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Quali-quantitative Analyses of Flavonoids of *Morus nigra* L. and *Morus alba* L. (Moraceae) Fruits

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Morus nigra L., belonging to the Moraceae family, is a decidious tree widely cultivated in Europe and West Asia. It has a long history of medicinal use in Chinese medicine, as a remedy for many kinds of diseases. The aim of the present study was to evaluate the profile of the phenolic constituents of *M. nigra* fruits and to compare their content with the fruits of another species of *Morus*, *Morus alba*, which is also very well known in folklore medicine. The fruits of black and white mulberries have been studied, and five compounds from the methanol extract have been identified by means of HPLC/PDA/ESI-MS. Four compounds (quercetin 3-*O*-glucoside, quercetin 3-*O*-rutinoside, kaempferol 3-*O*-rutinoside, and 5-*O*-caffeoylquinic acid) have been isolated by use of Sephadex LH-20 column chromatography and HPLC and characterized by means of NMR and ESI-MS. Furthermore, HPLC/PDA/ESI-MS analysis of the red pigment of *M. nigra* fruits revealed the presence of four anthocyanins recognized as cyanidin 3-*O*-glucoside, cyanidin 3-*O*-rutinoside, pelargonidin 3-*O*-glucoside, and pelargonidin 3-*O*-rutinoside. All of the compounds were quantified.

KEYWORDS: *Morus nigra*; *Morus alba*; flavonoids; anthocyanins; HPLC; ESI-MS/MS

INTRODUCTION

Naturally occurring flavonoids are polyphenolic compounds present in a variety of fruits, vegetables, and seeds. They are important for humans not only because they contribute to plant color but also because they show many biological and pharmacological activities, including antioxidative, antiinflammatory, and antiviral effects (*1*). Therefore, many consumers and food manufacturers have become interested in flavonoids for their medicinal properties, especially their potential role in the prevention of cancers and cardiovascular disease (*2*). Anthocyanins, belonging to the group of flavonoids and being responsible for the orange, red, and blue colors of flowers, fruits, and vegetables (*3*), are viewed as natural colorants to replace synthetic ones. Thus, new sources of these compounds are nowadays desired.

Moraceae is a family of flowering plants that comprises about 40 genera and over 1000 species. Belonging to this family, *Morus* is a genus of 10–16 species of deciduous trees native to warm, temperate, and subtropical regions of Asia, Africa, North America, and southern Europe (*4*). Mulberry trees are fast-growing when young, but soon become slow-growing and rarely exceed 10–15 m tall. Their fruits are multiplied, 2–3 cm long. In several species, mulberries begin as white to pale yellow with pink edges, becoming red during ripening and dark purple to black when fully ripened (*5, 6*). In most mulberry-growing countries, especially in China and India, their production is focused on enhancing the foliage. The leaves, being the sole food source of the silkworm (*Bombyx mori* L.), the cocoon of which is used to make silk, have a big ecological and economical importance (*7*). In Europe, they are appreciated more for their fruits than for the foliage (*8*). However, almost all of the parts of the tree are used for pharmacological actions all over the world (*9*). The leaves have been shown to possess diuretic, hypoglycemic, and hypotensive activities (*10, 11*), whereas the root bark of mulberry trees has long been used for antiinflammatory, antitussive, and antipyretic purposes (*12*). Mulberry fruits can be used as a warming agent, as a remedy for dysentery, and as a tonic, sedative, laxative, odontalgic, anthelmintic, expectorant, and emetic (*13, 14*). In Italy, the berries of *Morus nigra* and *Morus alba* are consumed as the fresh fruit or in the form of various confectionary products such as jam, marmalade, frozen desserts, pulp, juice, paste, ice cream, and wine.

Previous phytochemical studies on the fruits of these *Morus* species showed the presence of fats and fatty acids, vitamin C, minerals, phenolics, and flavonoids (*15*) and, in the case of black mulberry fruits, some organic acids (*16*) and anthocyanins (*17*). *M. nigra* berries showed also a high antioxidant activity in the β-carotene bleaching method and against liposome oxidation as reported by Hassimotto et al. in 2005 (*18*). Moreover, the identification and quantification of anthocyanins and flavonoids of mulberry cultivars from China and Korea are mentioned (*19, 20*). The aim of the present work was to evaluate for the first time the HPLC flavonoid profile of *M. nigra* and *M. alba* fruits

