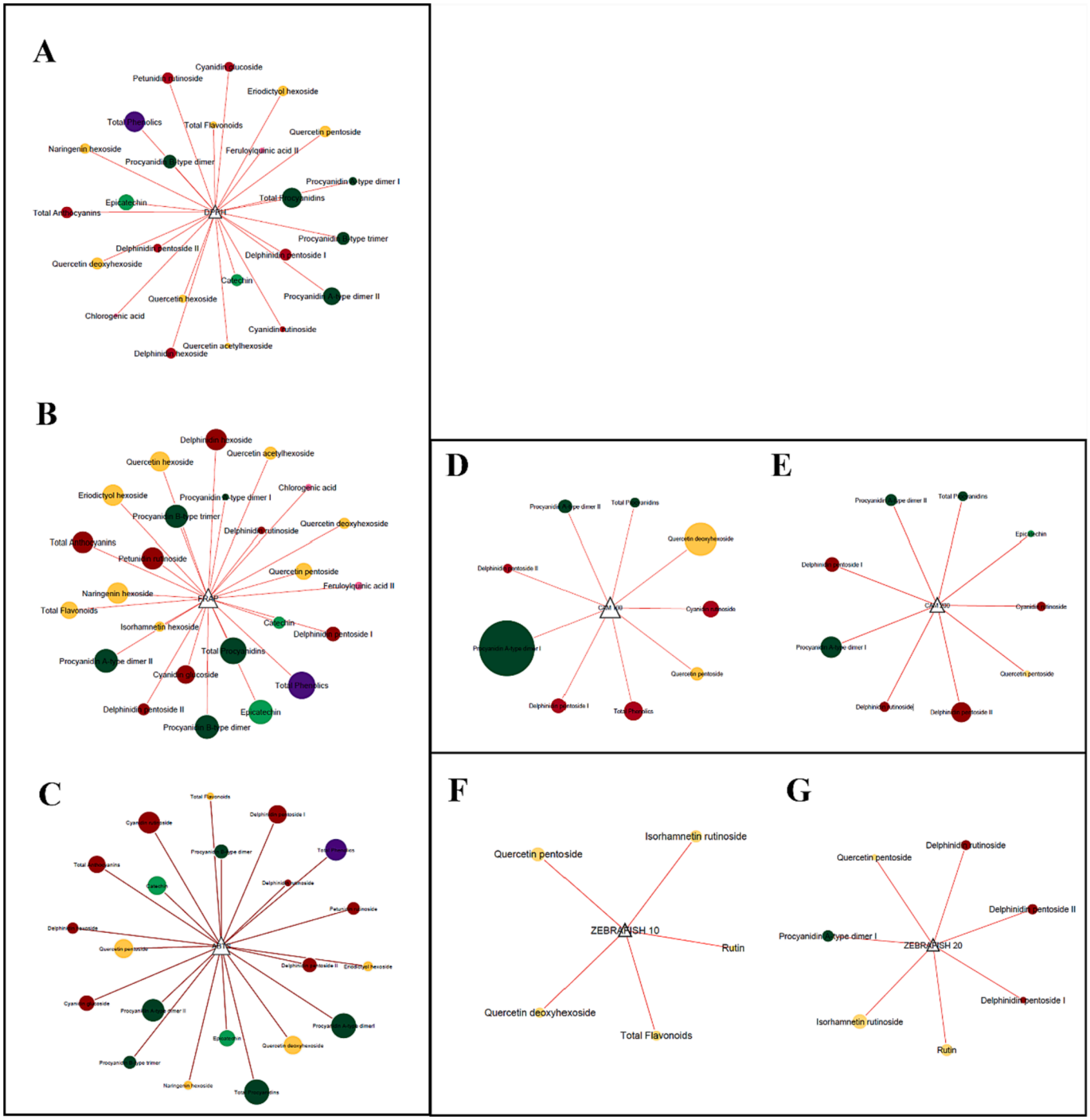

Fig. 4. Targeted correlation networks of antioxidant (A-B-C), antiangiogenic CAM 100–200 μg/egg (D-E, respectively) and zebrafish 10–20 μg/embryo (F-G, respectively) data of *Prunus* fruit extracts, with tests and different concentration points superimposed as singular central nodes. Nodes size is according the node strength (ns). Node color refers to different phenylpropanoid sub-classes (pink: phenolic acids and derivatives; yellow: flavonoids; green; procyanidins; dark red: anthocyanins; violet: total phenolics. Lines joining the nodes represent positive (red) and negative (blue) correlations, of width proportional to each corresponding |ρ|. Distances from the central hubs (equal to edge length) are inversely proportional to |ρ|. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

this group act early in the general pathway and can represent precursors of metabolites in the other, downstream, sub-classes. This hyopthesis is in agreement with previous studies showing differential accumulation between early and late metabolic products in the phenylpropanoid pathway (Li et al., 2018; Saha et al., 2021).

