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Short Communication

Anthocyanins from fruits of *Rubus pinnatus* and *Rubus rigidus*

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Abstract

The same anthocyanins, cyanidin 3-(6″-*O*-α-rhamnopyranosyl-β-glucopyranoside) (**1**), and cyanidin-3-*O*-β-glucopyranoside (**2**), were isolated from extracts of red fruits of *Rubus pinnatus* Willd. and purple-black fruits of *Rubus rigidus* Sm. using Amberlite XAD-7 column chromatography, Sephadex LH-20 gel filtration and preparative HPLC. Their structures were elucidated by a combination of chromatography, homo- and heteronuclear NMR techniques. The relative amounts of 1 and 2 in *R. rigidus* were 59.4% and 40.6%, respectively, while in *R. pinnatus*, the relative amounts were 58.6% and 41.4%, respectively.

Keywords: Fruits; *Rubus pinnatus*; *Rubus rigidus*; Rosaceae; Anthocyanins; Cyanidin 3-(6″-*O*-α-rhamnopyranosyl-β-glucopyranoside); Cyanidin 3-*O*-β-glucopyranoside

1. Introduction

The genus *Rubus* comprises about 250 species that are distributed worldwide (Brown, 2002; Sauer, 1993). Many species in the genus have fruits that are eaten fresh or as jam, jelly, syrup, wine, liqueur, etc. These fruits are known to be rich in vitamins A, B and C, while both the roots and leaves are claimed to have medicinal properties (Brown, 2002; Sauer, 1993). The dried leaves from the species of *Rubus* are made into tea and are used in herbal tea blends (Brown, 2002). In recent years the antioxidative capacities of a number of *Rubus* species have been investigated

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in vitro (Costantino et al., 1992; Deighton et al., 2000; Wang and Lin, 2000; Moyer et al., 2002a; b; Mullen et al., 2002; McGhie et al., 2002).

Several authors have characterized individual anthocyanins of various blackberry species and cultivars (Mazza and Miniati, 1993). There seems, however, to be discrepancy with respect to the nature of the individual anthocyanins; one or more of the 3-glucoside, 3-rutinoside, 3-xyloside and acylated derivatives of cyanidin, in addition to pelargonidin derivatives, have been reported (Mazza and Miniati, 1993; Seeram et al., 2001; Stintzing et al., 2002). Blackberries have generally been considered to contain an intermediate quantitative anthocyanin content, between the values for red and black raspberries (Torre and Barritt, 1977; Moyer et al, 2002b). The quantitative data presented by Deighton et al. (2000) revealed 4 and 0.2 g kg^{-1} (fresh weight) for black and red raspberry, respectively. In this context it is interesting to note that Melo et al. (2000) have shown that the colouration of raspberries is not based on co-pigmentation, but relies mainly on pH effects in the vacuoles. Jennings and Carmichael (1980) have reported on the inheritance of anthocyanins in various *Rubus* species, including blackberries.

The species *R. pinnatus* Willd. (South African blackberry) and *R. rigidus* Sm. (wild trailing blackberry) are widespread throughout tropical and subtropical Africa. However, the local languages make no distinction between them: Nkenene (Luganda), Luwambi (Lugisu), Encerere (Runyankore-Rukiga) and Amakerere (Runyoro and Lutoro) (Katende, et al., 1999). Both species have sweet edible fruits with colours ranging from red (*R. pinnatus*) to purple–black (*R. rigidus*). The fruits are collected from the wild (not cultivated) and eaten fresh (Katende, et al., 1999). Although anthocyanins contribute to the conspicuous colours of these fruits, their identities have not been previously reported. In this paper, we report the isolation and determination of the anthocyanins from these fruits. The methanolic extracts of *R. rigidus* have recently been found to inhibit the activity of both enzymes HIV1-reverse transcriptase (HIV1-RT) and TK p56(lck) (Tshibangu et al., 2002).

2. Materials and methods

2.1. Plant material

Fruits of *R. pinnatus* (300 g) were collected in Bushenyi District (Kabwohe) in Uganda, and fruits of *R. rigidus* (209 g) were collected in Kabale District (Ibumba), Uganda in February 2003. The identification of the two plants was carried out in the Botany Department at Makerere University, and voucher specimens were deposited in the herbarium of that Department. The fruits were kept in a freezer before analysis.

2.2. Isolation of pigments

The fruits of *R. pinnatus* and *R. rigidus* were separately extracted with 1% tetrafluoroacetic acid (TFA) in methanol. The filtered extract was concentrated under reduced pressure, purified by partition (several times) against ethyl acetate and applied to an Amberlite XAD-7 column. The anthocyanins adsorbed to the column were washed with water, and eluted from the column with methanol containing 1% TFA. The concentrated anthocyanin extract was purified by Sephadex

LH-20 chromatography using 50% aqueous methanol containing 1% TFA as eluent. The individual anthocyanins were separated using preparative HPLC.