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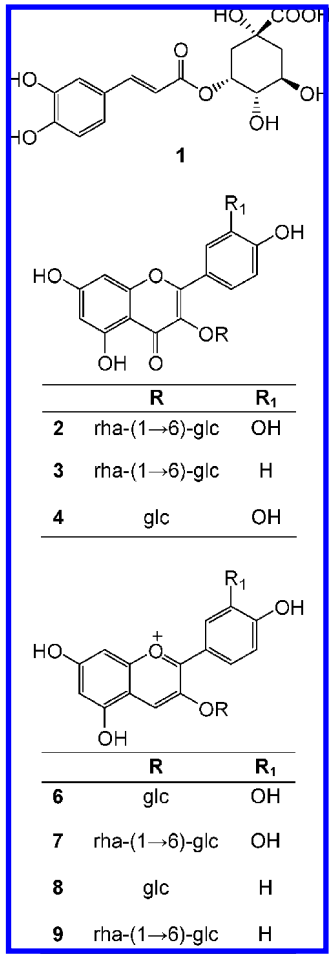

Figure 1. Chemical structures of phenolic compounds identified in *M. nigra* and *M. alba* fruits.

through a quali-quantitative comparative study in order to draw attention to these species and to contribute to the improvement of the potential value of these fruits as food.

MATERIALS AND METHODS

Standards and Solvents. The standard of quercetin 3-*O*-rutinoside was purchased from Merck (Darmstadt, Germany). Standards of cyanidin 3-*O*-glucoside and cyanidin 3-*O*-rutinoside were obtained from Extrasynthese (Genay, France). Standards of pelargonidin 3-*O*-glucoside and pelargonidin 3-*O*-rutinoside were kindly provided by Indena, Italy. HPLC grade acetonitrile (CH_3CN), acetic acid (CH_3COOH), and formic acid (HCOOH) were purchased from J. T. Baker (Baker Mallinckrodt, Phillipsburg, NJ). HPLC grade water (18 mΩ) was prepared by a Milli-Ω^{50} purification system (Millipore Corp., Bedford, MA).

General Experimental Procedures. An Avance Bruker 250 NMR spectrometer was used for NMR experiments. 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 a Shimadzu injector with a Waters μ-Bondapak C_{18} column (7.8 × 300 mm, 10 μm,Waters, Milford, MA). Thin-layer chromatography (TLC) was performed on precoated Kieselgel 60 F_{254} plates (Merck).

Plant Material. *M. nigra* L. and *M. alba* L. (Moraceae) fruits were collected in Benevento, Italy, in June 2005. The voucher specimens are deposited at Herbarium Horti Botanici Pisani, Pisa, Italy.

HPLC-PDA-ESI-MS Analyses. *Qualitative Analyses.* HPLC-PDA-ESI-MS analyses were performed using a Surveyor LC pump, a Surveyor autosampler, coupled with a Surveyor PDA detector, and an LCQ Advantage ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with Xcalibur 3.1 software. Analyses were performed using a 3.0 × 150 mm, 3.5 μm, Symmetry C_{18} column (Waters). In the case of flavonoid analyses, the eluent was a mixture of 0.1% acetonitrile solution of CH_3COOH (solvent A) and 0.1% aqueous solution of CH_3COOH (solvent B). The solvent gradient was as follows: 0–50 min, 8–40% (A). Elution was performed at a flow rate of 0.4 mL/min with a splitting system of 2:8 to MS detector (80 μL/min) and PDA detector (320 μL/min), respectively. Analyses were performed with an ESI interface in the negative mode. The ionization conditions were optimized, and the parameters were as follows: capillary temperature, 280 °C; capillary voltage, 50 V; tube lens offset, 40 V; sheath gas flow rate, 70 arbitrary units; auxiliary gas flow rate, 7 arbitrary units; spray voltage, 4.50 kV; scan range of *m*/*z* 200–800. PDA data were recorded over the 220–500 nm range with preferential channels 254 and 324 nm as the detection wavelengths. In the anthocyanin case, the eluent was a mixture of 1% acetonitrile solution of HCOOH (solvent A) and 1% aqueous solution of HCOOH (solvent B). The solvent gradient was as follows: 0–55 min, 5–50% (A). Elution was performed at a flow rate of 0.5 mL/min with a splitting system of 2:8 to

