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Flavonoids of *Zizyphus jujuba* L. and *Zizyphus spina-christi* (L.) Willd (Rhamnaceae) fruits

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ABSTRACT

The ripe edible fruits of jujube, *Zizyphus jujuba* Miller (syn. *Z. sativa* Gaertner, *Z. vulgaris* Lam.) and Christ's thorn jujube *Zizyphus spina-christi* (L.) Willd (Rhamnaceae family) were phytochemically investigated, comparing their quali-quantitative flavonoids profile. Twelve compounds from both methanol extracts have been recognized as quercetin, kaempferol, and phloretin derivatives by means of HPLC/ESI-MS analyses. Six major compounds have been purified by Sephadex LH-20 column chromatography followed by HPLC and were characterized using NMR spectroscopy. One *C*-glycoside, 3′,5′-di-*C*-*β*-D-glucosylphloretin, was detected in *Z. spina-christi*. The quantitative analysis of all compounds was also reported showing a higher content of flavonoids in *Z. jujuba*.

1. Introduction

Plant polyphenols are a wide group of secondary metabolites that can range from simple molecules, such as phenolic acids, to highly polymerized constituents such as tannins. They are compounds with a large number of derivatives in the plant kingdom, from fungi to Angiospermae, including food plants, and are common components in the human diet. All the phenolics, but especially flavonoids, have been reported to have multiple biological effects such as antioxidant activity (Bors & Saran, 1987; Larson, 1988), anti-inflammatory action (Moroney, Alcanaz, Forder, Carey, & Hoult, 1988; Vlaskovska, Drenska, & Ovcharov, 1990), inhibition of platelet aggregation (Van Wauwe & Goossens, 1983), inhibition of mast cell histamine release (Amellal et al., 1985), and antimicrobial activities (Pratt & Hudson, 1990). Moreover, 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 (Hertog, Feskens, Hollman, Katan, & Kromhout, 1993; Hertog, Sweetman, Fehily, Elwood, & Kromhout, 1997) and certain types of cancer (Hertog et al., 1995; Hertog, Feskens, Hollman, Katan, & Kromhout, 1994). In addition, flavonoids contribute the colors to flowers, fruits, and vegetables (Cooper-Driver, 2001) and could be used to replace toxic synthetic pigments.

Food chemists have proposed the replacement of synthetic antioxidants such as butylhydroxyanisole (BHA) and butylhydroxytoluene (BHT), with natural ones because the synthetic antioxidants are suspected to be carcinogenic (Branen, 1975; Ito, Fukushima, Hagiwara, Shibata, & Ogiso T., 1983). In summary, natural antioxidants are useful in the food industry as preservatives to increase the life of food products preventing the loss of their sensory and nutritional quality.

Zizyphus species (Rhamnaceae family) are commonly used in folklore medicine for the treatment of various diseases such as digestive disorders, weakness, liver complaints, obesity, urinary troubles, diabetes, skin infections, loss of appetite, fever, pharyngitis, bronchitis, anaemia, diarrhea, and insomnia (Han & Park, 1986; Kirtikar & Basu, 1984). They are widespread in the Mediterranean region, Africa, Australia, and tropical America. Previous phytochemical studies on the different species of the genus *Zizyphus* led to the isolation and characterization of cyclopeptide alkaloids, flavonoids, sterols, tannins, and triterpenoid saponins (Ikram, Ogihara, & Yamasaki, 1981; Nawwar, Ishak, Michael, & Buddrus, 1984). *Zizyphus jujuba* Miller (syn. *Z. sativa* Gaertner, *Z. vulgaris* Lam.) is a thorny small or medium sized tree widely cultivated in

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Italy for its edible orange–brown fruits, commonly known as "giuggiole" or "zizzole" (Pignatti, 1982). *Zizyphus spina-christi* (L.) Willd is a wild tree, with spiny branches and small, orange–yellow fruits, commonly found in Jordan, Israel, and Egypt, where it is used to treat the blisters, bruises, chest pains, dandruff, fractures, headache, and mouth problems (Ghazanfar, 1994).

The aim of this work was to evaluate and to compare the flavonoids profile of these edible and non-commercial fruits, making a quali-quantitative study of their content by HPLC/PDA/ESI-MS, in order to draw attention to these species and to contribute to the improvement of their potential value as food.

