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Polyphenolic constituents of blackcurrant seed residue

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Abstract

Chemical investigation of blackcurrant seed residue from oil extraction revealed the presence of an array of polyphenols which were dominated by four anthocyanins consisting of the rutinosides and glucosides of delphinidin and cyanidin. Also isolated were the glucosides and rutinosides of myricetin and quercetin, kaempferol-3-glucoside, dihydroquercetin and aureusidin, as well as the phenolic acids 1-cinnamoyl- and 1-*p*-coumaroyl-β-D-glucosides. This is the first report of aureusidin and 1-cinnamoyl-β-D-glucoside as blackcurrant constituents.

Keywords: Blackcurrant (*Ribes nigrum*); Grossulariaceae; Seed residue; Polyphenols; Anthocyanins; Flavonoids; Phenolic acids

1. Introduction

Blackcurrant (*Ribes nigrum*) berries are widely cultivated for use in beverages reputed to be excellent for health due to their high content of antioxidants (Costantino, Albasini, Rastelli, & Benvenuti, 1992; Costantino, Rastelli, Rossi, Bertoldi, & Albasini, 1993). The black coloration of the berries has been attributed to the exceptionally high level of anthocyanins present in the fruit (Demina, 1974; Le Lous, Majoie, Moriniere, & Wulfert, 1975). The fruit also contains considerable amounts of flavonoids (Hakkinen & Auriola, 1998; Koeppen & Herrmann, 1977), phenolic acids (Koeppen & Herrmann, 1977; Stoehr & Herrmann, 1975) and proanthocyanidins (Foo & Porter, 1981), which have also been reported to be present in the leaves (Calamita, Malinowski, & Strzelecka, 1983; Tits, Angenot, Poukens, Warin & Dierckxsens, 1992; Tits, Poukens, Angenot, & Dierckxsens, 1992) or buds (Rolland, Binsard, & Raynaud, 1977).

The seed of blackcurrant has attracted much interest (Zhao, Fu, Yu, & Liu, 1994) as it contains an exceptionally high level of nutritionally desirable polyunsaturated γ-linolenic acid (Traitler, Winter, Richli, & Ingenbleek, 1984). Polyunsaturated fatty acids (PUFA) are susceptible to oxidation, but somehow they are stable in the intact seeds, and this fact suggests the possible co-existence of potent natural antioxidants. No information about the nature of the seed constituents was available and it was therefore considered that a chemical investigation on the seed was warranted. As a result of this investigation two novel non-cyanogenic nitrile-containing compounds namely nigrumin-5-*p*-coumarate and nigrumin-5-ferulate, were successfully isolated from the seed residue after supercritical CO_2 extraction (Lu, Foo, & Wong, 2002). This report is a continuation of this study and describes chemical structure identification of an array of phenolic compounds, which have not been reported in blackcurrant seed previously.

2. Materials and methods

2.1. Extraction

Blackcurrant seed residue (100 g), obtained from supercritical CO_2 extraction, was soaked in 300 ml of 7:3 acetone/water overnight at ambient temperature. After filtration, the residue was extracted twice more with the same solvent (2×300 ml). The combined extract was concentrated on a rotary evaporator at 40 °C under reduced pressure and the aqueous residue defatted with hexane (3×100 ml), then concentrated and freeze-dried to afford 5.0 g red-coloured solid.

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2.2. Chromatographic separation

The freeze dried extract was fractionated on a Polyamide column (60×4 cm I.D.) to give a water fraction (predominantly sugars, discarded), an anthocyanin fraction obtained by eluting with up to 1:4 methanol/water containing 4% acetic acid and a phenolic fraction collected with methanol. The anthocyanin fraction was further chromatographed, first on Polyamide and then MCI-HP20 columns (40×1.8 cm I.D.), both eluting with water to 1:4 methanol/water containing 4% acetic acid, to yield pure individual anthocyanins. The phenolic fraction was subjected to repeated chromatographic treatment on MCI-HP20, eluting with water and then increasing methanol content to 1:1 until pure compounds were isolated. Sub-fractions were collected using a fraction collector and monitored by HPLC.

