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Enhanced Profiling of Flavonol Glycosides in the Fruits of Sea Buckthorn (*Hippophae rhamnoides*)

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ABSTRACT: Use of enhanced LC−MS/MS methods to identify common glycosyl groups of flavonoid glycosides enabled better characterization of the flavonoids in fruits of sea buckthorn (*Hippophae rhamnoides*). The saccharide moieties of 48 flavonol *O*-glycosides detected in a methanol extract were identified by these methods. Several of the flavonol glycosides were acylated, two of which were isolated and found to be new compounds. Their structures were determined using spectroscopic and chemical methods as isorhamnetin 3-*O*-(6-*O*-*E*-sinapoyl-*β*-D-glucopyranosyl)-(1→2)-*β*-D-glucopyranoside-7-*O*-*α*-L-rhamnopyranoside (**24**) and isorhamnetin 3-*O*-(6-*O*-*E*-feruloyl-*β*-D-glucopyranosyl)-(1→2)-*β*-D-glucopyranoside-7-*O*-*α*-L-rhamnopyranoside (**30**). Analysis of the acylated glycosyl groups of **24** and **30** by serial mass spectrometry provided evidence to suggest the acylation position of 11 other minor flavonol glycosides acylated with hydroxycinnamic or hydroxybenzoic acids. The nitric oxide scavenging activities of **24** and **30** were compared with those of other flavonoids and with ascorbic acid and the potassium salt of 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1*H*-imidazolyl-1-oxy-3-oxide (carboxy-PTIO).

KEYWORDS: *acylated flavonol glycoside, isorhamnetin, LC−MS, NMR, heteronuclear two-bond correlation spectroscopy, nitric oxide scavenging activity*

INTRODUCTION

Hippophae rhamnoides L. (sea buckthorn), which belongs to the family Elaeagnaceae, is a deciduous shrub or tree native to temperate regions of Asia and Europe. The fruits are considered to be a good source of nutrients and bioactive substances such as vitamins (A, C, E, K, riboflavin and folic acid), carotenoids, lipids, sterols, triterpenes and flavonoids.[1−3] Sea buckthorn is traditionally used in ethnomedicinal remedies for diseases of the skin and digestive system.[4] The fruit pulp of *H. rhamnoides* has been shown to promote wound healing activity in vivo.[5]

Flavonoid glycosides are among the predominant polyphenols present in the fruits of *H. rhamnoides* and have been analyzed by various techniques including high-speed countercurrent chromatography (HSCCC), liquid chromatography coupled to diode array detection and serial mass spectrometry (LC−UV−MS/MS), and a combination of these methods (HSCCC/LC−MS/MS).[6−8] LC−UV−MS/MS analysis of pomace extracts enabled more than 30 flavonol *O*-glycosides to be detected, comprising mono-, di- and triglycosides, many of which were acylated with hydroxycinnamic and hydroxybenzoic acids.[6] HSCCC/LC−MS/MS of juice concentrates revealed the presence of flavonol *O*-glycosides acylated with several groups, including malic and oxalic acids. However, for most of these acylated flavonol glycosides, and for many of the nonacylated compounds, the glycosidic linkages and position of any acylation have not been investigated fully.[6,8]

During a project conceived to investigate the potential of plant extracts in skincare products, we isolated two of the acylated flavonol glycosides from *H. rhamnoides* fruits. Both were derivatives of isorhamnetin (3,5,7,4′-tetrahydroxy-3′-methoxyflavone) and their structures, determined using NMR spectroscopy and chemical methods, had not been reported previously from any natural source. They appeared to correspond to two acylated flavonol glycosides detected in an earlier study of sea buckthorn fruits,[6] which were identified tentatively as derivatives of dimethyl ethers of quercetin (3,5,7,3′,4′-pentahydroxyflavone). Revision of these structures prompted reinvestigation of the minor flavonoids in *H. rhamnoides* fruits with the benefit of recently improved LC−MS/MS methods for characterizing common glycosyl groups of flavonol *O*-glycosides.[9,10] On this basis, the saccharide moieties of 48 flavonoid glycosides detected in an extract were determined. In addition to the two isolated compounds, evidence was also obtained to indicate the acylation position of 11 of the flavonoid glycosides that were acylated with hydroxycinnamic and hydroxybenzoic acids.

In order to evaluate the potential of flavonoids and flavonoid-containing fractions from sea buckthorn fruits as ingredients of dietary or health-care products, an in vitro nitric oxide (NO) scavenging assay was used. NO is a cell-signaling molecule generated at high levels by epithelial keratinocytes in response to interferon-gamma and tumor necrosis factor-alpha; it has both cytostatic and cytotoxic actions in skin and is involved in intercellular calcium dynamics.[11] It plays a vital role in host defense and immunity, including the modulation of inflammatory responses. Following exposure of skin to UV irradiation, NO plays a major role in the development of erythema, edema, and melanogenesis.[12] Excessive NO is produced during the course of a variety of rheumatic diseases.[13] The use of natural products to moderate or prevent the adverse effects of excessive NO production is therefore of considerable interest.

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Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data for 24 and 30 (MeOH-d_4, 30 °C)

