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NMR-based identification of the phenolic profile of fruits of *Lycium barbarum* (goji berries). Isolation and structural determination of a novel *N*-feruloyl tyramine dimer as the most abundant antioxidant polyphenol of goji berries

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ABSTRACT

Biological properties of fruits of *Lycium barbarum* (goji berries) have been ascribed to their high content of nutrients and phenolics. Comprehensive studies aimed at unambiguously identifying the phenolic components in goji berries are still lacking. In this paper, we report on the isolation and NMR-based identification of the major phenolics in commercially available goji berries. Together with already known phenolics, including caffeic acid, *p*-coumaric acid, rutin, scopoletin, *N-trans*-feruloyl tyramine, and *N-cis*-feruloyl tyramine, an unreported *N*-feruloyl tyramine dimer was characterized as the most abundant polyphenol isolated from the berries. Usually divalent molecules show enhanced biological activities than their corresponding monomers.

1. Introduction

Dietary intake of berry fruits is well known to have a beneficial impact on human health. An ever-growing body of scientific studies has ascertained the curative effects of berries against a broad selection of human diseases, including cardiovascular and neurodegenerative diseases, obesity and some types of cancers (Firuzi, Miri, Tavakkoli, & Saso, 2011). Biological properties of berries have been basically related to complementary, additive or synergistic interactions between the high content of nutrients (vitamins, minerals, folate, and fibers) and a diversity of phenolic-type phytochemicals. Flavonoids, tannins, stilbenoids, and phenolic acids are among the major phenolics detected in berries (Wang, Chang, Stephen, & Chen, 2010). Important biological properties are related to phenolics, including antioxidant activity, regulation of some metabolizing enzymes, and modulation of gene expression and subcellular signaling pathways (Seeram, Adams, Zhang, Sand, & Herber, 2006). However, conclusive studies on the chemistry of beneficial molecules contained in berries as well as on their mechanism of action at a molecular level are still lacking.

Among regularly consumed berries, fruits of *Lycium barbarum* (Solanaceae), also known as goji berries or wolfberries, have lately emerged as an interesting source of nutrients and beneficial molecules. Following the growing interest in traditional herbs and plant food supplements, the consume of goji berries – mostly popular in China – has spread also to subtropical regions from southeastern Asia to Europe and North America.

A number of dietary constituents of goji berries, essentially polysaccharides, zeaxanthin, a selection of vitamins, and antioxi-

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dant compounds have been characterized (Wang et al., 2010). So far, only a few studies have reported on the phenolic components of goji berries, at times presenting contradictory data. The favonol rutin, for example, has been characterized as the main flavonoid in *Lycium chinense* together with chlorogenic acid and protocatechuic acid (Qian, Liu, & Huang, 2004); but, it has not been found in *L. barbarum*, in which only three flavonols, kaempferol, quercetin and myricetin, have been identified by means of LC–MS (Le, Chiu, & Ng, 2007). Very recently, the knowledge on the antioxidant composition of phenolics in *L. barbarum* has been complemented by the discovery of some monomers and dimers of phenolic amides containing *N*-feruloyl tyramine units that have been termed lyciumamides (Gao et al., 2015).

In the present paper, we report on the isolation and NMR-based identification of the main phenolic compounds isolated from commercially available goji berries. Together with already known phenolics, including caffeic acid (**1**), *p*-coumaric acid (**2**), rutin (**3**), scopoletin (**4**), and both *N-trans*-feruloyl tyramine (**5**) and *N-cis*-feruloyl tyramine (**6**) (Fig. 1), an unreported *N*-feruloyl tyramine dimer (**7**) has been isolated, identified and characterized as the most abundant polyphenol contained in the analyzed goji berries (Fig. 2). Even if the chemical structure of this molecule resembles that of the already mentioned lyciumamides, none of the reported lyciumamides (Gao et al., 2015) was detected in our samples.

