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Phenolics of *Arbutus unedo* L. (Ericaceae) Fruits: Identification of Anthocyanins and Gallic Acid Derivatives

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Arbutus unedo L., the strawberry tree (Ericaceae family), is an evergreen shrub or small tree, typical of the Mediterranean fringe and climate. The aim of the present study was to evaluate the profile of the phenolic constituents of *A. unedo* fruits. Seven compounds were purified by Sephadex LH-20 column chromatography of the MeOH extract followed by HPLC and were characterized as arbutin, β-D-glucogalline, gallic acid 4-*O*-β-D-glucopyranoside, 3-*O*-galloylquinic acid, 5-*O*-galloylquinic acid, 3-*O*-galloylshikimic acid, and 5-*O*-galloylshikimic acid, by means of NMR and ESI-MS analyses. Moreover, LC-PDA-MS analysis of the red pigment of *A. unedo* fruits revealed the presence of three anthocyanins recognized as cyanidin 3-*O*-β-D-galactopyranoside, delphinidin 3-*O*-β-D-glucopyranoside, and cyanidin 3-*O*-β-D-arabinopyranoside. These pigments were also quantified.

KEYWORDS: ***Arbutus unedo*; anthocyanins; gallic acid derivatives; HPLC; ESI-MS**

INTRODUCTION

Plant polyphenols are a wide group of secondary metabolites that are a common component of our diet and can range from simple molecules, such as phenolic acids, to highly polymerized compounds such as tannins. During recent years, there has been a growing interest in plant phenols due to their wide range of biological effects, including antioxidant, antiinflammatory, antiallergic, and antibacterial properties. Antioxidant phenolics have been suggested to play a preventive role in the development of cancer and heart diseases. In fact, epidemiological studies have proved that there is a significant correlation between increased consumption of fruits and vegetables and reduced risk of cardiovascular diseases (*1*−*3*) and certain types of cancer (*4*).

Anthocyanins are one group of widespread natural phenolic compounds present in many flowers, fruits, and vegetables and are responsible for their orange, red, and blue color (*5*). They are nontoxic, water-soluble compounds and are of great interest in nutrition and medicine because of their potent antioxidant capacity and possible protective effects on human health (*6*). Recently, there has been increased interest in the development of colorants from natural sources to replace synthetic pigments by natural ones. Thus, new sources of these compounds are now desired.

Arbutus unedo L., the strawberry tree (Ericaceae family), is a native Mediterranean species but is also cultivated in other regions such as the Near East and Transcaucasia (*7*, *8*). The plant is an evergreen, ornamental shrub or tree, usually smaller than 4 m (*9*). Its fruits are spherical, about 2 cm in diameter, dark red, and tasty only when they fully ripen in autumn. The arbutus berries are rarely eaten as fresh fruits but have some importance in local agricultural economies that use them for the production of highly appreciated alcoholic beverages such as wines, liquors, and brandies. Other food applications such as preserves, jams, jellies, and marmalades (*8*) can also be obtained from strawberry tree fruits. It is also possible to incorporate the berries into yogurts and use as other fruits in confectionaries for pie and pastry fillings, cereals products, etc. *A. unedo* fruits are also well-known in folk medicine as antiseptics, diuretics, and laxatives, while the leaves of the plant are used as diuretic, urinary antiseptic, antidiarrheal, astringent, depurative, and antihypertensive (*10*).

Previous phytochemical studies on the plant showed the presence of three anthocyanins: delphinidin 3-*O*-galactoside, cyanidin 3-*O*-galactoglucoside, and cyanidin 3-*O*-galactoside (*11*), sugars, vitamins, organic acids, and some phenolic compounds (*12*, *13*). Strawberry tree fruits showed a good antioxidant activity in TEAC test as reported by García-Alonso et al. in 2004 (*14*). The purpose of the present study was to evaluate for the first time the profile of phenolic derivatives of *A. unedo* berries, in order to draw attention to this species and to contribute to the improvement of the potential value of this minor fruit as food.