		24		30	
atom		δ ^{1}H (J in Hz)	δ ^{13}C	δ ^{1}H (J in Hz)	δ ^{13}C
aglycone	2		158.7		158.7
	3		135.3		135.3
	4		179.9		179.9
	5		162.7		162.8
	6	6.30 d (2.2)	100.3	6.32 d (2.2)	100.5
	7		163.3		163.4
	8	6.38 d (2.2)	95.4	6.44 d (2.2)	95.5
	9		157.7		157.8
	10		107.4		107.5
	1′		122.8		122.8
	2′	7.68 m	114.2	7.69 m	114.1
	3′		148.9		148.9
	4′		151.7		151.6
	5′	6.91 d (8.8)	116.5	6.90 d (8.7)	116.5
	6′	7.69 m	125.3	7.70 m	125.3
	3′-OMe	3.96 s	57.2	3.95 s	57.2
3-*O*-Glc	1	5.31 d (7.6)	100.5	5.38 d (7.6)	100.6
	2	3.66 dd (9.1, 7.6)	85.3	3.68 dd (9.1, 7.6)	85.0
	3	3.56 t (9.0)	77.8	3.58 t (9.0)	77.8
	4	3.31 m	71.3	3.33 m	71.3
	5	3.11 ddd (9.8, 5.4, 2.3)	78.4	3.16 ddd (9.6, 5.4, 2.4)	78.5
	6	3.62 dd (12.0, 2.3)	62.4	3.64 dd (12.0, 2.4)	62.4
		3.45 dd (12.1, 5.4)		3.48 m	
2^{Glc}-*O*-Glc	1	4.68 d (7.6)	106.4	4.70 d (7.5)	106.2
	2	3.37 dd (9.2, 7.6)	76.4	3.37 dd (9.0, 7.5)	76.3
	3	3.44 t (9.1)	78.0	3.44 t (9.0)	77.9
	4	3.32 m	72.1	3.32 m	72.1
	5	3.64 ddd (9.8, 7.0, 2.3)	75.7	3.61 m	75.7
	6	4.44 dd (11.8, 7.1)	64.7	4.38 dd (11.8, 6.4)	64.8
		4.33 dd (11.8, 2.3)		4.33 dd (11.8, 2.7)	
7-*O*-Rha	1	5.50 d (1.8)	99.9	5.50 d (1.7)	99.9
	2	4.07 dd (3.4, 1.8)	71.8	4.05 dd (3.4, 1.7)	71.8
	3	3.82 dd (9.3, 3.5)	72.2	3.82 dd (9.4, 3.4)	72.2
	4	3.47 t (9.4)	73.8	3.47 t (9.4)	73.8
	5	3.56 dd (9.4, 6.1)	71.2	3.57 m	71.3
	6	1.23 d (6.1)	18.2	1.24 d (6.1)	18.2
O-Sin/Fer	α	7.27 d (15.8)	146.8	7.29 d (15.9)	146.7
	β	6.02 d (15.8)	115.4	6.00 d (15.9)	114.9
	CO		168.8		168.9
	1		126.3		127.4
	2	6.47 s	106.3	6.77 d (1.8)	111.2
	3		149.2		149.2
	4		139.5		150.7
	5		149.2	6.63 d (8.2)	116.4
	6	6.47 s	106.3	6.70 dd (8.2, 1.8)	124.0
	3-OMe	3.76 s	56.7	3.78 s	56.4
	5-OMe	3.76 s	56.7		

MATERIALS AND METHODS

General Instrumentation. LC−MS analysis was carried out with an Accela LC-system (autosampler, pump and photodiode array detector) coupled to a LTQ-Orbitrap XL hybrid linear ion trap-orbitrap mass analyzer fitted with an Ion Max electrospray ionization (ESI) source (Thermo Scientific, Waltham MA, USA). Samples (5 μL) were injected onto a RP C18 column (Phenomenex Luna C18(2), 150 × 3 mm i.d., 3 μm particle size) and eluted at 0.4 mL min^{-1} and 30 °C using a linear gradient of MeOH, H_2O and MeCN with 1% formic acid (0:90:10 − 40:50:10 v/v over 30 min) followed by a 5 min column wash (90:0:10) and equilibration in start conditions for 3 min before the next injection. In initial analyses, MS1 spectra at 30 000 resolution were recorded in the range m/z 250−2000 by the orbitrap in either positive or negative modes in separate analyses. Simultaneously with the high resolution analysis, the linear ion-trap recorded low resolution MS1 (m/z 125−2000), MS2 and MS3 spectra in both positive and negative modes. For MS2 spectra, precursor ions were automatically selected by the instrument control software (Xcalibur 2.0) in order of abundance, while MS3 spectra were requested on the three most abundant ions in each MS2 spectrum. More focused analyses in negative mode were performed with manual programming to acquire the MS^n spectra needed to identify certain 3-*O*-glycosides (the rutinosides, sophorosides, 6-rhamnosylsophorosides, 2-glucosylrutinosides and various acylated sophorosides) of isorham-

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netin, kaempferol, myricetin and quercetin and their derivatives bearing an additional monosaccharide at C-7. In positive mode, required MS^n spectra of the sodiated species of these flavonoids were also acquired by manual programming, as were spectra of other flavonol glycosides noted in the initial analyses for which the required MS^n spectra had not been obtained automatically. MS^n spectra in both positive and negative modes were recorded using an ion isolation width of ±2 *m/z* and a normalized collision energy of 35%.

NMR spectra were acquired in MeOH-d_4 at 30 °C on a Bruker Avance 400 MHz instrument or a Bruker Avance II$^+$ 700 MHz instrument equipped with a TCI-Cryoprobe. Standard pulse sequences and parameters were used to obtain 1D ^{1}H, 1D ^{13}C, 1D selective NOE, COSY, TOCSY, HSQC, H2BC and HMBC spectra. For the TOCSY experiments, mixing times of 60 and 100 ms were used. Chemical shift referencing was carried out using the internal solvent resonances at δ_H 3.31 and δ_C 49.1 (calibrated to TMS at 0.00 ppm).

Semipreparative HPLC was carried out on a Waters system comprising a 717plus autosampler, 2996 photodiode array detector and 600 controller (Waters, Milford MA, USA), fitted with a SUPELCO Discovery RP C18 column of 250 × 10 mm i.d. and 5 μm particle size (Sigma-Aldrich, Gillingham, U.K.). For compound isolation, isocratic elution of MeOH:H_2O at 40:60 v/v over 30 min was used with a flow rate of 4 mL min^{-1}, and a column temperature of 30 °C.

Sample Preparation and Isolation. The fruits of *Hippophae rhamnoides* L. were acquired from the living collections at the Royal Botanic Gardens, Kew (RBG Kew) in November 2009; accession no. 1979-6576. The tree was verified by Brian Stannard (RBG Kew), and voucher material of the fruit (BI-19216) has been retained at the Jodrell Laboratory, RBG Kew. Fresh fruits of *H. rhamnoides* (300 g) were blended and exhaustively extracted with 2 L of MeOH three times (24 h per time) to yield a crude extract (12 g), which was dissolved in H_2O and partitioned against $CHCl_3$. The H_2O layer was concentrated by rotary evaporation and further fractionated by Biotage flash chromatography (70 g of isolute sorbent C18 No. 9451-1000, 95 × 37 mm i.d., particle size 40−70 μm) using a linear gradient of MeOH and H_2O (20:80 to 100:0 v/v; 40 mL/fraction). Eight fractions were collected, and further purification of the fractions was by semipreparative HPLC. Fraction 2 yielded **24** (4.1 mg), **30** (3.4 mg) and **29** (2 mg) as yellow amorphous solids, while fraction 3 yielded **45** (4.0 mg) and **46** (1.6 mg). Because of the complexity of the extract, the maximum purity achieved for **24** and **30** was approximately 80%.