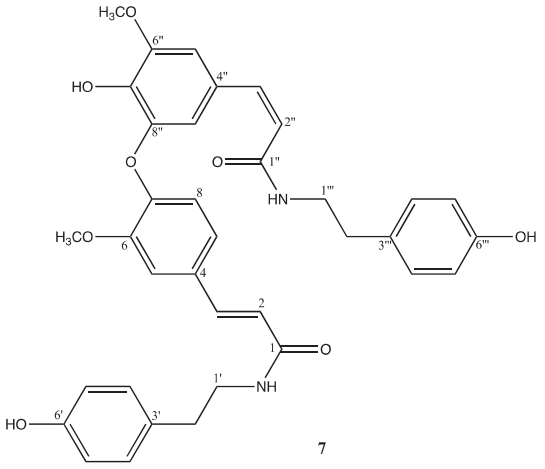

Fig. 2. Chemical structure of **7**, the most abundant phenolic compound identified in commercially available goji berries.

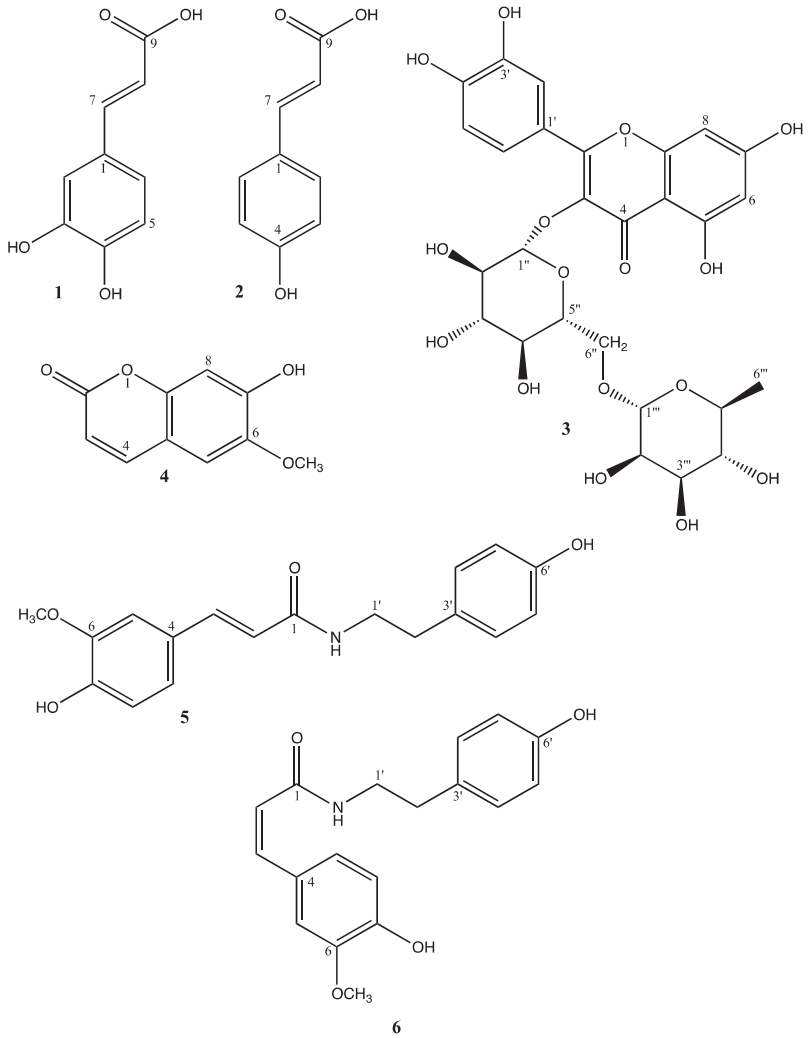

Fig. 1. Chemical structures of the main phenolic compounds identified in commercially available goji berries. Caffeic acid (**1**), *p*-coumaric acid (**2**), rutin (**3**), scopoletin (**4**), *N-trans*-feruloyl tyramine (**5**), and *N-cis*-feruloyl tyramine (**6**).

2. Materials and methods

2.1. Biological material

Five 80-g packages of fruits of *L. Barbarum* were bought at a local store.

2.2. Extraction and purification procedures

Fruits (400 g) were homogenized and extracted with 2 L of H_2O:MeOH 2:8 (v/v) overnight. The obtained extract was concentrated and partitioned twice with EtOAc.