2. Materials and methods

2.1. Plant materials

Zizyphus jujuba Miller fruits were collected in Pisa, Italy, in September 2005. The specimen was further identified and authenticated by Dr. Fabiano Camangi, Scuola Superiore S. Anna di Studi Universitari e di Perfezionamento di Pisa, Pisa, Italy. *Z. spina-christi* (L.) Willd fruits were collected near Adasiyyeh, Jordan, in June 2005, and characterized by Professor Ammar Bader, Faculty of Pharmacy, Al-Zaytoonah Private University of Jordan, Amman, Jordan.

2.2. Reagents

Standards of quercetin 3-*O*-rutinoside was obtained from Merck (E. Merck, Darmstadt, Germany). Standards of kaempferol 3-*O*-robinobioside, kaempferol 3-*O*-rutinoside, quercetin 3-*O*-β-D-galactoside, and quercetin 3-*O*-β-D-glucoside were previously isolated from vegetable material and fully characterized in our laboratory. Standards of HPLC grade acetonitrile (ACN) and acetic acid (CH_3COOH) were purchased from J.T. Baker (Baker Mallinckrodt, Phillipsburg, NJ, USA). HPLC grade water (18 mΩ) was prepared by a Mill-Ω^{50} purification system (Millipore Corp., Beddford, MA, USA).

2.3. General methods

An Avance Bruker 250 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 a Shimadzu injector with a Waters μ-Bondapak C_{18} column (7.8 × 300 mm, 10 μm,Waters, Milford, MA, USA). Thin-layer chromatography (TLC) was performed on precoated Kieselgel 60 F_{254} plates (E. Merck, Darmstadt, Germany). 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 3.0 × 150 mm, 3.5 μm, Symmetry® C_{18} column (Waters, Milford, MA). The eluent was a mixture of 0.1% methanol 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. The volume of the injection was 20 μl. 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, −12 V; tube lens offset, −35 V; sheath gas flow rate 60 arbitrary units; auxiliary gas flow rate, 6 arbitrary units; spray voltage, 4 kV; scan range of *m/z* 200–800. N_2 was used as the sheath and auxiliary gas. PDA data were recorded with 220–500 nm range with the preferential channels 254 and 324 nm as the detection wavelengths. HPLC-PDA quantitative 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 Millenium^{32} Chromatography Manager Software. The experimental conditions (solvent gradient, PDA channels, and column) were the same as described above. The volume of the injection was 25 μl. Quantitative determination was carried using calibration curves of the standards. Quercetin 3-*O*-rutinoside and 3′,5′-di-*C*-β-glucosylphloretin were selected as the external standards of calibration for *O*-glycoside and *C*-glycoside flavonoids, respectively. Standard calibration curves were prepared in a concentration range 0.0005–0.025 mg/ml, with six different concentration levels (0.0005, 0.001, 0.0025, 0.005, 0.01, and 0.025 mg/ml) for quercetin 3-*O*-rutinoside and for 3′,5′-di-*C*-β-glucosylphloretin in a range of 0.001–0.01 mg/ml with four concentration levels (0.001, 0.0025, 0.005, and 0.01 mg/ml). Triplicate injections were made for each level, and a weighed linear regression was generated. The calibration curves with an external standard were obtained using concentration (mg/ml) with respect to the area obtained from the integration of the PDA peaks at a wavelength of 254 nm for *O*-glycoside and 285 nm for *C*-glycoside flavonoids. The relation between variables was analysed using linear simple correlation. For the linear regression of the external standard, R^2 was 0.9995 for quercetin 3-*O*-rutinoside and 0.9996 for 3′,5′-di-*C*-β-glucosylphloretin. For the quantification of the compounds, a GraphPad Software Prism 3.0 was used. The amount of the compounds was finally expressed in mg/10 g of dried fruits.

2.4. Extraction, isolation, and identification

Lyophilized fruits of *Z. jujuba* (560 g) and *Z. spina-christi* (240 g) were defatted at room temperature with *n*-hexane, and extracted with MeOH by exhaustive maceration (5 × 500 ml) to yield 380 g and 150 g of residue, respectively, which were successively dissolved in water and partitioned with EtOAc and *n*-BuOH. The dried *n*-butanol extract of *Z. jujuba* (2 g) and *Z. spina-christi* (6 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 seven (A–G) and twelve (A–K) fractions for *Z. jujuba* and *Z. spina-christi*, respectively, by TLC analysis on silica 60 F_{254} gel-coated glass sheets developed with *n*-BuOH–AcOH–H_2O (60:15:25) as the eluent. By means of RP-HPLC with MeOH–H_2O (45:55) on a 300 mm × 7.8 mm i.d., C_{18} μ-Bondapak column (flow rate 2.0 ml/min) compounds **1** (5.7 mg, t_R = 18 min), **2** (3.4 mg, t_R = 19 min), and **5** (2.0 mg, t_R = 23 min) were isolated from *Z. jujuba* fraction G (27.6 mg). *Z. spina-christi* fractions G (76 mg) and I (50.6 mg) were separately purified with MeOH–H_2O (35:65) for fraction G and MeOH–H_2O (45:55) for fraction J, to afford compounds **1** (1.6 mg, t_R = 31 min), **11** (3.3 mg, t_R = 38), and **12** (3.7 mg, t_R = 57 min) from fraction G, and compound **6** (2.1 mg, t_R = 25 min) from fraction J, respectively.