MATERIALS AND METHODS

Standards and Solvents. Standards of cyanidin 3-*O*-glucoside and delphinidin 3-*O*-galactoside high-performance liquid chromatography (HPLC) grade were obtained from Extrasynthèse (Genay, France). The standard of cyanidin 3-*O*-arabinoside was kindly provided by Indena, Italy. HPLC grade methanol (MeOH) and formic acid (HCOOH) were purchased from J. T. Baker (Baker Mallinckrodt, Phillipsburg, NJ). HPLC grade water (18 mΩ) was prepared by a Mill-Ω^{50} purification

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system (Millipore Corp., Bedford, MA). Hydrochloric acid (37% HCl) was purchased from Carlo Erba (Carlo Erba Reagenti SpA, Rodano, Italy).

General Experimental Procedures. A Bruker AC200 NMR spectrometer was used for NMR experiments; chemical shifts are expressed in δ (parts per million) referenced to the solvent peaks δ_H 3.34 and δ_C 49.0 for CD_3OD. Column chromatography was performed over Sephadex LH-20 (Pharmacia, Uppsala, Sweden); HPLC separations were conducted on a Shimadzu LC-8A (Shimadzu Corp., Kyoto, Japan) series pumping system equipped with a Waters R401 refractive index detector and Shimadzu injector with a Waters μ-Bondapak C_{18} column (Waters, Milford, MA). Thin-layer chromatography (TLC) was performed on precoated Kieselgel 60 F_{254} plates (Merck, Darmstadt, Germany).

Plant Material. *Arbutus unedo* L. (Ericaceae) fruits were collected in Marina di Vecchiano, Pisa, Italy, in November 2004. A voucher specimen (N. 6221 *Arbutus unedo*/28) was deposited in Nuove Acqusizioni at Herbarium Horti Botanici Pisani, Pisa, Italy.

HPLC-PDA-ESI-MS Analyses of Anthocyanins. *Qualitative Analyses.* HPLC-PDA-ESI-MS analyses were performed using a Surveyor LC pump, a Surveyor autosampler, coupled with a Surveyor PDA detector, and a LCQ Advantage ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with Xcalibur 3.1 software. Analyses were performed using a 4.6 × 250 mm, 4 μm, Synergi POLAR-RP 80A column (Phenomenex Corp., Torrance, CA). The eluent was a mixture of 0.5% methanolic solution of HCOOH (solvent A) and 0.5% aqueous solution of HCOOH (solvent B). The solvent gradient was as follows: 0−20 min, 35−90% (A). Elution was performed at flow rate of 1.0 mL/min with a splitting system of 2:8 to MS detector (200 μL/min) and PDA detector (800 μL/min), respectively. The volume of the injection was 20 μL. Analyses were performed with an ESI interface in the positive mode. The ionization conditions were optimized, and the parameters used were as follows: capillary temperature, 280 °C; capillary voltage, 11.00 V; tube lens offset, 15.00 V; sheath gas flow rate, 60.00 arbitrary units; auxiliary gas flow rate, 6.00 arbitrary units; spray voltage, 3.50 kV; scan range of *m*/*z* 300−700. N_2 was used as the sheath and auxiliary gas. PDA data were recorded with 220−600 nm range with a preferential channel of 525 nm as the detection wavelength.

Quantitative Analyses. HPLC-PDA analyses were performed using a Waters 600E multisolvent delivery system, a Waters 717plus autosampler, and a Waters 996 PDA detector (Waters, Milford, MA) equipped with Millennium^{32} Chromatography Manager software. The experimental conditions (solvent gradient, PDA channel, column) were the same as described above. The volume of the injection was 10 μL.

Extraction and Identification of Anthocyanins. Ten grams of fresh fruits of *A. unedo* was homogenized in 30 mL of 2% methanolic solution of HCl. The solution was filtered on a Buchner funnel, and the filtrate was used for HPLC analyses. The anthocyanins present in the extract were identified by comparison of their retention times and mass fragmentation pattern with the pure compounds.

Calibration, Quantification, and Statistical Analysis of Anthocyanins. Cyanidin 3-*O*-glucoside was selected as the external standard of calibration for anthocyanins. Standard curve calibration was prepared in a concentration range 0.00125−0.05 mg/mL of standard dissolved in 2% methanolic solution of HCl, with six different concentration levels (0.00125, 0.0025, 0.005, 0.0125, 0.025, 0.05 mg/mL). Triplicate injections were made for each level, and a weighed linear regression was generated. The curve of calibration with the external standard was obtained using concentration (mg/mL) with respect to the area obtained from the integration of the PDA peaks. The relation between variables was analyzed using linear simple correlation. For the linear regression of the external standard, R^2 was 0.9996. For the quantification of the compounds, a GraphPad Software Prism 3.0 was used.