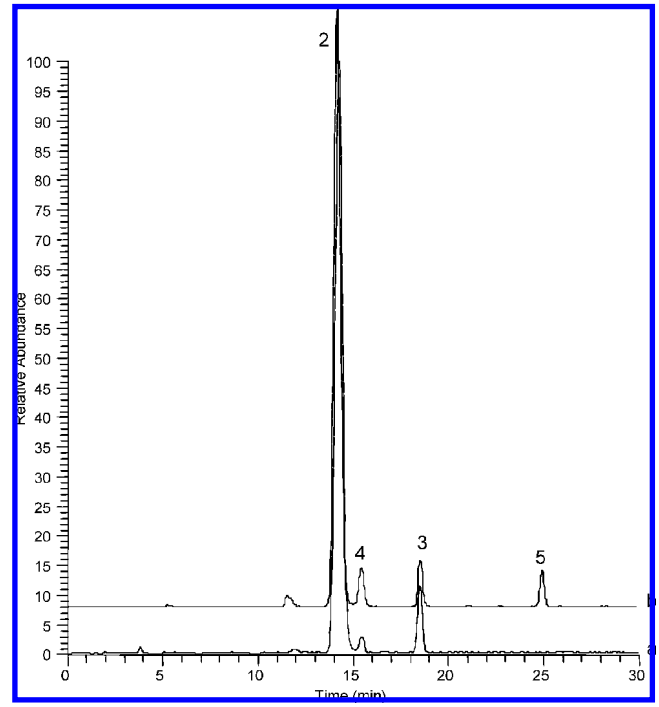

Figure 2. Qualitative HPLC-ESI-MS comparison between flavonoid glycosides of *M. nigra* (a) and *M. alba* (b) fruits.

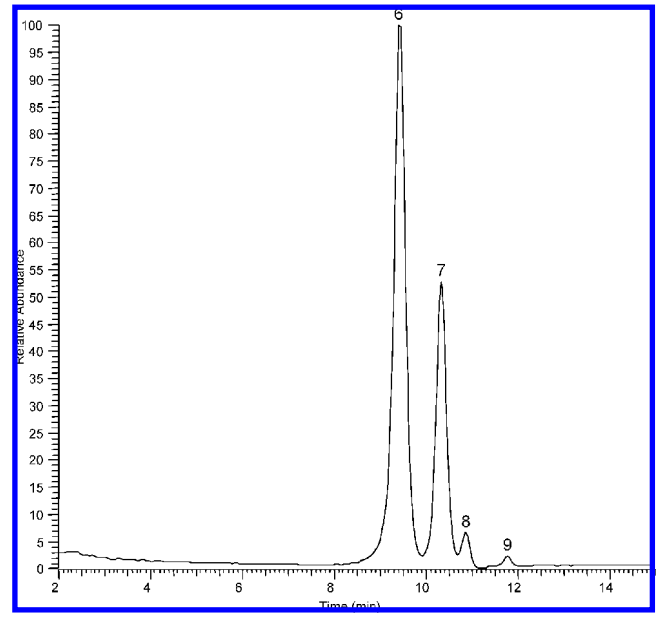

Figure 3. HPLC-PDA chromatogram recorded at 515 nm corresponding to the anthocyanin extract of *M. nigra* fruits.

Table 1. Chromatographic, Spectroscopic, and Spectrometric Data and Quantitative Amount of Flavonoids Found in *M. nigra* and *M. alba* Fruits

compound						amount[a] (mg/10 g of dried material)	
no.	name	t_R	$[M - H]^-$ (*m/z*)	MS/MS fragments (*m/z*)	λ_{max}	*M. nigra*	*M. alba*
2	quercetin 3-*O*-rutinoside	14.3	609	463, 343, 301	255, 355	32.9	12.7
3	kaempferol 3-*O*-rutinoside	18.6	593	447, 327, 285	265, 340	1.5	2.7
4	quercetin 3-*O*-glucoside	15.6	463	343, 301	255, 350	3.4	2.9
5	unidentified	25.0	505	463, 301	255, 305, 350		3.6

[a] Values are means ($n = 3$): the relative standard deviations for all compounds were <2%.

Table 2. Chromatographic, Spectroscopic, and Spectrometric Data and Quantitative Amount of Anthocyanins Identified in *M. nigra* Fruits

compound						
no.	name	t_R (min)	$[M + H]^+$ (*m/z*)	MS/MS fragments (*m/z*)	λ_{max}	amount[a] (mg/10 g of fresh fruits)
6	cyanidin 3-*O*-glucoside	9.4	449	287	280, 525	17.9
7	cyanidin 3-*O*-rutinoside	10.3	595	449, 287	275, 525	7.5
8	pelargonidin 3-*O*-glucoside	10.9	433	271	280, 500	1.2
9	pelargonidin 3-*O*-rutinoside	11.8	579	433, 271	280, 505	0.4

[a] Values are means ($n = 3$): the relative standard deviations for all compounds were <1%.