During the purification steps, the antioxidant activity of the obtained fractions was detected by the DPPH assay. The ethyl acetate extract was first separated on a Silica 40 g column and eluted with a gradient elution with changing ratio of EtOAc:MeOH, from 100:0 to 0:100 in 70 min (10 mL/min flow). From this column two fractions with antioxidant properties were collected, namely A and B.

The A fraction, collected between 10 and 15 min of the gradient elution, was further separated on a C18 10 μm HPLC column by gradient elution with changing ratios of H_2O:MeOH from 20:80 to 0:100 in 20 min. The antioxidant compound (**7**) deriving from the A fraction was collected after 6.20 min and for a whole minute.

The B fraction was collected between 20 and 40 min. This fraction was further separated on a Gemini C 18.5 μm HPLC column by gradient elution with changing ratios of H_2O:MeOH from 10:90 to 0:100 in 15 min. From this fraction, compounds **1**, **2**, **3**, **4**, **5** and **6** were purified.

2.3. NMR and MS experiments

NMR spectra were recorded on a Varian Unity Inova 700 spectrometer equipped with a ^{13}C Enhanced HCN Cold Probe. Shigemini 5 mm NMR tubes were used. CD_3OD was selected as deuterated solvent (δ_H 3.31; δ_C 49.0 ppm). Standard Varian Pulse Sequences were employed for the respective classes of spectra. All of the NMR data were reported in the paper were derived from ^1H NMR, ^{13}C NMR, COSY, z-filtered TOCSY, ROESY, phase-sensitive HMBC and HSQC experiments.

High Resolution (HR)-MS and HR-MS/MS analyses were performed on a linear ion trap LTQ Orbitrap XL Fourier transform mass spectrometer (FTMS) equipped with an ESI ION MAX source. The following source settings were used for both HR-MS (*m*/*z* 150–700) and HR collision induced dissociation (CID) MS/MS (*m*/*z* 170–700): spray voltage 4.5 kV, capillary temperature 350 °C, capillary voltage 0 V, sheath gas 20 and auxiliary gas 21 (arbitrary units), and tube lens voltage 60 V. HR CID MS/MS experiment was acquired on $[M+H]^+$ ion of **7** by using 25% collision energy. Calculation of elemental formulas in MS and CID MS/MS spectra were performed by using the monoisotopic ion peak of **7**. Mass tolerance of 5 ppm was used.

2.4. DPPH test

The measurement of the DPPH radical scavenging activity was performed according to the methodology procedure (Brand-Williams, Cuvelier, & Berset, 1995). In more detail, 20 μL of each sample was added to 2 mL of DPPH solution. The mixture was shaken vigorously and allowed to stand in the dark at room temperature and observed over a 2 h period. BHA was used as positive control.

2.5. Identification of the isolated compounds

Caffeic acid (**1**) (1.7 mg): HRESIMS monoisotopic ion peak at *m*/*z* 181.0282 $[M+H]^+$ (calcd for $C_9H_9O_4$, 181.0295). ^1H and ^{13}C NMR data in CD_3OD (700 MHz): H2 7.15; H5 7.04; H6 6.79; H7 7.56; H8 6.27. C1 127.37; C2 115.90; C3 149.23; C4 150.04; C5 117.02; C6 124.00; C7 146.40; C8 116.03; C9 171.42.

p-Coumaric acid (**2**) (2.5 mg): HRESIMS monoisotopic ion peak at *m*/*z* 165.0507 $[M+H]^+$ (calcd for $C_9H_9O_3$, 165.0546). ^1H and ^{13}C NMR data in CD_3OD (700 MHz): H2, H6 7.42; H3, H5 6.78; H7 7.56; H8 6.26. C1 127.46; C2, C6 131.20; C3, C5 116.94; C4 161.28; C7 146.51; C8 115.94; C9 171.49.