Extraction, Isolation, and Identification of Other Phenolics. Fresh fruits of *A. unedo* (1.5 kg) were lyophilized, defatted at room temperature with *n*-hexane, and extracted with MeOH by exhaustive maceration (5 × 500 mL) to yield 189 g of residue which was successively dissolved in water and partitioned with EtOAc and *n*-BuOH. The dried butanolic extract (2.5 g) was subjected to fractionation on a Sephadex LH-20 column, using MeOH as eluent at a flow rate 0.8 mL/min; 88 fractions of 8 mL were collected and grouped into six major fractions (A−F) by TLC analysis on silica 60 F_{254} gel-coated glass sheets developed with *n*-BuOH−AcOH−H_2O (60:15:25) as eluent. Fractions B (70.2 mg), C (94.6 mg), and F (58.7 mg) were separately purified by RP-HPLC on a 300 mm × 7.8 mm i.d., C_{18} μ-Bondapak column at a flow rate at 2.0 mL/min with MeOH−H_2O (1:9) for fractions B and C, and MeOH−H_2O (5:95) for fraction F, to afford compounds **4** (4.6 mg, t_R = 10 min), **5** (1.4 mg, t_R = 12 min), **6** (6.6 mg, t_R = 16 min), and **7** (0.8 mg, t_R = 18 min) from fraction B, compounds **5** (3 mg, t_R = 8 min), **8** (4.1 mg, t_R = 9 min), and **6** (29.3 mg, t_R = 22 min) from fraction C, and compounds **8** (1.5 mg, t_R = 7 min), **9** (2.3 mg, t_R = 9 min), and **10** (5.3 mg, t_R = 10 min) from fraction F, respectively.

Arbutin (**4**) was obtained as a white amorphous powder. ESI-MS, *m*/*z* 271 $[M - H]^-$. ^{1}H NMR (CD_3OD, 200 MHz): δ 4.73 (1H, d, J = 7.3 Hz, H-1′), 6.69 (2H, d, J = 8.9 Hz, H-3 and H-5), 6.97 (2H, d, J = 8.9 Hz, H-2 and H-6). ^{13}C NMR (CD_3OD, 200 MHz): δ 62.6 (C-6′), 71.5 (C-4′), 75.0 (C-2′), 78.1 (C-3′ and C-5′), 103.0 (C-1′), 116.6 (C-2 and C-6), 118.6 (C-3 and C-5), 152.5 (C-1), 153.8 (C-4) (*15*).

β-D-Glucogalline (**5**) was obtained as a pale yellow amorphous powder. ESI-MS, *m*/*z* 331 $[M - H]^-$. ^{1}H NMR (CD_3OD, 200 MHz): δ 5.60 (1H, d, J = 7.5 Hz, H-1′), 7.12 (2H, s, H-2 and H-6). ^{13}C NMR (CD_3OD, 200 MHz): δ 62.4 (C-6′), 71.1 (C-4′), 74.1 (C-2′), 78.2 (C-3′), 78.8 (C-5′), 96.0 (C-1′), 110.6 (C-2 and C-6), 120.8 (C-1), 140.4 (C-4), 146.5 (C-3 and C-5), 167.1 (C-7) (*16*).

3-*O*-Galloylquinic acid (**6**) was obtained as a brownish amorphous powder. ESI-MS, *m*/*z* 343 $[M - H]^-$. ^{1}H NMR (CD_3OD, 200 MHz): δ 1.95−2.30 (4H, m, H-2 and H-6), 3.78 (1H, dd, J = 7.8 and 3.0 Hz, H-4), 4.20 (1H, m, H-5), 5.40 (1H, m, H-3), 7.06 (2H, s, H-2′ and H-6′). ^{13}C NMR (CD_3OD, 200 MHz): δ 38.3 (C-6), 38.9 (C-2), 71.5 (C-5), 72.4 (C-4), 73.6 (C-3), 76.5 (C-1), 110.3 (C-2′ and C-6′), 121.1 (C-1′), 139.8 (C-4′), 146.4 (C-3′ and C-5′), 167.4 (C-7′), 178.0 (C-7) (*17*).