MS detector, respectively. Analyses were performed with an ESI interface in the positive mode. Cyanidin 3-*O*-glucoside was used to optimize the ionization and fragmentation conditions, and the parameters were as follows: capillary temperature, 220 °C; capillary voltage, 30 V; tube lens offset, 45 V; sheath gas flow rate, 55 arbitrary units; auxiliary gas flow rate, 3 arbitrary units; spray voltage, 4.50 kV; scan range of *m/z* 200–800. PDA data were recorded over the 220–600 nm range with a preferential channel 515 nm as the detection wavelength. In both cases N_2 was used as the sheath and auxiliary gas. The volumes of the injections were 20 μL.

Quantitative Analyses. HPLC-PDA analyses were performed using a Waters 600E multisolvent delivery system, a Waters 717plus autosampler, and a Waters 996 PDA detector equipped with Millenium[32] Chromatography Manager software. The experimental conditions (solvent gradients, columns, injection volumes) were the same as described above. The detection wavelengths used for PDA signal complete integration were 254 and 515 nm for flavonoids and anthocyanins, respectively.

Extraction, Isolation, and Identification of Flavonoids. Lyophilized fruits of *M. nigra* (115 g) and *M.alba* (200 g) were defatted at room temperature with *n*-hexane and extracted with MeOH by exhaustive maceration (5 × 500 mL) to yield 76 and 110 g of residue, respectively, which were successively dissolved in water and partitioned with EtOAc and *n*-BuOH. The dried *n*-butanol extracts of *M. nigra* (8 g) and *M. alba* (1 g) were separately subjected to fractionation on a Sephadex LH-20 column, using MeOH as eluent at a flow rate of 0.8 mL/min. Fractions of 8 mL were collected and grouped into 17 (A−R) and 7 (A−G) for *M. nigra* and *M. alba*, respectively, by TLC analysis on silica 60 F_{254} gel-coated glass sheets developed with *n*-BuOH/AcOH/H_2O (60:15:25) as eluent. *M. nigra* fractions O (45.1 mg), P (33.2 mg), and Q (66.3 mg) were separately purified by RP-HPLC on a C_{18} μ-Bondapak column at a flow rate of 2.0 mL/min with MeOH/H_2O (3:7) for fraction O, MeOH/H_2O (2:3) for fraction Q, and MeOH/H_2O (45:55) for fraction P, to afford compounds **1** (7 mg, $t_R = 14$ min) and **3** (2.9 mg, $t_R = 60$ min) from fraction O and compound **2** (3.7 mg, $t_R = 19$ min; and 14 mg, $t_R = 25$ min) from fractions P and Q, respectively. Compound **4** (3.9 mg, $t_R = 31$ min) was obtained by purification of fraction G of *M. alba* with MeOH/H_2O (2:3).

5-*O*-caffeoylquinic acid (**1**) was obtained as a brownish amorphous powder: ESI-MS, *m/z* 353 $[M - H]^-$ (*21*).

Quercetin 3-*O*-rutinoside (**2**) was obtained as a yellow amorphous powder: ESI-MS, *m/z* 609 $[M - H]^-$ (*22*).

Kaempferol 3-*O*-rutinoside (**3**) was obtained as a yellow amorphous powder: ESI-MS, *m/z* 593 $[M - H]^-$ (*23*).

Quercetin 3-*O*-glucoside (**4**) was obtained as a yellow amorphous powder: ESI-MS, *m/z* 463 $[M - H]^-$ (*23*).

Extraction and Identification of Anthocyanins. Ten grams of fresh fruits of *M. nigra* were homogenized in 60 mL of 2% HCl methanol solution. The solution was filtered on a Büchner funnel, and the filtrate was used for HPLC analyses. Identifications were made by comparing MS, PDA/UV, and retention data recorded for anthocyanin standard.

Quantitative Analyses. Quercetin 3-*O*-rutinoside and cyanidin 3-*O*-glucoside were selected as external standards of calibration for flavonoids and anthocyanins, respectively. Standard calibration curves were prepared over a concentration range of 0.5–50 μg/mL with five concentration levels (0.5, 1, 5, 10, and 50 μg/mL) for quercetin 3-*O*-rutinoside and for cyanidin 3-*O*-glucoside in a range 2.5–500 μg/mL with six different concentration levels (2.5, 5, 10, 50, 100, and 500 μg/mL). Triplicate injections were made for each level, and a weighed linear regression was generated for both external standards of calibration. The curve of calibration with external standard was obtained using concentration (μg/mL) with respect to area obtained from integration of the PDA peaks. The relationship between variables was analyzed using linear simple correlation. For the linear regression of the external standards, R^2 values were 0.9996 and 0.9999 for quercetin 3-*O*-rutinoside and cyanidin 3-*O*-glucoside, respectively. For the quantification of the compounds, GraphPad Software Prism 3.0 was used. The amount of the compounds was expressed as milligrams per 10 g of dried fruits for flavonoids and milligrams per 10 g of fresh fruits for anthocyanins.