Rutin (**3**) (4.1 mg): HRESIMS monoisotopic ion peak at *m*/*z* 611.4121 $[M+H]^+$ (calcd for $C_{27}H_{31}O_{16}$, 611.4127). ^1H and ^{13}C NMR data in CD_3OD (700 MHz): H6 6.21; H8 6.37; H2′ 767; H5′ 6.88; H6′ 7.63; H1″ 5.11; H2″ 3.47; H3″ 3.41; H4″ 3.26; H5″ 3.32; H6″a 3.39; H6″b 3.80; H1‴ 4.52; H2‴ 3.63; H3‴ 3.54; H4‴ 3.28; H5‴ 3.45; H6‴ 1.12. C2 158.50; C3 135.72; C4 179.41; C5 163.00; C6 100.01; C7 166.13; C8 95.00; C9 159.44; C10 105.72; C1′ 123.72; C2′ 117.63; C3′ 145.92; C4′ 149.92; C5′ 116.01; C6′ 123.65; C1″ 104.72; C2″ 75.84; C3″ 78.02; C4″ 71.11; C5″ 77.52; C6″ 68.60; C1‴ 102.54; C2‴ 72.32; C3‴ 72.44; C4‴ 73.91; C5‴ 69.80; C6‴ 18.04.

Scopoletin (**4**) (2.8 mg): HRESIMS monoisotopic ion peak at *m*/*z* 193.0493 $[M+H]^+$ (calcd for $C_{10}H_9O_4$, 193.0495). ^1H and ^{13}C NMR data in CD_3OD (700 MHz): H3 6.18; H4 7.84; H5 7.10; H8 6.75; 6-OMe 3.88; C2 164.22; C3 112.83; C4 146.32; C5 110.12; C6 147.24; C7 153.10; C8 104.14; C9 151.62; C10 112.71; 6-OMe 56.97.

N-trans-feruloyl tyramine (**5**) (1.3 mg): HRESIMS monoisotopic ion peak at *m*/*z* 314.1384 $[M+H]^+$ (calcd for $C_{18}H_{20}O_4N$, 314.1387). ^1H and ^{13}C NMR data in CD_3OD (700 MHz): H2 6.39 (doublet *J* 15.4 Hz); H3 7.41 (doublet *J* 15.4 Hz); H5 7.10; H8 7.00; H9 6.77; H1′ 3.45; H2′ 2.73; H4′, H8′ 7.03; H5′, H7′ 6.70; 6-OMe 3.86; C1 169.23; C2 118.70; C3 143.00; C4 128.21; C5 112.00; C6 149.27; C7 149.51; C8 119.50; C9 122.85; C1′ 42.73; C2′ 35.99; C3′ 131.12; C4′, C8′ 130.92; C5′, C7′ 116.42; C6′ 157.02; 6-OMe 53.64.

N-cis-feruloyl tyramine (**6**) (0.8 mg): HRESIMS monoisotopic ion peak at *m*/*z* 314.1384 $[M+H]^+$ (calcd for $C_{18}H_{20}O_4N$, 314.1387). ^1H and ^{13}C NMR data in CD_3OD (700 MHz): H2 5.79 (doublet *J* 12.7 Hz); H3 6.59 (doublet *J* 12.7 Hz); H5 7.35; H9 6.74; H1′ 3.36; H2′ 2.69; H4′, H8′ 7.00; H5′, H7′ 6.68; 6-OMe 3.85; C1 170.48; C2 113.72; C3 138.50; C4 128.01; C5 113.02; C6 148.72; C7 148.51; C8 120.22; C9 124.81; C1′ 42.23; C2′ 35.56; C3′ 131.40; C4′, C8′ 130.80; C5′, C7′ 116.36; C6′ 156.85; 6-OMe 53.73.

(**7**) (5.9 mg): HRESIMS monoisotopic ion peak at *m*/*z* 625.2543 $[M+H]^+$ (calcd for $C_{36}H_{37}O_8N_2$, 625.2544) ($\Delta = -0.228$ ppm). ^1H and ^{13}C NMR data in CD_3OD (700 MHz) are reported in Table 2. Diagnostic HMBC correlations: H2/C1, H2/C4, H3/C1, H5/C6, H5/C7, H8/C4, H8/C6, H8/C7, H1′/C1, H2′/C3′, H2′/C4′, H4′/C6′, H5′/C6′, H2″/C1″, H2″/C4″, H3″/C4″, H3″/C9″, H5″/C6″, H5″/C7″, H9″/C8″, H9″/C7″, H1‴/C1″, H1‴/C3‴, H2‴/C3‴, H2‴/C4‴, H4‴/C6‴.