Gallic acid 4-*O*-β-D-glucopyranoside (**7**) was obtained as a brownish amorphous powder. ESI-MS, *m*/*z* 331 $[M - H]^-$. ^{1}H NMR (CD_3OD, 200 MHz): δ 4.68 (1H, d, J = 7.8 Hz, H-1), 7.06 (2H, s, H-2 and H-6). ^{13}C NMR (CD_3OD, 200 MHz): δ 62.0 (C-6′), 70.7 (C-4′), 75.1 (C-2′), 77.4 (C-3′), 78.6 (C-5′), 106.4 (C-1′), 110.4 (C-2 and C-6), 122.0 (C-1), 141.8 (C-4), 145.4 (C-3 and C-5), 175.0 (C-7) (*18*).

5-*O*-Galloylquinic acid (**8**) was obtained as a brownish amorphous powder. ESI-MS, *m*/*z* 343 $[M - H]^-$. ^{1}H NMR (CD_3OD, 200 MHz): δ 1.90−2.25 (4H, m, H-2 and H-6), 3.74 (1H, dd, J = 9.5 and 3.5 Hz, H-4), 4.13 (1H, m, H-3), 5.40 (1H, m, H-5), 7.09 (2H, s, H-2′ and H-6′) (*17*).

5-*O*-Galloylshikimic acid (**9**) was obtained as a brownish amorphous powder. ESI-MS, *m*/*z* 325 $[M - H]^-$. ^{1}H NMR (CD_3OD, 200 MHz): δ 2.17 (1H, dd, J = 13.0 and 5.5 Hz, H-2_a), 2.82 (1H, dd, J = 13.0 and 4.5 Hz, H-2_b), 5.65 (1H, t, J = 4.0 Hz, H-5), 6.53 (1H, d, J = 4.0 Hz, H-6), 7.05 (2H, s, H-2′ and H-6′) (*19*).

3-*O*-Galloylshikimic acid (**10**) was obtained as a brownish amorphous powder. ESI-MS, *m*/*z* 325 $[M - H]^-$. ^{1}H NMR (CD_3OD, 200 MHz): δ 2.37 (1H, dd, J = 13.0 and 6.0 Hz, H-2_a), 2.91 (1H, dd, J = 13.0 and 5.0 Hz, H-2_b), 3.87 (1H, dd, J = 8.0 and 4.0 Hz, H-4), 4.37 (1H, t, J = 4.0 Hz, H-5), 5.31 (1H, m, H-3), 6.57 (1H, d, J = 4.0 Hz, H-6), 7.07 (2H, s, H-2′ and H-6′) (*20*).

RESULTS AND DISCUSSION

Qualitative−Quantitative Anthocyanins Profile. The anthocyanin profile of strawberry tree berries was carried out by means of HPLC-PDA-ESI-MS analyses. The chromatogram of the anthocyanins extract, recorded at 520 nm, is shown in **Figure 1**. As can be seen, there are three peaks (**1**, **2**, **3**) in the chromatogram and all of them were identified as anthocyanins (**Figure 2**) by comparison of their HPLC retention times, elution orders, ESI-MS spectrometric data, and photodiode array PDA/UV/vis with anthocyanin standards (**Table 1**). Peak **1** was identified as delphinidin 3-*O*-galactoside with a λ_{max} 535 nm and a mass spectrum consisting of a $[M]^+$ at *m*/*z* 465 and a