Sugar Analysis. Acid hydrolysis of **24** and **30** (1 mg in 20 μL MeOH) was carried out by standard procedures (0.5 mL 2 M HCl, 100 °C, 2 h). After cooling, particulates were spun down by microcentrifugation, and the supernatant was removed and dried under a stream of N_2. The absolute configuration of the constituent monosaccharides released by acid hydrolysis was determined from GC−MS analysis of trimethylsilylated thiazolidine derivatives, which were prepared using the method described by Ito and co-workers.[14] Conditions for GC were as follows: capillary column, DB5-MS (30 m × 0.25 mm × 0.25 μm); oven temp program, 180−300 °C at 6 °C/min; injection temp, 350 °C; carrier gas, He at 1 mL/min. The acid hydrolysates of **24** and **30** gave L-rhamnose and D-glucose (L-rhamnose, t_R = 10.27 min; D-glucose, t_R = 12.04 min; identical to authentic standards).

*Isorhamnetin 3-O-(6-O-E-sinapoyl-β-D-glucopyranosyl)-(1→2)-β-D-glucopyranoside-7-O-α-L-rhamnopyranoside (**24**).* Yellow amorphous solid: UV (LC−PDA) λ_{max} (nm) 243, 267sh, 333; LC−HRESIMS *m/z* 991.2711 [M − H]$^-$ (calcd for $C_{45}H_{51}O_{25}^-$ = 991.2725); ion trap MS/MS of *m/z* 991 [M − H]$^-$, *m/z* (rel. int.) 845 [(M − H) − Rha]$^-$ (100); ion trap MS3 (*m/z* 991 → 845), *m/z* (rel. int.) 653 (100), 639 (loss of sinapoyl) (97), 621 (loss of sinapic acid) (10), 515 (23), 459 (10); 329 (14), 315 [isorhamnetin − H]$^-$ (19), 314 [isorhamnetin − 2H]$^-$ (10); ^{1}H and ^{13}C NMR (MeOH-d_4) see Table 1.

*Isorhamnetin-3-O-β-D-glucopyranoside-7-O-α-L-rhamnopyranoside (**29**).* Yellow amorphous solid: UV (LC−PDA) λ_{max} (nm) 255, 265sh, 353; LC−HRESIMS *m/z* 623.1614 [M − H]$^-$ (calcd for $C_{28}H_{31}O_{16}^-$ = 623.1618); ion trap MS/MS of *m/z* 623 [M − H]$^-$, *m/z* (rel. int.) 477 [(M − H) − Rha]$^-$ (100), 461 [(M − H) − Glc]$^-$ (40), 315 [isorhamnetin − H]$^-$ (10); ion trap MS3 (*m/z* 623 → 477), *m/z* (rel. int.) 357 (20), 315 [isorhamnetin − H]$^-$ (20), 314 [isorhamnetin − 2H]$^-$ (100); ion trap MS3 (*m/z* 623 → 461), *m/z* (rel. int.) 315 [isorhamnetin − H]$^-$ (100); ^{1}H NMR data in agreement with literature.[6]

*Isorhamnetin 3-O-(6-O-E-feruloyl-β-D-glucopyranosyl)-(1→2)-β-D-glucopyranoside-7-O-α-L-rhamnopyranoside (**30**).* Yellow amorphous solid: UV (LC−PDA) λ_{max} (nm) 253, 268sh, 331. LC−HRESIMS *m/z* 961.2618 [M − H]$^-$ (calcd for $C_{44}H_{49}O_{24}^-$ = 961.2619); ion trap MS/MS of *m/z* 961 [M − H]$^-$, *m/z* (rel. int.) 815 [(M − H) − Rha]$^-$ (100); ion trap MS3 (*m/z* 961 → 815), *m/z* (rel. int.) 653 (80), 639 (loss of feruloyl) (100), 621 (loss of ferulic acid) (12), 485 (20), 459 (14); 329 (11), 315 [isorhamnetin − H]$^-$ (22), 314 [isorhamnetin − 2H]$^-$ (10); ^{1}H and ^{13}C NMR (MeOH-d_4) see Table 1.

*Isorhamnetin-3-O-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**45**).* Yellow amorphous solid: UV (LC−PDA) λ_{max} (nm) 255, 265sh, 300sh, 355; LC−HRESIMS *m/z* 623.1613 [M − H]$^-$ (calcd for $C_{28}H_{31}O_{16}^-$ = 623.1618); Ion trap MS/MS of *m/z* 623 [M − H]$^-$, *m/z* (rel. int.) 315 [isorhamnetin − H]$^-$ (100), 300 (22), 271 (9), 255 (5); ^{1}H NMR data in agreement with literature.[15]

*Isorhamnetin-3-O-β-D-glucopyranoside (**46**).* Yellow amorphous solid: UV (LC−PDA) λ_{max} (nm) 255, 265sh, 300sh, 354; LC−HRESIMS *m/z* 477.1036 [M − H]$^-$ (calcd for $C_{22}H_{21}O_{12}^-$ = 477.1039); ion trap MS/MS of *m/z* 477 [M − H]$^-$, *m/z* (rel. int.) 357 (22), 315 [isorhamnetin − H]$^-$ (27), 314 [isorhamnetin − 2H]$^-$ (100), 299 (6), 285 (8), 271 (6); ^{1}H NMR data in agreement with literature.[16]