Quercetin 3-*O*-robinobioside (**1**): yellow amorphous powder. Negative ESI-MS: *m/z* 609 $[M-H]^-$. ^1H and ^{13}C data are consistent with the previously published data (Rastrelli, Saturnino, Schettino, & Dini, 1995).

Quercetin 3-*O*-rutinoside (**2**): yellow amorphous powder. Negative ESI-MS: *m/z* 609 $[M-H]^-$. ^1H and ^{13}C data are consistent with the previously published data (Rastrelli et al., 1995).

Quercetin 3-*O*-α-L-arabinosyl-(1→2)-α-L-rhamnoside (**5**): yellow amorphous powder. Negative ESI-MS: *m/z* 579 $[M-H]^-$. ^1H

and ^{13}C data are consistent with the previously published data (Nielsen, Olsen, & Møller B.L., 2005).

Quercetin 3-*O*-*β*-D-xylosyl-(1→2)-α-L-rhamnoside (**6**): yellow amorphous powder. Negative ESI-MS: *m/z* 579 $[M-H]^-$. ^1H and ^{13}C data are consistent with the previously published data (Nielsen et al., 2005).

3′,5′-di-*C*-*β*-D-Glucosylphloretin (**11**): yellow amorphous powder. Negative ESI-MS: *m/z* 597 $[M-H]^-$. ^1H and ^{13}C data are consistent with the previously published data (Ogawa et al., 2001).

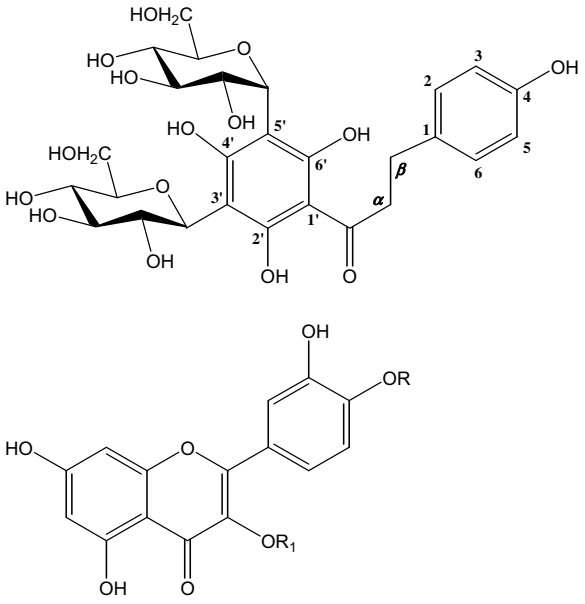
Quercetin 3-*O*-*β*-D-xylosyl-(1→2)-α-L-rhamnoside-4′-*O*-α-L-rhamnoside (**12**): yellow amorphous powder. Negative ESI-MS: *m/z* 725 $[M-H]^-$. ^1H and ^{13}C data are consistent with the previously published data (Shahat et al., 2001).

3. Results and discussion

The methanol extracts of the fruits of *Z. jujuba* and *Z. spina-christi* were partitioned with EtOAc and *n*-BuOH. The *n*-BuOH partitions were subjected to fractionation with an initial separation by Sephadex LH-20 column chromatography. Subsequent purification by semipreparative HPLC led to the isolation of six compounds. Compound **1** was recognized as quercetin 3-*O*-robinobioside, **2** as quercetin 3-*O*-rutinoside, **5** as quercetin 3-*O*-α-L-arabinosyl-(1→2)-α-L-rhamnoside, **6** as quercetin 3-*O*-*β*-D-xylosyl-(1→2)-α-L-rhamnoside, **11** as 3′,5′-di-*C*-*β*-D-glucosylphloretin, and **12** as quercetin 3-*O*-*β*-D-xylosyl-(1→2)-α-L-rhamnoside-4′-*O*-α-L-rhamnoside, respectively (Fig. 1). The structures of the isolated components were established by different spectroscopic methods (ESI-MS, ^1H, ^{13}C, HMQC, and HMBC NMR experiments) and confirmed by comparison with those reported in the literature.