3. Results and discussion

3.1. NMR characterization of the main phenolics isolated from goji berries (L. barbarum)

Fruits of *L. barbarum* were homogenized and extracted with a hydromethanolic solution. After concentration, the obtained extract was partitioned against EtOAc. The DPPH-guided purification of the ethyl acetate extract was conducted as reported in Section 2. Eventually, seven antioxidant compounds were isolated and subjected to both MS- and NMR-based analysis for their identifica-

tion. A complete list of the purified compounds is reported in Table 1. In more detail, compounds **1**, **2**, **3**, **4**, **5** and **6** were identified as caffeic acid (Ha et al., 2012), *p*-coumaric acid (Swislocka, Kowczyk-Sadowy, Kalinowska, & Lewandowski, 2012), rutin (Lallemand & Duteil, 1977), scopoletin (Darmawan, Kosela, Kardono, & Syah, 2012), *N-trans*-feruloyl tyramine, and *N-cis*-feruloyl tyramine (Gao et al., 2015) (Fig. 1), respectively, by comparison of their MS and NMR data with those reported in literature. The quantities reported in Table 1 refer to the isolated amount of each compound and do not correspond to the initial content of each molecule in the ethyl acetate extract. In fact, some quantities of the isolated compounds may have been lost during the isolation and purification procedures. However, Table 1 realistically reflects the natural relative ratio of the major phenolics contained in commercially available goji berries.

3.2. Structural elucidation of 7

Unlike the other above reported phenolics, compound **7** appeared to be a new molecule. Thus, in-depth MS- and NMR-based analyses were undertaken, in order to define its chemical structure.

The high-resolution (HR) full MS spectrum of **7** contained a monocharged ion peak $[M+H]^+$ at *m/z* 625.2543 accompanied by the ion peak $[M+Na]^+$ at *m/z* 647.2363, corresponding to the molecular formula $C_{36}H_{36}O_8N_2$ ($\Delta = -0.228$ ppm).

The NMR spectra of the compound revealed two sets of slightly different spin systems suggesting that the molecule existed as a dimer. The cross-interpretation of the ^1H NMR, COSY, and z-TOCSY experiments of **7** led to the identification of two *para*-disubstituted phenyl rings along with two more phenyl rings, 1,2,3,5-tetrasubstituted and 1,2,4-trisubstituted, respectively. Two double bonds and two $-CH_2CH_2-$ segments were also identified. In accordance with the molecular formula of **7**, in the ^{13}C NMR spectrum 36 carbons, including 2 carbonyls, 26 sp^2 carbons (24 involved in phenyl rings and 4 in two sets of double bonds), 2 methoxyls, and 4 methylenes were detected. By means of the ps-HSQC spectrum, each proton was associated with the respective carbon (Table 2). Key HMBC correlations (Section 2) allowed us to determine that our molecule was constituted by two *N*-feruloyl tyramine monomers (Fig. 2) identified as *N-trans*-feruloyl tyramine and *N-cis*-feruloyl tyramine, respectively. In more detail, H3 was long-range coupled to C4, C5 and C9. So, the 1,2,4-trisubstituted aromatic ring was found to be part the *N-trans*-feruloyl tyramine unit. Likewise, H3″, involved in a *cis* double bond with H2″, appeared long-range coupled to C4″, C5″ and C9″ of the 1,2,3,5-tetrasubstituted aromatic ring. As a result, the whole of the obtained NMR data led us to univocally connect the *N-trans*-feruloyl tyramine unit and the *N-trans*-feruloyl tyramine unit by an oxygen bridge between C7 and C8″ (Fig. 2).

Table 1
Antioxidant compounds identified in the ethyl acetate extract of dried goji berries (350 g).

Compound	mg
Caffeic acid (**1**)	1.7
p-Coumaric acid (**2**)	2.5
Rutin (**3**)	4.1
Scopoletin (**4**)	2.8
N-trans-feruloyl tyramine (**5**)	1.3
N-cis-feruloyl tyramine (**6**)	0.8
N-feruloyl tyramine dimer (**7**)	5.9

N.B. The reported quantity corresponds to the isolated amount of each compound and does not constitute the total initial quantity of the molecules in the ethyl acetate extract.