Nitric Oxide Scavenging Assay. The in vitro NO scavenging assay was adapted from a published protocol that uses photolysis of sodium nitroprusside (SNP) to produce NO,[17] in a 96-well microwell format. The NO released is competitively captured by the fluorescent indicator DAF-FM (4-amino-5-methylamino-2′,7′-difluorofluorescein) in the presence of various concentrations of a plant extract, fractions or compounds, under aerobic conditions.[18] A reduction in fluorescence yields can be correlated with the scavenging effect of the samples. Briefly, 50 μL of test sample in 4% aqueous DMSO or only the DMSO solvent were placed in duplicate in an opaque 96-well microplate. Samples were tested at 20, 80, 160, 400, and 800 μM for compounds or 2 μg/mL to 3.2 mg/mL for extracts. In addition to the DMSO solvent blanks, wells were prepared to measure background fluorescence; these contained 50 μL of 4% DMSO and 100 μL of water.[18] To all the wells was then added 50 μL of 3 μM DAF-FM in 0.05% aqueous DMSO. The wells containing test samples and the solvent blank received 100 μL each of 20 mM SNP in water. The plate was shaken to mix the reagent solutions and left in ambient temperature and light, supplemented with a fluorescent lamp, for 11 min without a lid. The fluorescence emission at 538 nm from the DAF-FM−NO adduct ("DAF-FM T")[18] was measured immediately after this period, with excitation at 485 nm, using an infinite M200 microplate reader (Tecan; Reading, U.K.). The percentage reduction in the fluorescence yields (percentage NO scavenging) for each sample concentration was calculated using the following equation:

$$\text{percentage NO scavenging} = [(F_{DMSO} - F_{sample})/(F_{DMSO} - F_{blank})] \times 100$$

where F_{sample}, F_{DMSO}, and F_{blank} are average readings for test samples at a given concentration, DMSO solvent blanks and background fluorescence blanks, respectively. The activities of tested samples were expressed as the half maximal effective concentration (EC_{50}). EC_{50} values and 95% confidence intervals were calculated with Sigmoidal dose−response curve fitting performed using GraphPad Prism 5 for Windows, GraphPad Software, San Diego, CA, USA.

A NO-trapping agent 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1*H*-imidazolyl-1-oxy-3-oxide, monopotassium salt (carboxy-PTIO) was used as an assay control with EC_{50} = 6.8 μg/mL (see Figure 1 and Table 2). In addition, myricetin was used as a test substance to compare its free radical scavenging activity with that of

flavonoids isolated from *H. rhamnoides*.[19] Compounds were tested at different maximum concentrations subject to their solubility in water.

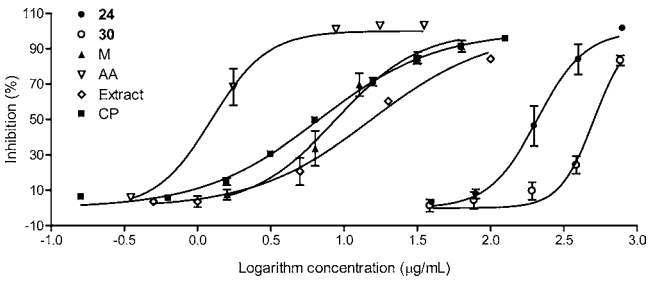

Figure 1. Sigmoidal dose–response curves of semipurified (ca. 80%) **24** and **30** compared with myricetin (M), ascorbic acid (AA), a crude MeOH extract of *H. rhamnoides* and the positive control, carboxy-PTIO (CP).

Table 2. Nitric Oxide Scavenging Activities of Isolated Constituents and Selected Compounds

compounds	EC_{50} (μg/mL)[a]
AA	1.2 (1.0–1.6)
CP	6.8 (6.2–7.3)
M	9 (7–11)
extract[b]	15 (12–20)
24	208 (177–246)
30	509 (458–567)

[a]EC_{50} values were determined graphically using sigmoidal dose–response curve fitting performed by GraphPad Prism 5; 95% confidence intervals are given in parentheses; AA = ascorbic acid, CP = carboxy-PTIO, M = myricetin. [b]Crude MeOH extract of *H. rhamnoides* fruits.

RESULTS AND DISCUSSION

Flavonoids Detected by LC–MS. Negative ion LC–MS analysis of an 80:20 MeOH/H_2O extract of fruits revealed numerous flavonoids (Table 3), of which three were very abundant (**29**, **45** and **46**) and a further six (**6**, **8**, **24**, **25**, **30** and **34**) were recorded above 1% relative abundance (calculated from the ion count of the molecular ion species). The major flavonoids were isorhamnetin-3-*O*-glucoside-7-*O*-rhamnoside (**29**), isorhamnetin 3-*O*-rutinoside (**45**) and isorhamnetin 3-*O*-glucoside (**46**), as identified previously.[6] Of the other six more abundant flavonoids, **6** and **8** were identified as kaempferol 3-*O*-sophoroside-7-*O*-rhamnoside and isorhamnetin 3-*O*-sophoroside-7-*O*-rhamnoside,[9,10] with the assignment of **6** being supported by coelution with a standard. The molecular formulas (calculated from $[M - H]^-$) of **24**, **25**, **30** and **34**, and their UV spectra (where these were obtained without interference from coeluting compounds), indicated that these flavonol glycosides were acylated with hydroxycinnamic acids.[20] The *m*/*z* values of their deprotonated molecules, *m*/*z* 991 (**24**), 961 (**25** and **30**) and 931 (**34**), corresponded to acylated flavonoids for which tentative structures were suggested by Rösch et al.[6] Of these, **24** and **30** were isolated, and their structures were determined by NMR spectroscopy as described in the following section. From an understanding of the MS^n fragmentation behavior gained from these two compounds, tentative structures were assigned to **25** and **34** (Table 3).

The negative ion LC–MS analysis of the fruit extract also detected numerous minor flavonoids at <1% relative abundance, of which 39 were investigated further. The molecular formulas of 25 of these, together with positive ion MS^n data, indicated that they were nonacylated flavonol glycosides, and the majority could be assigned structures using recently published enhanced LC–MS methods for the identification of common glycosyl groups of flavonoids,[9,10] although the location of the glycosyl groups at C-3 and C-7 had to be assumed as it was not possible to obtain diagnostic UV spectra. The distribution of glycosyl groups, at one or two positions, could however be determined from MS2 analyses of $[M + Na]^+$, and for di-*O*-glycosides the predominant loss from $[M - H]^-$ following MS2 indicated the type of sugar linked at C-7.[9] Hexose residues were assigned as glucose (Glc) from the findings of Rösch et al.,[6] who reported that Glc was the only hexose sugar released following hydrolysis of a total flavonoid preparation of *H. rhamnoides* fruits; the identification of Glc as the hexose residue in *O*-linked disaccharides was further supported by the product ion spectra of the sodiated glycosyl group.[10] The structures assigned to the minor flavonoid glycosides **3**, **9**, **10**, **20**, **35**, **37**, **42** and **44** were further supported by comparison of analytical data with standards.