To evaluate the phytochemical profile of flavonoids of the methanol extracts of *Z. jujuba* and *Z. spina-christi* fruits, HPLC–PDA–MS analyses were carried out using the "on-line" method. The LC–MS Base Peak chromatograms of both methanol extracts are shown in Fig. 2. The retention times t_R, $[M-H]^-$, MS/MS fragments, and UV λ_{max} of the identified flavonoids are shown in Table 1.

HPLC–PDA–MS analyses of the plant extracts revealed the presence of some compounds not isolated. Four of them were recognized as kaempferol 3-*O*-robinobioside (**3**), kaempferol 3-*O*-rutinoside (**4**), quercetin 3-*O*-*β*-D-galactoside (**9**), and quercetin 3-*O*-*β*-D-glucoside (**10**) by comparison of their HPLC retention times, elution orders, ESI-MS spectrometric data, and photodiode array PDA/UV/Vis with reference standards. Two other constituents, **7** and **8**, remained not identified, but it was possible to hypothesize their structures. The tandem mass spectrum, produced by compound **8**, at *m/z* 613, with fragment ions at *m/z* 523 and 493, showed the pattern typical for a *C*-glycoside derivative, namely $[M-H-90]^-$ and $[M-H-120]^-$ (Waridel et al., 2001). Considering also, that the molecular weight of compound **8** was 16 u higher than the one of compound **11** and its absorption maximum (λ_{max}) in the UV spectrum was similar to that of **11**, it allowed thinking that compound **8** differs from **11** by the presence of one additional hydroxyl group in position α, *β*, or 3. The MS/MS spectrum of



	R	R_1
1	H	rha-(1→6)-gal
2	H	rha-(1→6)-glc
5	H	ara-(1→2)-rha
6	H	xyl-(1→2)-rha
12	rha	xyl-(1→2)-rha

Fig. 1. Chemical structures of phenolic compounds isolated from *Z. jujuba* and *Z. spina-christi* fruits.

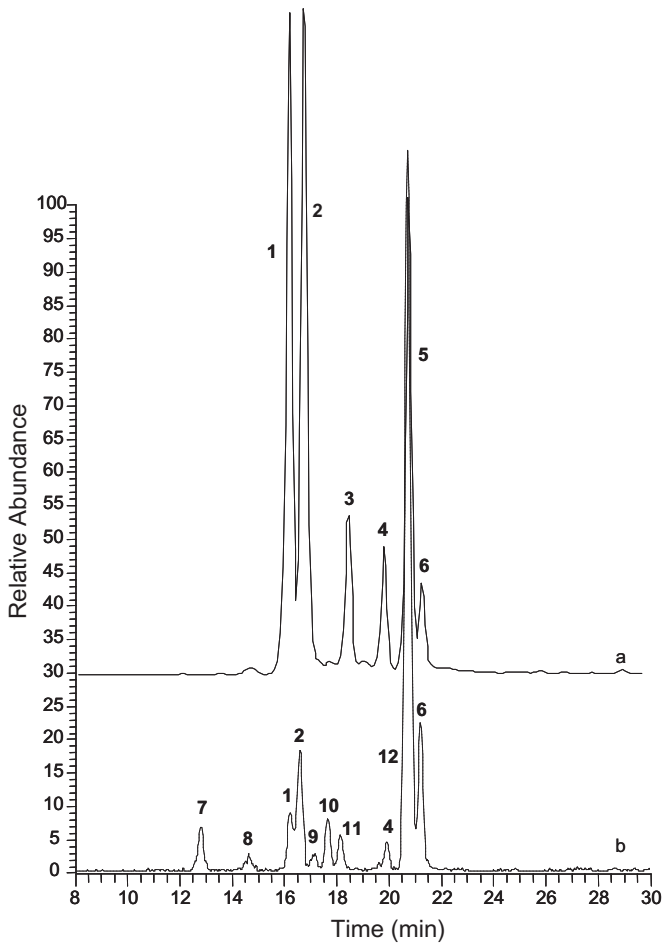


Fig. 2. LC–MS comparison between flavonoid glycosides of *Z. jujuba* (a) and *Z. spina-christi* (b) fruits.