2.3. High performance liquid chromatography

Preparative HPLC was performed with an ODS Hypersil column (25 × 2.2 cm; i.d.; 5 μm) using a Gilson pump (305/306) equipped with a UV 6000LP detector. Two solvents were used for elution: A = HCO_2H–H_2O (1:9; v/v) and B = HCO_2H–H_2O–MeOH (1:4:5; v/v). The elution profile consisted of a linear gradient from 10% to 100% B for 30 min, isocratic elution (100% B) for the next 9 min, followed by a linear gradient from 100% to 10% B for 1 min. The flow rate was 14 mL/min for 40 min and aliquots of 500 μL were injected.

The analytical HPLC results were obtained with an HP-1050 system (Hewlett Packard) using an ODS Hypersil column (25 × 0.4 cm; i.d.; 5 μm). The elution profile for analytical HPLC consisted of isocratic elution (90%A, 10% B) for 4 min, linear gradient from 10% to 100%B during the next 17 min and isocratic elution (100%B) for 4 min using solvents HCOOH-H_2O (1:9; v/v) (A) and HCOOH-H_2O-MeOH (1:4:5; v/v) (B). Aliquots of 15 μL were injected and the flow rate was 1.3 mL/min. Prior to injection, all samples were filtered through a 0.45 μm Millipore membrane filter. Pigments 1 and 2 were co-chromatographed with authentic cyanidin 3-rutinoside (14.6 min) and cyanidin 3-glucoside (14.2 min) from blackcurrant (*Ribes nigrum*), respectively (Frøytlog et al., 1998).

2.4. Spectroscopy

UV–VIS absorption spectra were recorded on-line during HPLC analysis, and the spectral measurements were made over the wavelength range 200–600 nm in steps of 2 nm. The NMR experiments were obtained at 600.13 and 150.92 MHz for ^1H and ^{13}C, respectively, on a Bruker DRX–600 instrument at 25°C. The deuterio-methyl ^{13}C signal and the residual ^1H signal of the solvent, CF_3COOD–CD_3OD (95:5; v/v), were used as secondary references (δ 49.0 and δ 3.4 ppm from tetramethylsilane for ^1H and ^{13}C, respectively (Fossen et al., 1998). The 1D ^1H NMR and the 2D HMBC, HSQC, COSY and TOCSY experiments were obtained with the 5 mm TB1 probe.

3. Results and discussion

The HPLC chromatogram of the weakly acidified methanolic extract of fruits of *R. pinnatus* detected in the visible spectral region revealed two anthocyanins, pigments 1 and 2. The UV–VIS spectra of both anthocyanins recorded on-line during HPLC analysis showed visible maxima around 520 nm, and their $A_{440}/A_{VIS\text{-}Max}$ were around 24%, indicating cyanidin or peonidin 3-glycosides (Andersen, 1987). The pigments were purified by partition against ethyl acetate and Amberlite XAD-7 column chromatography, and separated by Sephadex LH-20 column chromatography and preparative HPLC.

The downfield part of the 1D ^1H NMR spectrum of 1 showed a singlet at 9.03 ppm (H-4), a 3H AMX system at 8.35 ppm (*dd*, 8.5 Hz, 2.3 Hz; H-6′), 8.12 ppm (*d*, 2.3 Hz; H-2′) and 7.10 ppm (*d*, 8.5 Hz; H-5′) and an unresolved 2H AB system at 6.98 ppm (H-8) and 6.76 ppm (H-6), respectively

Table 1
^{1}H NMR spectra data for cyanidin 3-(6″-*O*-α-rhamnopyranosyl-β-glucopyranoside) (1) and cyanidin 3-*O*-β-glucopyranoside (2) in CD_3OD:CF_3COOD (95:5; v/v) at 25°C

Aglycone	δ (ppm), multiplicity & J_{HH} (Hz)	
	1	Pigment 2
4	9.03 *s*	9.11 *s*
6	6.76 *d* 2.0	6.75 *d* 2.0
8	6.98 *s* (broad)	6.98 *s* (broad)
2′	8.12 *d* 2.3	8.14 *d* 2.2
5′	7.10 *d* 8.8	7.12 *d* 8.6
6′	8.35 *dd* 8.5, 2.3	8.36 *dd* 8.5, 2.3
3-β-glucopyranoside		
1″	5.37 *d* 7.9	5.38 *d* 7.7
2″	3.74 *m*	3.76 *m*
3″	3.60 *m*	3.65 *m*
4″	3.48 *m*	3.53 *m*
5″	3.79 *m*	3.82 *m*
6A″	4.15 *dd* 10.1, 1.9	4.01 *dd* 9.8, 2.0
6B″	3.68 *m*	3.79 *m*
6″-α-rhamnopyranosyl		
1‴	4.75 *d* 1.7	
2‴	3.88 *m*	
3‴	3.72 *m*	
4‴	3.42 *m*	
5‴	3.68 *m*	
6‴	1.25 *d* 6.2	