The majority of the minor components detected were glycosides of either kaempferol, quercetin or isorhamnetin, although as noted by Rösch et al.,[6] the rutinosides of myricetin (**12**) and aglycones whose molecular masses suggested mono- and dimethylethers of myricetin were also present. The product ion spectra of the protonated aglycone, obtained by MS^n of $[M + H]^+$, of **38** and **47** matched the spectra of laricitrin (myricetin 3′-methyl ether) and syringetin (myricetin 3′,5′-dimethyl ether), respectively. However we were unable to compare these with the spectra of mearnsetin (myricetin 4′-methyl ether) and myricetin 3′,4′-dimethyl ether, which are likely to be similar to those of laricitrin and syringetin, respectively; thus, the aglycone assignments are tentative.

Of the 14 minor acylated flavonol glycosides investigated, nine were assigned as being acylated with hydroxycinammic acids, while five were considered to be acylated with malic acid (Table 3). Evidence for the latter came from accurate mass MS2 of $[M - H]^-$, which recorded the neutral loss of $C_4H_4O_4$, for which the most likely candidate is malyl.[8] The malylated compounds were all derivatives of isorhamnetin diglycosides, and fragmentation of the $[(M - H) - malyl]^-$ ion suggested that the aglycone bore a 3-*O*-rutinosyl group in three of the compounds (**41**, **43**, **48**) but was substituted at two positions with 3-*O*-Glc and 7-*O*-Rha in the other two (**36**, **40**). The location of the malyl group could not be determined by MS.

Structural Elucidation of Compounds 24 and 30. The UV spectra of **24** (λ_{max}: 243, 267sh, 333 nm) and **30** (λ_{max}: 253, 268sh, 331 nm) and the corresponding MS^n data suggested that these compounds were acylated isorhamnetin triglycosides. In negative mode, both showed a predominant neutral loss of 146 Da following MS^2, which was determined as $C_6H_{10}O_4$ by high resolution measurements, suggesting the presence of a rhamnose moiety (Rha). Following MS3, the Rha-loss ion showed neutral losses of 206 Da in **24** and 176 Da in **30**, suggesting losses of sinapoyl and feruloyl, respectively. Further stages of serial MS^9 gave spectra typical of deprotonated isorhamnetin 3-*O*-sophoroside. Analysis of the aromatic proton resonances in the 1D ^1H NMR spectrum of **24**, the corresponding ^{13}C NMR chemical shifts obtained by HSQC, and long-range ^1H–^{13}C connectivities detected by HMBC confirmed that the aglycone was isorhamnetin. The site of *O*-methylation on the B-ring was also established from a ROE

Table 3. Flavonoids Detected in an 80:20 MeOH/H_2O Extract of Dried Fruits of *Hippophae rhamnoides* by Negative Ion LC–MS^n Analyses[a]