2.3. HPLC analysis

HPLC analysis was performed on a Hewlett Packard series 1100 equipped with a DAD detector and a LiChrospher® 100 RP-18 (5 μm) column (125×4 mm) held at 30 °C. The solvent program started from 4% B (2% HOAc in CH$_3$CN) in solvent A (2% HOAc in H$_2$O) up to 12% B in 20 min, to 20% B in 30 min and to 50% B in 45 min. Flow rate was set at 1 ml/min and compounds were monitored by UV absorption set at 280 nm for phenolic acids, 350 nm for flavonoids and 520 nm for anthocyanins.

2.4. Identification

Purified phenolic compounds (see Fig. 1 for chemical structures) were identified by NMR spectroscopy on a Bruker Avance 300 instrument and chemical shifts (δ in ppm) were referenced to TMS (^{1}H) or solvent signal (^{13}C).

2.4.1. Delphinidin-3-rutinoside (*1*)

HPLC-Rt: 19.3 min, on-line UV $\lambda_{\max}$: 526, 276, 232 nm. ^{1}H NMR (CD$_3$OD/TFA, 9:1): δ 1.18 (d, *J* = 6.2 Hz, H-6‴), 3.31–3.86 (m, sugar-H), 4.09 (d, *J* = 11.0 Hz, H-6″a), 4.69 (br s, H-1‴), 5.30 (d, *J* = 7.7 Hz, H-1″), 6.67 (d, *J* = 1.9 Hz, H-6), 6.84 (d, *J* = 1.2 Hz, H-8), 7.70 (s, H-2′, 6′), 8.83 (s, H-4). ^{13}C NMR: see Table 1.

2.4.2. Delphinidin-3-glucoside (*2*)

HPLC-Rt: 17.4 min, on-line UV $\lambda_{\max}$: 526, 276, 232 nm. ^{1}H NMR (CD$_3$OD/TFA, 9:1): δ 3.45–3.80 (m, sugar-H), 3.95 (dd, *J* = 12.0, 2.0 Hz, H-6″a), 5.33 (d, *J* = 7.7 Hz, H-1″), 6.65 (d, *J* = 2.0 Hz, H-6), 6.86 (d, *J* = 1.2 Hz, H-8), 7.74 (s, H-2′, 6′), 8.94 (s, H-4). ^{13}C NMR: see Table 1.

2.4.3. Cyanidin-3-rutinoside (*3*)

HPLC-Rt: 23.4 min, on-line UV $\lambda_{\max}$: 518, 280, 232 nm. ^{1}H NMR (CD$_3$OD/TFA, 9:1): δ 1.18 (d, *J* = 6.3 Hz, H-6‴), 3.38–3.92 (m, sugar-H), 4.08 (d, *J* = 9.9 Hz, H-6″a), 4.68 (d, *J* = 1.2 Hz, H-1‴), 5.29 (d, *J* = 7.6 Hz, H-1″), 6.68 (d, *J* = 1.9 Hz, H-6), 6.87 (d, *J* = 1.2 Hz, H-8), 6.99 (d, *J* = 8.8 Hz, H-5′), 7.98 (d, *J* = 2.2 Hz, H-2′), 8.22 (dd, *J* = 8.7, 2.2 Hz, H-6′), 8.89 (s, H-4). ^{13}C NMR: see Table 1.

Table 1
^{13}C NMR data of anthocyanins **1**–**4** in CD$_3$OD/TFA (9:1) and flavonol glycosides **5**–**9** in DMSO-d$_6$