Table 1
Chromatographic, spectroscopic, and spectrometric data of components found in *Z. jujuba* and *Z. spina-christi* fruits

Peak	Compound	t_R (min)	$[M-H]^-$ (*m/z*)	MS/MS fragments (*m/z*)	λ_{max}
1	Quercetin 3-*O*-robinobioside	16.2	609	463, 343, 301	255, 355
2	Quercetin 3-*O*-rutinoside	16.8	609	463, 343, 301	255, 355
3	Kaempferol 3-*O*-robinobioside	18.6	593	447, 285	265, 345
4	Kaempferol 3-*O*-rutinoside	19.9	593	447, 285	265, 345
5	Quercetin 3-*O*-α-L-arabinosyl-(1→2)-α-L-rhamnoside	20.8	579	447, 429, 301	255, 350
6	Quercetin 3-*O*-β-D-xylosyl-(1→2)-α-L-rhamnoside	21.4	579	447, 429, 301	255, 350
7	Unidentified	12.8	755	609, 489, 343, 301	255, 355
8	Unidentified	14.6	613	595, 523, 493	295
9	Quercetin 3-*O*-β-D-galactoside	17.2	463	343, 301	255, 355
10	Quercetin 3-*O*-β-D-glucoside	17.6	463	343, 301	255, 355
11	3′,5′-Di-*C*-β-D-glucosylphloretin	18.1	597	579, 507, 477	285
12	quercetin 3-*O*-β-D-xylosyl-(1→2)-α-L-rhamnoside-4′-*O*-α-L-rhamnoside	20.7	725	579, 447, 301	265, 340

compound **7** obtained for the ion at *m/z* 755, showing the fragment ions at *m/z* 609 $[M-H-146]^-$ and 301 $[M-H-146-162-146]^-$, corresponded to the loss of one deoxyhexose unit (146 u), followed by one hexose and one deoxyhexose (162 + 146 u), respectively. Furthermore, its *m/z* was 146 u higher than the one of constituents **1** and **2** and its UV spectrum with λ_{max} at 255 and 355 nm was typical of flavonol derivatives (Wollenweber, 1982), confirming the hypothesis that compound **7** was a glycosilated flavonol, probably a quercetin derivative.

Methanol extracts of *Z. jujuba* and *Z. spina-christi* fruits were considered, from both, qualitative and quantitative point of view. *Z. jujuba* presented a rich flavonol glycosides composition that includes 6 compounds. They were all *O*-flavonol glycosides with quercetin and kaempferol as aglycones and oligosaccharides moieties as disaccharides linked at the 3-OH position. The methanol extract of *Z. spina-christi* was richer in the number of flavonoids, also with major structural variability. There were 10 compounds, both *O*- and *C*-glycosides. Except for quercetin and kaempferol as aglycons, two dihydrochalcone derivatives were present. Finally, the glycosilated flavonoids contained one, two, or three sugar moieties. Quercetin 3-*O*-robinobioside (**1**), quercetin 3-*O*-rutinoside (**2**), kaempferol 3-*O*-rutinoside (**4**), and quercetin 3-*O*-β-D-xylosyl-(1→2)-α-L-rhamnoside (**6**) were common components of both fruits extracts.

Results obtained from quantitative analyses are shown in Table 2. *Z. jujuba* fruits demonstrate a higher quantitative content of flavonoids than the fruits of *Z. spina-christi* (214.4 versus 65.1 mg/10 g of dried fruits) and had compound **2**, followed by **1** and **6** as the major constituents, while the most abundant flavonoid for *Z. spina-christi* fruits was component **11**.

The differences in flavonoids 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 (Zadernowski, Naczk, & Nesterowicz, 2005).

Finally, it can be concluded that the studied *Zizyphus* spp. fruits are valuable horticultural products, based on their rich and nutrient composition. The quantity of flavonoids, which are considered to have possible protective effects on human health, as suggested by the recent epidemiological and experimental studies, is a promising source for future industrial research of *Zizyphus* ssp. phytochemicals with potential human health benefits.

Table 2
Quantitative amount[a] (mg/10 g of dried material) of flavonoids found in *Z. jujuba* and Z. spina-christi fruits

Compound	*Z. jujuba*	*Z. spina-christi*
1	74.6	5.2
2	81.4	7.3
3	6.8	/
4	4.8	1.9
5	40.7	/
6	6.1	8.7
7	/	2.6
8	/	7.3
9	/	5.1
10	/	8.9
11	/	8.1
12	/	10.0
Total	214.4	65.1

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

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