no.	t_R (min)	$[M-H]^-$ (m/z)	$[M-H]^-$ formula	rel pk ht (%)	agly[b]	3-*O*-glycoside[c]	7-*O*-glycoside	det[d]
1	8.21	917.2568	$C_{39}H_{49}O_{25}$	<0.1	Q	β-Glc(1→2)[α-Rha(1→6)]-β-Glc-	α-Rha-	MS
2	9.07	771.1989	$C_{33}H_{39}O_{21}$	0.7	Q	β-Glc(1→2)-β-Glc-	α-Rha-	MS[e]
3	10.10	901.2633	$C_{39}H_{49}O_{24}$	0.1	K	β-Glc(1→2)[α-Rha(1→6)]-β-Glc-	α-Rha-	Std
4	10.58	639.1571	$C_{28}H_{31}O_{17}$	0.2	I	β-Glc-	β-Glc-	MS
5	10.91	931.2720	$C_{40}H_{51}O_{25}$	0.2	I	β-Glc(1→2)[α-Rha(1→6)]-β-Glc-	α-Rha-	MS
6	10.96	755.2034	$C_{33}H_{39}O_{20}$	2.3	K	β-Glc(1→2)-β-Glc-	α-Rha-	Std[e]
7	10.96	785.2149	$C_{34}H_{41}O_{21}$	0.6	I	α-Rha(1→6)-β-Glc-	β-Glc-	MS
8	11.92	785.2141	$C_{34}H_{41}O_{21}$	6.8	I	β-Glc(1→2)-β-Glc-	α-Rha-	MS[e]
9	12.50	771.1996	$C_{33}H_{39}O_{21}$	0.2	Q	β-Glc(1→2)[α-Rha(1→6)]-β-Glc-		Std
10	14.83	755.2037	$C_{33}H_{39}O_{20}$	0.3	Q	α-Rha(1→2)[α-Rha(1→6)]-β-Glc-		Std
11	14.99	609.1466	$C_{27}H_{29}O_{16}$	0.2	Q	β-Glc-	α-Rha-	MS
12	15.21	625.1417	$C_{27}H_{29}O_{17}$	0.1	M	α-Rha(1→6)-β-Glc-		MS
13	15.58	755.2046	$C_{33}H_{39}O_{20}$	0.1	K	β-Glc(1→2)[α-Rha(1→6)]-β-Glc-		MS
14	15.75	785.2153	$C_{34}H_{41}O_{21}$	0.3	I	β-Glc(1→2)[α-Rha(1→6)]-β-Glc-		MS[e]
15	16.07	905.2367	$C_{41}H_{45}O_{23}$	0.2	I	6-*O*-hydroxybenzoyl-β-Glc(1→2)-β-Glc-	α-Rha-	MS
16	16.50	977.2583	$C_{44}H_{49}O_{25}$	0.4	Q	6-*O*-sinapoyl-β-Glc(1→2)-β-Glc-	α-Rha-	MS[e]
17	17.10	947.2466	$C_{43}H_{47}O_{24}$	0.1	Q	6-*O*-feruloyl-β-Glc(1→2)-β-Glc-	α-Rha-	MS[e]
18	17.21	917.2365	$C_{42}H_{45}O_{23}$	0.1	Q	6-*O*-*p*-coumaroyl-β-Glc(1→2)-β-Glc-	α-Rha-	MS[e]
19	17.48	639.1575	$C_{28}H_{31}O_{17}$	0.1	I	β-Glc(1→2)-β-Glc-		MS[e]
20	17.69	739.2094	$C_{33}H_{39}O_{19}$	0.3	K	α-Rha(1→2)[α-Rha(1→6)]-β-Glc-		Std[e]
21	18.02	769.2196	$C_{34}H_{41}O_{20}$	0.9	I	α-Rha(1→2)[α-Rha(1→6)]-β-Glc-		MS[e]
22	18.02	1137.3321	$C_{51}H_{61}O_{29}$	0.8	I	6-*O*-sinapoyl-β-Glc(1→2)[α-Rha(1→6)]-β-Glc-	α-Rha-	MS[e]
23	18.39	593.1513	$C_{27}H_{29}O_{15}$	0.9	K	β-Glc-	α-Rha-	MS
24	18.39	991.2711	$C_{45}H_{51}O_{25}$	9.6	I	6-*O*-sinapoyl-β-Glc(1→2)-β-Glc-	α-Rha-	NMR[e]
25	18.44	961.2618	$C_{44}H_{49}O_{24}$	1.4	K	6-*O*-sinapoyl-β-Glc(1→2)-β-Glc-	α-Rha-	MS[e]
26	18.50	739.2096	$C_{33}H_{39}O_{19}$	0.1	K	α-Rha(1→6)-β-Glc-	α-Rha-	MS
27	18.61	1107.3218	$C_{50}H_{59}O_{28}$	0.6	I	6-*O*-feruloyl-β-Glc(1→2)[α-Rha(1→6)]-β-Glc-	α-Rha-	MS[e]
28	18.82	1077.3104	$C_{49}H_{57}O_{27}$	0.3	I	6-*O*-*p*-coumaroyl-β-Glc(1→2)[α-Rha(1→6)]-β-Glc-	α-Rha-	MS[e]
29	19.07	623.1614	$C_{28}H_{31}O_{16}$	14.8	I	β-Glc-	α-Rha-	NMR
30	19.07	961.2618	$C_{44}H_{49}O_{24}$	1.4	I	6-*O*-feruloyl-β-Glc(1→2)-β-Glc-	α-Rha-	NMR[e]
31	19.14	931.2520	$C_{43}H_{47}O_{23}$	0.1	K	6-*O*-feruloyl-β-Glc(1→2)-β-Glc-	α-Rha-	MS[e]
32	19.32	901.2413	$C_{42}H_{45}O_{22}$	0.1	K	6-*O*-*p*-coumaroyl-β-Glc(1→2)-β-Glc-	α-Rha-	MS[e]
33	19.38	769.2195	$C_{34}H_{41}O_{20}$	0.9	I	α-Rha(1→6)-β-Glc-	α-Rha-	MS[e]
34	19.38	931.2507	$C_{43}H_{47}O_{23}$	5.6	I	6-*O*-*p*-coumaroyl-β-Glc(1→2)-β-Glc-	α-Rha-	MS[e]
35	19.43	609.1461	$C_{27}H_{29}O_{16}$	0.5	Q	α-Rha(1→6)-β-Glc-		Std[e]
36	19.97	739.1729	$C_{32}H_{35}O_{20}$	0.5	I	malyl-β-Glc-	α-Rha-	MS
37	20.13	463.0885	$C_{21}H_{19}O_{12}$	0.6	Q	β-Glc-		Std
38	20.45	639.1575	$C_{28}H_{31}O_{17}$	0.1	MeM	α-Rha(1→6)-β-Glc-		MS[e]
39	20.94	593.1514	$C_{27}H_{29}O_{15}$	0.2	I	Pentose	α-Rha-	MS
40	21.05	739.1737	$C_{32}H_{35}O_{20}$	0.1	I	malyl-β-Glc-	α-Rha-	MS
41	22.50	739.1737	$C_{32}H_{35}O_{20}$	0.1	I	malyl-[α-Rha(1→6)-β-Glc]-		MS
42	23.58	593.1512	$C_{27}H_{29}O_{15}$	0.6	K	α-Rha(1→6)-β-Glc-		Std[e]
43	23.80	739.1737	$C_{32}H_{35}O_{20}$	0.1	I	malyl-[α-Rha(1→6)-β-Glc]-		MS
44	24.02	447.0937	$C_{21}H_{19}O_{11}$	0.3	K	β-Glc-		Std
45	24.27	623.1613	$C_{28}H_{31}O_{16}$	25.1	I	α-Rha(1→6)-β-Glc-		NMR[e]
46	24.60	477.1036	$C_{22}H_{21}O_{12}$	20.4	I	β-Glc-		Std
47	24.74	653.1726	$C_{29}H_{33}O_{17}$	0.2	diMeM	α-Rha(1→6)-β-Glc-		MS[e]
48	25.28	739.1732	$C_{32}H_{35}O_{20}$	0.6	I	malyl-[α-Rha(1→6)-β-Glc]-		MS

[a]Data provided are HPLC retention times (t_R), accurate masses and determined ionic formulas of $[M-H]^-$, relative ion abundance of $[M-H]^-$ (or $[M+HCOO]^-$ for **1**–**3**, **5**–**8**) at maximum peak height (rel pk ht), identity of aglycones (agly) and *O*-linked sugars at C-3 and C-7, and structural assignments (det), noting whether these are supported by data from NMR, MS or comparison with standards (Std). [b]Aglycones: I = isorhamnetin, K = kaempferol, M = myricetin, MeM = myricetin 3′-methyl ether (laricitrin) or myricetin 4′-methyl ether (mearnsetin), diMeM = myricetin 3′,5′-dimethyl ether (syringetin) or myricetin 3′,4′-dimethyl ether, Q = quercetin. [c]The primary sugar (*O*-linked at C-3) is the last listed in each entry. [d]Comparative data for standards were obtained either from purchased compounds or by analysis of the leaf or fruit extract of *Styphnolobium japonicum*, from which they had been isolated and fully characterized by NMR.[28,29] [e]The 3-*O*-glycosyl groups of these flavonoids were additionally supported by serial MS of the sodiated molecule;[10] otherwise, all MS determinations of the 3-*O* glycosyl group were supported by serial MS of the deprotonated molecule.[9]

correlation between H-2′ (δ_H 7.68) and OCH_3 (δ_H 3.96). The ^{13}C NMR resonances assigned for the aglycone of **24** were similar to literature values for isorhamnetin acquired in MeOH-d_4,[21] except for the downfield shifts of C-2 ($\Delta\delta_C$ +11.6), C-4 ($\Delta\delta_C$ +3.6), C-6($\Delta\delta_C$ +1.7), C-8 ($\Delta\delta_C$ +1.5) and upfield shifts of C-3 ($\Delta\delta_C$ −0.8) and C-7 ($\Delta\delta_C$ −1.1), which were consistent with glycosylation at both C-3 and C-7 (see Table 1).