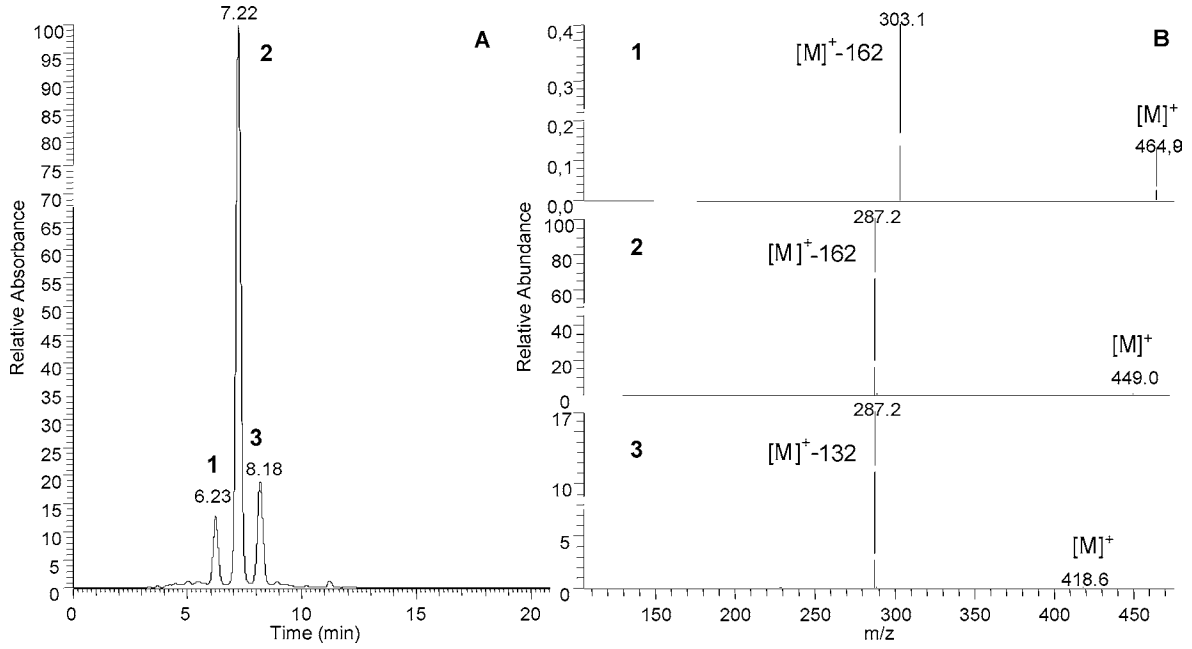

Figure 1. (**A**) HPLC-PDA-ESI-MS chromatogram recorded at 520 nm corresponding to the anthocyanins extract of *Arbutus unedo* fruits and (**B**) MS/MS fragmentation of parent ions **1**, **2**, **3**.

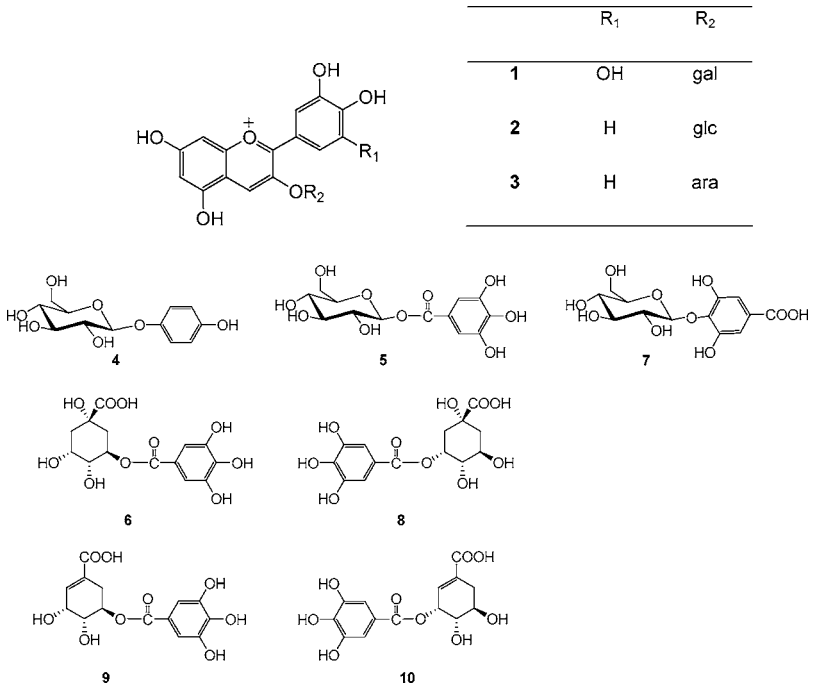

Figure 2. Chemical structures of the anthocyanins identified in *Arbutus unedo* fruits. gal = galactose, glc = glucose, ara = arabinose and structures of the isolated phenolic compounds.