Finally, network correlation analyses were exploited to identify the metabolites mostly involved in the antioxidant and antiangiogenic properties of the *Prunus* extracts (Figs. 4 and 4S). Notably, either single network with the activity of interest placed as central "hub", as well as cumulative networks for each of the two bioactivities under study (antioxidant: DPPH + FRAP + ABTS; antiangiogenic: CAM + zebrafish at both tested concentrations) were performed (Figs. 4 and 4S, respectively). In addition, node strength (ns), defined as the average of all the Pearson correlation coefficients (ρ) each node owes with all the others, was calculated, as index to evidence the "strongest" nodes in the dataset under investigation. In this way, it was possible to identify metabolites which specifically correlated with a test and/or a concentration (e.g. isorhamnetin hexoside with FRAP; *p*-coumaroyl hexoside in CAM at 100 μg/egg; catechin in zebrafish at 10 μg/embryo). These variabilities are not surprising, since it is known the occurrence of specificity of a test or a concentration in the generation or the absence of a bioactivity (Apak et al., 2007). However, these analyses also allowed for revealing metabolites consistently and significantly correlated with different tests for the same property (antioxidant) or at both tested concentrations (CAM and zebrafish). In order to better highlight these compounds, cumulative networks were produced and analyzed (Fig. 4S). Thus, it was possible to identify a large group of flavonoids (eriodictyol hexoside, quercetin pentoside, quercetin deoxyhexoside), procyanidins (catechin and epicatechin, procyanidin B-type trimer, procyanidin A-type dimer I and II, procyanidin B-type dimer, and total procyanidins) and anthocyanins (cyanidin glucoside, delphinidin hexoside, delphinidin pentoside I and II, petunidin rutinoside, and total anthocyanins), together with total phenolics, being significantly correlated with all the three antioxidant tests (DPPH, FRAP, and ABTS) (Fig. 4S-A). Similarly, quinic acid and icariside F_2 resulted positively correlated with CAM at both 100 and 200 μg/egg; whereas, on the contrary, no metabolites being correlated with zebrafish at 10 and 20 μg/embryos were observed (Fig. 4S-B). The analysis of the ns' enabled, ultimately, to evidence higher contribution of total procyanidins and phenolics (0.85 and 0.83, respectively), followed by procyanidin A-type dimer II (0.77) and epicatechin (0.71) regarding the antioxidant activity network; and of quinic acid, procyanidin A-type dimer, and icariside F_2 (0.53, 0.52 and 0.44, respectively) for the antiangiogenic activity network. Although at mathematical-based level, these analyses provide clues about the involvement of these compounds in the detected bioactivities, also supported by other studies which confirm the strong antioxidant and antiangiogenic properties of these molecules, acting as single or as total (phytocomplex) levels (De Leo et al., 2021; Navarro et al., 2018; Wang et al., 2022).

4. Conclusions

The phytochemical and biological investigation of eleven plum fruits varieties herein reported highlighted that all the studied extracts were rich in bioactive phenolic constituents being a potential alternative to the commonly commercial ones. LC-HR-MS results showed a moderate extent of changes at metabolite level within the variety collection under study, in the absence, up to date, of corresponding genetic characterization able to more adequately infer the genotype- and genotype × environment-specific alterations. For this reason, a series of bioinformatic analyses was applied aimed to indicate the relationships within the different varieties; both PCA and HCL highlighted 'Rossa Casa Velasco' and 'Marisa' as the varieties which mostly diverged by the others. In addition, a high coordination of phenylpropanoid metabolism was found, as revealed by metabolite-metabolite correlation matrix, whereas correlation network analyses suggested the detected antioxidant and antiangiogenic properties might be due to the activity of a group of compounds acting alone (e.g. quinic acid, procyanidin A-type dimer, and icariside F_2 for the antiangiogenic activity) or as phytocomplex (in the case of the antioxidant activity).

Presently, increasing world population and the importance of varying diet are crucial points for the future of the agri-food chain. To answer to this growing request more diversified agricultural systems and cultivars could be implemented. The underutilized and neglected vegetable and/or fruit varieties with potential to improve the food security and population health and also to enhance biodiversity should be a good source. In general, the underexploited fruits and vegetables could give the possibility to diversify the agri-food system and to make the production in agricultural chain less responsive to the climate changes as these plants generally showed to be hardy and resilient to these changes, being able to grow in soil poor of nutrients and water and to overcome new and stressed climate conditions. The implementation of the varieties under study to expand plum branch, to help local farmers, and also to strengthen traditional food factories, presently has never received attention due to the absence of data on fruits quality. The phytochemical and biological studies of these ancient varieties could be crucial for the new plum cultivars selection and to extend their cultivation in the agri-food chain. Meanwhile, the multidisciplinary approach used in this study, including chemical, biological, and bioinformatic datasets obtained by reproducible and affordable methods could be useful in food research to deeply investigate edible plant sources particularly when characterized by a rich biodiversity. Furthermore, the potential properties as antioxidant and antiangiogenic agents make these fruits a valuable source of health-promoting substances to counteract oxidative stress related conditions.

Finally, our results provide evidence to overcome the loss of plums biodiversity through action oriented on the advancement of new fruit products and biodiversity-centered agri-food strategies.

CRediT authorship contribution statement

Emily Cioni: Formal analysis, Investigation, Data curation. **Marinella De Leo:** Data curation, Writing – original draft, Methodology, Validation. **Anna Cacciola:** Investigation, Methodology. **Valeria D'Angelo:** Formal analysis, Data curation. **Maria Paola Germanò:** Conceptualization, Writing – review & editing. **Fabiano Camangi:** Resources, Methodology. **Dorotea Ricci:** Software, Investigation, Visualization. **Eleonora Fabene:** Validation, Investigation, Visualization. **Gianfranco Diretto:** Conceptualization, Data curation, Writing – original draft. **Nunziatina De Tommasi:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **Alessandra Braca:** Funding acquisition, Methodology, Project administration, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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"Potenziamento strutture di ricerca e creazione di campioni nazionali di R&S su alcune Key Enabling Technologies" UE – Next Generation EU and to National Center 2 "AgriTech" (code CN00000022).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.137574.

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