The ^1H NMR spectra of **24** and **30** each contained three anomeric proton resonances; in **24** they appeared at δ_H 5.31 (1H, d, J = 7.6 Hz, δ_C 100.5), δ_H 4.68 (1H, d, J = 7.6 Hz, δ_C 106.4), and δ_H 5.50 (1H, d, J = 1.8 Hz, δ_C 99.9); in **30** the corresponding resonances were at δ_H 5.38 (1H, d, J = 7.6 Hz, δ_C 100.6), δ_H 4.70 (1H, d, J = 7.5 Hz, δ_C 106.2) and δ_H 5.50 (1H, d, J = 1.7 Hz, δ_C 99.9). Acid hydrolysis of **24** and **30** followed by determination of absolute configuration for the constituent monosaccharides using GC−MS confirmed that these comprised D-Glc and L-Rha only. The magnitudes of the coupling constants for the anomeric protons in the ^1H NMR spectra of **24** and **30** indicated that the D-Glc and L-Rha residues were in the β- and α-configurations, respectively;[22] thus, each compound contained two β-D-Glc residues and one α-L-Rha residue.

The assignments of the glycosidic resonances for **24** and **30** were based on detailed interpretation of COSY, TOCSY, HSQC, HMBC and H2BC experiments (see Table 1). The multiplicities and coupling constants for the ^1H resonances of the sugar residues were as expected for β-D-Glc*p* and α-L-Rha*p* (see Table 1). Full assignment of the two β-D-Glc*p* residues was not straightforward, because their H-4 resonances at δ_H 3.31 (1H, m, δ_C 71.3) and δ_H 3.32 (1H, m, δ_C 72.1) were almost coincident. This ambiguity was resolved by using a heteronuclear two-bond correlation (H2BC) pulse sequence, which gave only $^2J_{HC}$ correlations between protons and proton-attached carbons, rather than the mixture of $^2J_{HC}$ and $^3J_{HC}$ correlations routinely observed in HMBC spectra.[23] As such the H2BC spectrum of **24** gave unequivocal $^2J_{HC}$ connectivities for each β-D-Glc*p* moiety from H-1 to C-2, H-2 to C-1 and C-3, H-3 to C-2 and C-4, and H-5 to C-4 and C-6, allowing C-4 and thus H-4 to be assigned unambiguously. A similar pattern of $^2J_{HC}$ connectivities was observed in the H2BC spectrum of **30**. The interglycosidic linkage defining the sophorose moiety of **24** was determined using HMBC data. Thus long-range correlations were detected from H-2 (δ_H 3.66) of primary Glc*p* to C-1 of terminal Glc*p* (δ_C 106.4), and from H-1 (δ_H 4.68) of terminal Glc*p* to C-2 (δ_{C2} 85.3) of primary Glc*p*. A similar pattern of correlations was detected for **30**.

Direct evidence for 7-*O*-glycosylation of the aglycone of **24** came from the long-range correlation detected in the HMBC spectrum between the anomeric proton resonance of α-L-Rha*p* at δ_H 5.50 (1H, d, J = 1.8 Hz, δ_C 99.9) and C-7 of isorhamnetin at δ_C 163.3; similarly between δ_H 5.50 and δ_C 163.4 of **30**. ROE correlations were detected between H-1 of α-L-Rha*p* (δ_H 5.50) and both H-6 (δ_H 6.30) and H-8 (δ_H 6.38) in **24**, and similarly between H-1 (δ_H 5.50) and both H-6 (δ_H 6.32) and H-8 (δ_H 6.44) in **30**. The sophorosyl moiety of both **24** and **30** was therefore *O*-linked at C-3 of the isorhamnetin aglycones.

The ^1H and ^{13}C NMR resonances assignments (see Table 1) of the hydroxycinnamoyl moiety of **24** were those of a sinapoyl residue,[24] as predicted by MS. The site of acylation was determined from the long-range correlations detected between 6-CH_2 (δ_H 4.44, 4.33) of the terminal β-D-Glc*p* residue of the 3-*O*-sophorosyl moiety and the carbonyl carbon of the sinapoyl residue (δ_C 168.8), in an HMBC experiment. Thus **24** was isorhamnetin 3-*O*-(6-*O*-*E*-sinapoyl-β-D-glucopyranosyl)-(1→2)-β-D-glucopyranoside-7-*O*-α-L-rhamnopyranoside) (Figure 2).

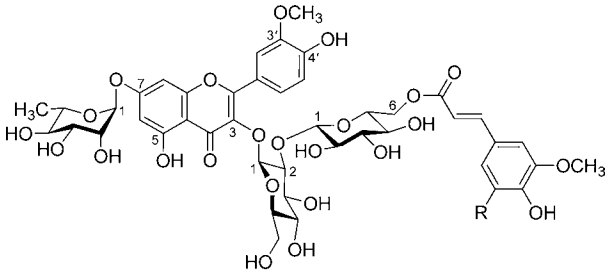

Figure 2. Acylated isorhamnetin triglycosides **24** and **30** from the fruits of *Hippophae rhamnoides*.

The NMR spectra of **30** were similar to those of **24** except for the resonances of the hydroxycinnamoyl moiety. In the ^1H NMR spectrum, the resonances at δ_H 6.77 (1H, d, J = 1.8 Hz), 6.70 (1H, dd, J = 8.2, 1.8 Hz), 6.63 (1H, d, J = 8.2 Hz), δ_H 7.29 (1H, d, J = 15.9 Hz), δ_H 6.00 (1H, d, J = 15.9 Hz) and δ_H 3.78 (3H, s) corresponded to an (*E*)-feruloyl moiety.[25] The site of acylation was determined from the long-range correlations observed between 6-CH_2 (δ_H 4.38, 4.33) of the terminal β-D-Glc*p* residue of the 3-*O*-sophorosyl moiety and the carbonyl carbon of the sinapoyl residue (δ_C 168.9), in an HMBC experiment. Compound **30** was therefore isorhamnetin 3-*O*-(6-*O*-*E*-feruloyl-β-D-glucopyranosyl)-(1→2)-β-D-glucopyranoside-7-*O*-α-L-rhamnopyranoside) (Figure 2). Compounds **24** and **30** are isolated from a natural source for the first time.