RESULTS AND DISCUSSION

Isolation and Structure Determination of Compounds 1–4. The isolation procedure of the methanol extracts of *M. nigra* and *M. alba* fruits was undertaken, and four compounds were purified (see **Figure 1**) and characterized as 5-*O*-caffeoylquinic acid (**1**), quercetin 3-*O*-rutinoside (**2**), kaempferol 3-*O*-rutinoside (**3**), and quercetin 3-*O*-glucoside (**4**). The structure of the isolated compounds was established by ^1H and ^{13}C NMR data and confirmed by comparison with those reported in the literature (*21–23*).

Comparative Study of the Composition of Flavonoids of *M. nigra* and *M. alba* Fruits. Both methanol extracts of *M. nigra* and *M. alba* fruits were analyzed by HPLC-ESI/MS-PDA analyses under the same gradient conditions. The LC-MS base peak chromatograms and all of the chromatographic, spectroscopic, and spectrometric data and quantitative amounts of the identified compounds are reported in **Figure 2** and **Table 1**, respectively. From a qualitative point of view *M. alba* fruits are richer in the number of different flavonoids. Apart from the three compounds quercetin 3-*O*-rutinoside (**2**), kaempferol 3-*O*-rutinoside (**3**), and quercetin 3-*O*-glucoside (**4**), which are present also in *M. nigra* fruits, they contain another constituent,

5, which is unidentified. From the UV−vis spectrum, having its absorption maxima (λ_{max}) typical of a flavonol derivative (*24*), between 250 and 270 nm and between 315 and 365 nm, and LC-MS/MS spectrum, which consists of a $[M - H]^-$ at *m/z* 505 and a fragment ion at 301 ($[M - H]^- - 42 - 162$]) showing also the loss of a hexose moiety, we can hypothesize that it is a glycosylated flavonol, probably a quercetin derivative. The loss of 42 amu could be due to the presence of an aliphatic chain in the molecule. Results obtained from the quantitative analysis show higher total amount of flavonoids in *M. nigra* fruits (37.8 mg/10 g in *M. nigra* vs 19.9 mg/10 g in *M. alba*), which contain compound **2** as the major constituent, followed by **3** and a smaller amount of compound **4**. Compound **2** is also the major component of *M. alba* fruits, **3** and **4** being almost in equivalent amounts. Compound **5** in black mulberries is absent. Besides, *M. nigra* fruits contain anthocyanins, which belong to the class of flavonoids, increasing the total amount of these compounds.

Quali-quantitative Anthocyanins Profile. The anthocyanin composition of *M. nigra* was determined by means of HPLC-PDA-ESI-MS analyses. The chromatogram of anthocyanin extract, acquired at 515 nm, is shown in **Figure 3**. Anthocyanins in black mulberries from Italian cultivars are a mixture of five different anthocyanidin glycosides identified some years ago (*17*). We identified four of them, cyanidin 3-*O*-glucoside (**6**), cyanidin 3-*O*-rutinoside (**7**), pelargonidin 3-*O*-glucoside (**8**), and pelargonidin 3-*O*-rutinoside (**9**), by comparison of their HPLC retention times, elution orders, ESI-MS spectrometric data, and photodiode array PDA/UV−vis with authentic reference compounds (see **Figure 1**). Chromatographic, spectroscopic, spectrometric, and quantitative data of anthocyanins are listed in **Table 2**. The total amount of anthocyanins is 27 mg/10 g of fresh berries. Cyanidin 3-*O*-glucoside is the major one (66.3%), and it is followed by cyanidin 3-*O*-rutinoside (27.8%), pelargonidin 3-*O*-glucoside (4.4%), and pelargonidin 3-*O*-rutinoside (1.5%).

Our results are not completely in agreement with those reported by Lee et al. and Chen et al. (*19, 20*). The differences in flavonoid composition of the fruits depended not only on the species but also on the growing conditions, such as soil, geographical and environmental conditions during fruit development, degree of maturity at harvest, and genetic differences. The results show that *M. nigra* and *M. alba* are appreciable sources of flavonoids, which are considered to have possible protective effects on human health, as suggested by recent epidemiological and experimental studies. Taking together the findings of this study and those of previous investigations (*15–20*) into phenolic compounds and their activity, it becomes evident that mulberry fruits are a promising source of pigments and potent natural antioxidants which should be considered for future exploitation.

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