(Table 1), in accordance with the anthocyanin, cyanidin. After the chemical shifts of the protons of 1 assigned, the chemical shifts of the corresponding carbons (Table 2) were assigned from the HSQC experiment. The remaining quaternary C-atoms were assigned using the HMBC spectrum, which was optimized for $^2J_{CH}$ and $^3J_{CH}$ couplings (Table 2). The two anomeric cross peaks at 5.37/102.52 ppm and 4.75/101.25 ppm in the HSQC spectrum of 1 indicated two monosaccharides. Starting from the doublet at 5.37 ppm ($J = 7.9$ Hz, H-1″) the observed cross peak with the signal at 3.74 ppm in the COSY spectrum permitted the assignment of H-2″. The chain of coupled protons H-2″, H-3″, H-4″, H-5″ and H-6A″ and 6B″ was thereafter assigned (Table 1) from cross peaks in the same spectrum. Subsequently, the chemical shifts of the corresponding carbon atoms (Table 2) were assigned from the HSQC spectrum, which together with ^{1}H–^{1}H coupling constants were in agreement with a β-linked glucopyranose. Similarly, the proton and carbon chemical shifts (Tables 1 and 2) of the other monosaccharide having the anomeric proton at 4.75 ppm ($J = 1.3$ Hz) were in accordance with an α−rhamnopyranosyl moiety. In cases where several sugar protons showed similar chemical shifts, the assignments were assisted by cross peaks in the TOCSY experiment of 1. The cross peak in the HMBC experiment at 5.37/144.49 ppm between the anomeric glucoside proton and C-3 of the aglycone, showed that the sugar moiety was linked to the aglycone 3-position. The linkage point between the two sugar units was indicated to be at C-6″ by the cross peak between the anomeric rhamnosyl proton and C-6″ at 4.75/66.56 ppm.

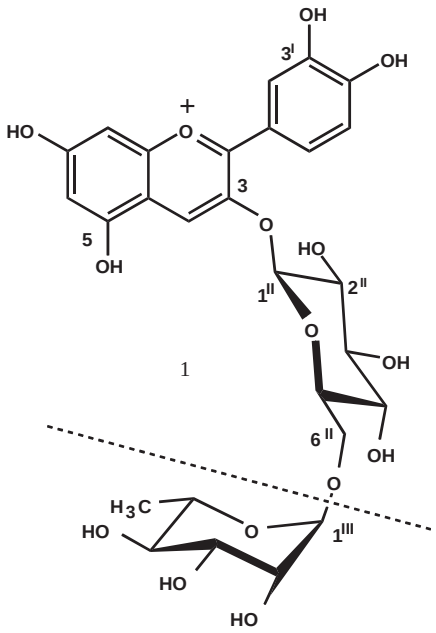
Table 2
^{13}C NMR spectra data for cyanidin 3-(6″-*O*-α-rhamnopyranosyl-β-glucopyranoside) (1) and cyanidin 3-*O*-β-glucopyranoside (2) in CD_3OD:CF_3COOD (95:5; v/v) at 25°C

aglycone	1 (ppm)	2 (ppm)
2	163.52	163.51
3	144.49	144.49
4	135.41	136.01
5	158.55	158.45
6	102.52	102.52
7	170.46	170.46
8	94.17	94.17
9	156.99	157.12
10	112.08	112.10
1′	120.09	120.09
2′	118.25	118.25
3′	146.52	146.54
4′	155.01	155.03
5′	114.80	114.81
6′	127.11	127.11
3-β-glucopyranoside		
1″	102.52	102.20
2″	73.75	73.73
3″	77.11	77.12
4″	70.60	70.61
5″	76.51	76.50
6″	66.56	61.36
6″-α-rhamnopyranosyl		
1‴	101.25	
2‴	70.82	
3‴	71.55	
4‴	73.09	
5‴	68.58	
6‴	17.21	

Thus, the identity of 1 was determined to be cyanidin 3-(6″-*O*-α-rhamnopyranosyl-β-glucopyranoside) (Fig. 1).

The 1D and 2D NMR spectra of 2 showed many similarities with the corresponding spectra of 1. However, the HSQC spectrum revealed only seven cross peaks in the sugar region in accordance with one monosaccharide, glucopyranose. The cross peak between anomeric proton H-1″ (δ 5.38, $J = 7.9$ Hz) and C-3 (δ 144.49) in the heteronuclear multiple HMBC spectrum of 2 established that the sugar was attached to the aglycone 3-position. Thus, the identity of 2 was found to be cyanidin 3-*O*-β-glucopyranoside (Fig. 1).

The HPLC profile of the acidified methanolic extract of the fruits of *R. rigidus* detected in the visible spectral region, indicated the same anthocyanins, 1 and 2, as found in *R. pinnatus*. After pigments isolation, the identities of these anthocyanins were confirmed by their UV-Vis and NMR spectral data in a similar manner as described above to. The relative amounts of 1 and 2 in



1

2

Fig. 1. The structure of cyanidin 3-(6″-*O*-α-rhamnopyranosyl-β-glucopyranoside), 1, and cyanidin-3-*O*-β-glucopyranoside, 2, isolated from *Rubus pinnatus* and *R. rigidus*.

R. rigidus were pigments 59.4% and 40.6%, respectively, while in *R. pinnatus*, the relative amounts were 58.6% and 41.4%.

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