Assignments of Minor Flavonoid Glycosides Acylated with Hydroxycinnamic Acids by MS^n Analysis. The sodiated species $[M + Na]^+$ of **24** and **30** showed neutral losses of Rha and then isorhamnetin following MS^2 and MS^3 to give a sodiated ion of the acylglycosyl group. Following MS^4, this ion showed neutral losses of water and 90, 102, 144, and 162 Da arising from ring cleavage of Glc or cleavage of the glycosidic bond.[10] Essentially this pattern of ions was the same for both **24** and **30** with each ion in the spectrum of **24** being m/z 30 more than that in **30**; i.e., all the main fragments retained the acyl group. This ion pattern was readily observed following fragmentation of the sodiated acylglycosyl group of the acylated isorhamnetin glycoside **34**, with each ion being 30 m/z units less than in the spectrum of **30**. This indicated, together with relevant supporting data from negative ion MS^n analysis, that **34** was the coumaroyl analogue of **30**. Compound **25** showed the same sodiated acylglycosyl group spectrum as **24**, but its molecular mass was 30 Da less and it showed the neutral loss of kaempferol following MS3 of $[M + Na]^+$, with kaempferol as the aglycone being confirmed by MS^n analysis of $[M + H]^+$. This was assigned as the kaempferol analogue of **24**, again with supporting evidence from negative ion spectra to indicate the 7-*O*-Rha substitution and the 3-*O*-sophorosyl moiety. Among the minor flavonoids, the kaempferol (**31**) analogue of **30** and coumaroyl (**34**) analogue of **24** were evident, as were an analogous set of three acylated quercetin 3-*O*-sophoroside-7-*O*-rhamnosides (**16**−**18**).

Another set of three acylated isorhamnetin glycosides (**22**, **27** and **28**) was also noted among the minor flavonoids that had

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molecular masses 146 Da (Rha) greater that each of **24**, **30** and **34**. Following the loss of the 7-*O*-Rha and the acyl group through MS^2 and MS^3 of $[M - H]^-$, fragmentation of the resulting ion indicated a Glc-(1→2)-[Rha-(1→6)]-Glc moiety.[9] Serial neutral losses of Rha and then isorhamnetin were observed following MS^2 and MS^3 of $[M + Na]^+$ of **22**, **27** and **28**, and the resulting acylglycosyl group showed single losses of Rha and acylsophorosyl following MS^4, indicating that the acyl group was not on Rha. Fragmentation of the Rha-loss ion gave the same neutral losses as observed in the spectra of the sodiated acylglycosyl groups of **24**, **30** and **34**, respectively, although the ion abundances were different. Thus we cautiously suggest the same site of acylation and tentatively assign **22**, **27** and **28** as derivatives of **24**, **30** and **34** having an additional Rha at C-6 of the primary Glc.

A final acylated isorhamnetin glycoside (**15**) was noted, which essentially fragmented in a similar manner to **24** in positive and negative ion MS^n analysis, except that the data indicated a mass of 120 Da ($C_7H_4O_2$) for the acyl residue, which was tentatively assigned as hydroxybenzoyl.

Nitric Oxide Scavenging Activity. In addition to flavonols and flavonol glycosides, ascorbic acid was tested in the nitric oxide scavenging assay,[26] because it was reported to be the principal vitamin constituent of sea buckthorn fruits, with an average content of approximately 400 mg/100 g.[27] As seen in Figure 3, ascorbic acid was the most active component

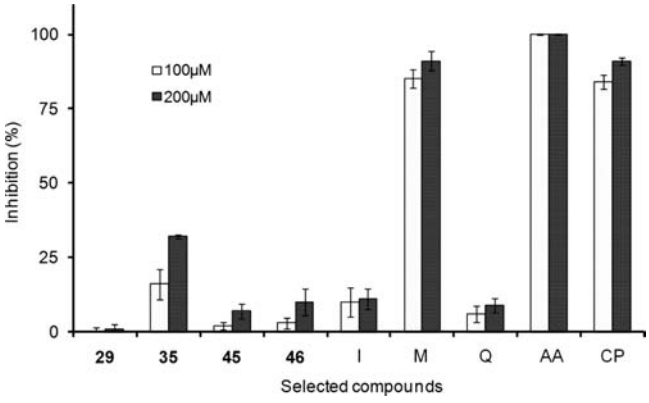

Figure 3. Comparison of NO scavenging activities of selected compounds (I = isorhamnetin, M = myricetin, Q = quercetin, AA = ascorbic acid, CP = carboxy-PTIO) (n = 3; ± SEM).

and gave 100% inhibition at 100 μM, while myricetin (3,5,7,3′,4′,5′-hexahydroxyflavone) showed comparable activities to carboxy PTIO (CP) with 91% inhibition at 200 μM. However, all the other flavonols showed no more than 32% inhibition at 200 μM. Because of the low solubility of some isolated flavonols in aqueous solution, only **24** and **30** were tested up to 800 μg/mL. Compounds **45** and **46** gave no more than 36% inhibition at 800 μM (data not shown), while **24** and **30** exhibited mild activity with EC_{50} values of 208 and 509 μg/mL, respectively, which were less than that of the MeOH extract (see Table 2). In terms of the aglycones, the most outstanding difference between myricetin and the other flavonols tested (quercetin and isorhamnetin) is the presence of three adjacent hydroxy groups on the B ring. This feature seems to be a key structure−activity determinant for NO scavenging activity of flavonols. However, a myricetin glycoside present in fruits of *H. rhamnoides* (Table 2) was only detected as a trace component (relative peak height of 0.1%). On the basis of the present observations, ascorbic acid is the principal contributor to the NO scavenging activity of the fruits of *H. rhamnoides*.

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Notes

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ABBREVIATIONS USED

AA, ascorbic acid; CP, carboxy-PTIO; diMeM, myricetin 3′,5′-dimethyl ether (syringetin) or myricetin 3′,4′-dimethyl ether; I, isorhamnetin; K, kaempferol; M, myricetin; MeM, myricetin 3′-methyl ether (laricitrin) or myricetin 4′-methyl ether (mearnsetin); Q, quercetin

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