Table 2
Chemical shifts data in CD_3OD (δ 3.31; 49.0 ppm) of **7**.

Position	7		
	^{13}C	^1H	$J_{H\text{-}H}$ (Hz); multiplicity[a]
1	169.36	–	–
2	118.89	6.37	15.4; d
3	142.19	7.41	15.4; d
4	128.44	–	–
5	111.68	7.10	bs
6	149.48	–	–
7	150.01	–	–
8	118.89	6.78	8.1; d
9	123.38	7.00	8.1; d
1′	42.78	3.44	7.7; t
2′	36.02	2.73	7.7; t
3′	131.20	–	–
4′	130.92	7.03	7.2; d
5′	116.45	6.70	7.2; d
6′	157.02	–	–
7′	116.45	6.70	7.2; d
8′	130.92	7.03	7.2; d
6-OMe	53.51	3.87	s
1″	170.51	–	–
2″	113.79	5.79	12.7; d
3″	138.53	6.59	12.7; d
4″	128.69	–	–
5″	125.00	6.71	bs
6″	149.50	–	–
7″	148.67	–	–
8″	148.73	–	–
9″	128.30	7.35	bs
1‴	42.23	3.37	7.7; t
2‴	35.55	2.67	7.7; t
3‴	131.36	–	–
4‴	130.72	6.98	7.2; d
5‴	116.36	6.67	7.2; d
6‴	156.91	–	–
7‴	116.36	6.67	7.2; d
8‴	130.72	6.98	7.2; d
8″-OMe	54.93	3.82	s

[a] d doublet; s singlet; bs broad singlet; t triplet.

Indirect proofs supporting the structural determination of **7** were obtained by HR-MS/MS experiments acquired in positive ion mode. The CID HR-MS/MS spectrum for the ion peak at *m/z* 625.2543 ($C_{36}H_{37}O_8N_2$; RDB 19.5) contained ion peaks deriving from a single cleavage on the molecule backbone along with ion peaks due to two simultaneous cleavages, as described in Fig. 3.

The fragmentation behavior of **7** confirmed the presence of the two *p*-OH phenylethylamine units at both ends of the molecule. In fact, the ion peak at *m/z* 488.1693 ($C_{28}H_{26}O_7N$; RDB: 16.5) was consistent with the loss of one *p*-OH phenylethylamine unit from the precursor ion (*m/z* 625) (cleavage #1; Fig. 3); while, the ion peak at *m/z* 351.0859 ($C_{20}H_{15}O_6$; RDB: 13.5) was the result of the loss of two *p*-OH phenylethylamine units from both ends of the molecule (cleavages #1 and 4; Fig. 3). Finally, the ion peak at *m/z* 325.1066 ($C_{19}H_{17}O_5$; RDB: 11.5) was assigned to the internal fragment deriving from the simultaneous cleavages #1 and 3 (Fig. 3).

Eventually, the chemical structure of **7** was defined as shown in Fig. 2.

It is worth underlining that the proposed structure for **7** is consistent with the reported mechanism of radical reactions leading to dimeric products (Gazak et al., 2009).

3.3. Antioxidant activity of 7

The antioxidant activity of **7** was assessed by the DPPH assay. Fig. 4 reports the effect of **7** on DPPH absorption. Values are expressed as the mean ± SD ($n = 5$). **7** showed enhanced ability in scavenging DPPH free radicals than both BHA, used as a control,