Table 1. Chromatographic, Spectroscopic, and Spectrometric Characteristics and Quantitative Amount (mg/100 g of Fresh Fruits) of the Anthocyanins Found in *Arbutus unedo* Fruits

peak no. (in **Figure 1**)	t_R (min)	λ_{max} (nm)	$[M]^+$ (*m/z*)	$[M]^+$ – sugar (*m/z*)	amount	peak assignment
1	6.23	535	465	303	0.044	delphinidin 3-*O*-galactoside
2	7.22	520	449	287	0.39	cyanidin 3-*O*-glucoside
3	8.18	520	419	287	0.073	cyanidin 3-*O*-arabinoside

fragment ion at *m/z* 303 ($[M]^+ - 162$) resulting from the loss of the galactose moiety. The major peak **2** was recognized as cyanidin 3-*O*-glucoside and presented a λ_{max} at 520 nm and a mass spectrum comprising the $[M]^+$ at *m/z* 449 and a fragment ion at *m/z* 287 ($[M]^+ - 162$) resulting from the loss of the glucose molecule. Peak **3**, identified as cyanidin 3-*O*-arabinoside, had a λ_{max} at 520 nm and presented a mass spectrum $[M]^+$ at *m/z* 419 with a fragment ion at *m/z* 287 ($[M]^+ - 132$) resulting from the loss of the arabinose residue. The $abs_{440}/abs_{\lambda max}$ ratio values calculated for each anthocyanin indicated a substitution in the C-3 position of the flavylium ring (*21*). It is well-known that anthohocyanins with glycosidic substitution at position 3 exhibit a ratio of the absorbance at 400−440 nm to the absorbance at the visible maximum wavelength (520 nm) almost twice that of anthocyanins with glycosidic substitution at position 5 or both 3 and 5 (*22*). Our results of the anthocyanin profile of *A. unedo* fruits are not completely in accordance with

those reported by Maccarone et al. (*11*). Only the presence of delphinidin 3-*O*-galactoside is in agreement with the latter analysis. The coupling of HPLC techniques ESI-MS and MS/MS analyses was useful for the unambiguous identification of the detected anthocyanins. The total amount of anthocyanins (**Table 1**) in *A. unedo* fruits, determined on the cyanidin 3-*O*-glucoside basis, was 0.51 mg/100 g of fresh berries. Cyanidin 3-*O*-glucoside was the predominant anthocyanin (76.9%), followed by cyanidin 3-*O*-arabinoside (14.4%). Delphinidin 3-*O*-galactoside was the least abundant anthocyanin (8.7%).

Gallic Acid Derivatives. The fractionation of the methanolic extract (see Materials and Methods) afforded seven pure compounds that were characterized as arbutin (**4**), β-D-glucogalline (**5**), 3-*O*-galloylquinic acid (**6**), gallic acid 4-*O*-β-D-glucopyranoside (**7**), 5-*O*-galloylquinic acid (**8**), 5-*O*-galloylshikimic acid (**9**), and 3-*O*-galloylshikimic acid (**10**) (**Figure 2**). The structures of the isolated compounds were established by ESI-MS, ^{1}H, and ^{13}C NMR data and confirmed by comparison with those reported in the literature (*14*−*19*). Gallic acid and its derivatives are widely distributed in the plant kingdom and represent a large family of plant secondary polyphenolic metabolites that are natural antioxidants. Esters of gallic acid have a diverse range of industrial uses: in food, in cosmetics, and in the pharmaceutical industries. In fact, they are used as food additives to prevent oxidation. Gallic acid derivatives have also been found in many phytomedicines with a number of biological and pharmalogical activities, including scavenging free radicals (*23*, *24*), inducing apoptosis of cancer cells (*25*), inhibiting squalene epoxidase (*26*), and interfering with the signal pathways involving Ca^{2+} and oxygen free radicals (*27*). In addition, gallic acid is employed as a source material for inks, paints, and color developers.

From our results it can be concluded that *A. unedo* berry, a so-called "minor fruit", is an appreciable source of phenolic constituents. Phenolic compounds identified in strawberry tree fruits belong to the classes of gallic acid derivatives and flavonoids, which are the most active as antioxidants (*28*). Gallic acid derivatives are the main constituents, but also quinic acids are present and their conversion to hippuric acid in the human body, which is thought to have antibacterial effects (*29*), may explain why *A. unedo* has proven useful in urinary infections (*30*). Taking also into the consideration the excellent cultivation of *A. unedo* and the great abundance in the Mediterranean region, its berries could become a potential source of health-promoting fruits in industrial use.

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