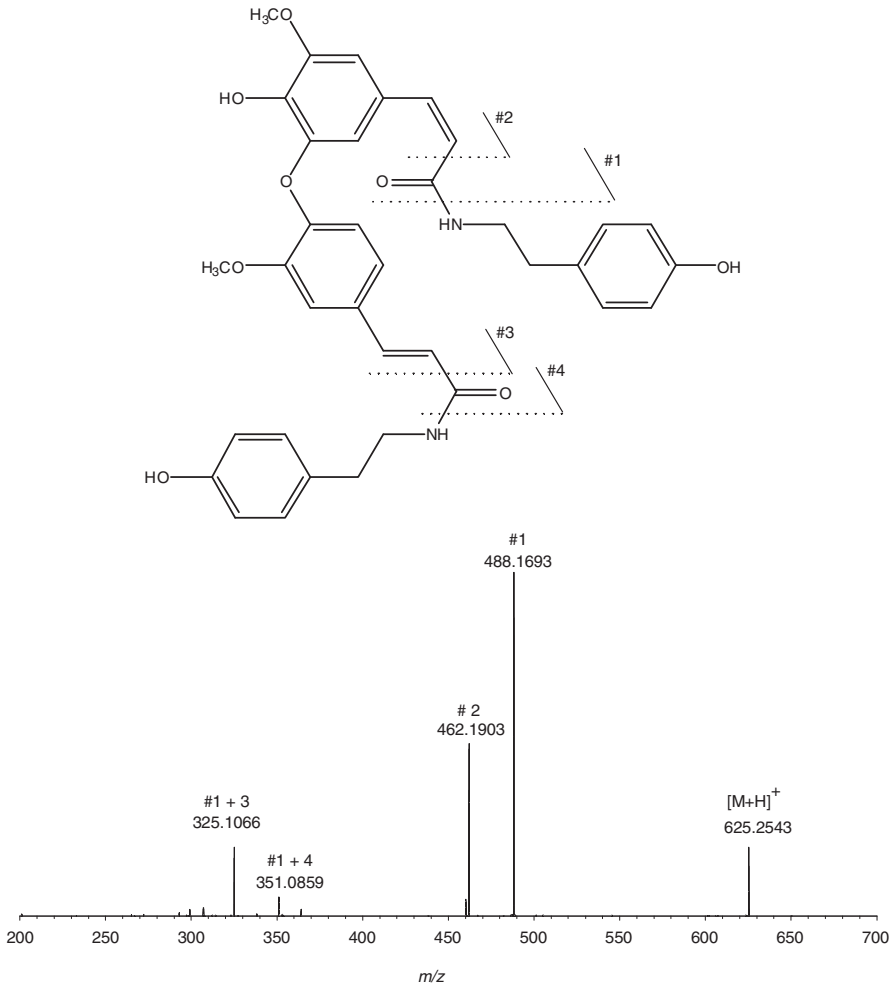

Fig. 3. Cleavages occurring during the positive ESI-MS/MS experiment of **7** (top) and associated spectrum (bottom).

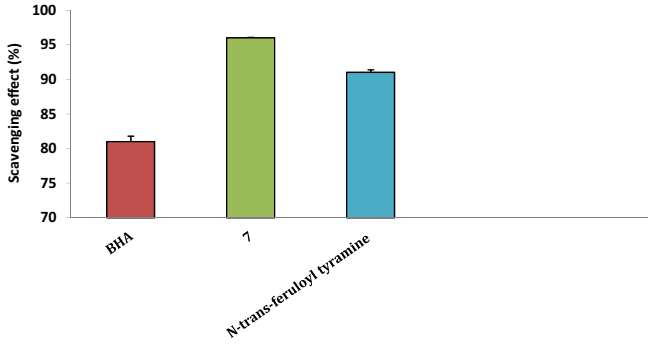

Fig. 4. Scavenging effects of BHA (used as positive control), compound **7** and *N-trans*-feruloyl tyramine. Values are expressed as the mean ± SD (n = 5).

and a standard of *N-trans*-feruloyl tyramine, used in the assay to compare.

3.4. Conclusions

Commercially available goji berries were analyzed and their phenolic profile characterized. Seven major phenolics were isolated and identified by means of NMR experiments. Six of such compounds turned out to be already known molecules. The remaining, and most abundant, antioxidant compound constituted an unreported molecule. It was identified as a dimer of *N*-feruloyl tyramine.

The discovery of a dimer as the major antioxidant compound in commercially available goji berries is of great interest. Divalent molecules in fact show enhanced biological activities when compared to their corresponding monomers. Hence, more in-depth studies on the bioactivities of *N*-feruloyl tyramine dimers need to be conducted, since the occurrence of such molecules might amplify the nutritive value of goji berries, whose consume has been steadily growing across the planet.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.08.129.

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