No	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
2	163.85	164.32	164.30	164.44	156.71	156.61	156.61	156.55	156.66
3	146.01	146.18	145.96	145.96	133.78	133.87	133.66	133.69	133.70
4	135.67	136.45	136.47	137.17	177.67	177.75	177.75	177.79	177.81
5	157.82	157.92	157.91	157.99	161.59	161.60	161.60	161.59	161.59
6	103.95	103.72	103.91	103.81	98.96	98.98	98.98	99.01	99.01
7	170.82	170.65	170.82	170.90	164.35	164.44	164.44	164.46	164.45
8	95.61	95.45	95.73	95.55	93.75	93.70	93.92	93.86	93.88
9	159.35	159.10	159.39	159.68	156.71	156.61	156.96	156.67	156.66
10	113.51	113.59	113.59	113.72	104.30	104.32	104.32	104.34	104.35
1′	120.30	120.36	121.52	121.57	120.46	120.41	121.54	121.53	121.58
2′	113.01	112.98	118.77	118.82	108.94	108.91	116.62	116.57	131.23
3′	147.76	147.85	147.76	147.73	145.70	145.73	145.08	145.14	115.46
4′	145.05	145.09	156.23	156.13	136.97	136.99	148.74	148.79	160.28
5′	147.76	147.85	117.87	117.81	145.70	145.73	115.58	115.56	115.46
6′	113.01	112.98	128.83	128.63	108.94	108.91	121.94	121.95	131.23
1″	103.59	104.01	103.91	104.11	101.33	101.29	101.55	101.26	101.25
2″	74.99	75.16	75.09	75.17	74.28	74.29	74.29	74.45	74.46
3″	77.82	78.45	77.85	78.51	76.46	76.93	76.26	76.86	76.79
4″	71.63	71.45	71.64	71.47	70.41	70.29	70.29	70.29	70.29
5″	78.32	79.17	78.42	79.14	76.88	77.94	76.93	77.88	77.88
6″	68.17	62.72	68.21	62.75	67.43	61.45	67.14	61.34	61.34
1‴	102.52		102.55		101.04		101.08		
2‴	72.23		72.26		70.74		70.72		
3‴	72.86		72.85		70.86		70.91		
4‴	74.33		74.33		72.22		72.21		
5‴	70.15		70.16		68.58		68.57		
6‴	18.19		18.30		18.06		18.05		

2.4.4. Cyanidin-3-glucoside (*4*)

HPLC-Rt: 21. 3 min, on-line UV $\lambda_{\max}$: 518, 280, 232 nm. ^{1}H NMR (CD$_3$OD/TFA, 9:1): δ 3.41–3.77 (m, sugar-H), 3.85 (dd, *J* = 12.0, 1.5 Hz, H-6″a), 5.20 (d, *J* = 7.7 Hz, H-1″), 6.55 (d, *J* = 1.9 Hz, H-6), 6.76 (br s, H-8), 6.92 (d, *J* = 8.5 Hz, H-5′), 7.91 (br s, H-2′), 8.11 (d, *J* = 8.5 Hz, H-6′), 8.88 (s, H-4). ^{13}C NMR: see Table 1.

2.4.5. Myricetin-3-rutinoside (*5*)

HPLC-Rt: 21.1 min, on-line UV $\lambda_{\max}$: 356, 260, 230 nm. ^{13}C NMR: see Table 1.

2.4.6. Myricetin-3-glucoside (*6*)

HPLC-Rt: 21.7 min, on-line UV $\lambda_{\max}$: 356, 260, 230 nm. ^{13}C NMR: see Table 1.

2.4.7. Quercetin-3-rutinoside (*7*)

HPLC-Rt: 26.1 min, on-line UV $\lambda_{\max}$: 354, 256, 230 nm. ^{13}C NMR: see Table 1.

2.4.8. Quercetin-3-glucoside (*8*)

HPLC-Rt: 26.5 min, on-line UV $\lambda_{\max}$: 354, 256, 230 nm. ^{13}C NMR: see Table 1.

2.4.9. *Kaempferol-3-glucoside (**9**)*

HPLC-Rt: 31.1 min, on-line UV λ_{max}: 348, 264 nm. ^{13}C NMR: see Table 1.

2.4.10. *Dihydroquercetin (**10**)*

HPLC-Rt: 19.4 min, on-line UV λ_{max}: 288, 234 nm. ^{13}C NMR (DMSO-d$_6$): δ 71.91 (C-3), 83.39 (C-2), 95.32 (C-8), 96.32 (C-6), 100.82 (C-10), 115.46 (C-5′), 115.68 (C-2′), 119.72(C-6′), 128.38 (C-1′), 145.26 (C-3′), 146.09 (C-4′), 162.89 (C-9), 163.66 (C-5), 167.13 (C-7), 198.06 (C-4).

2.4.11. *Aureusidin (**11**)*

HPLC-Rt: 30.1 min, on-line UV λ_{max}: 400 nm. ^{1}H NMR (CD$_3$OD): δ 6.09 (d, *J* = 1.5 Hz, H-5), 6.20 (d, *J* = 1.5 Hz, H-7), 6.47 (s, α-H), 6.83 (d, *J* = 8.2 Hz, H-5′), 7.18 (dd, *J* = 8.2, 1.6 Hz, H-6′), 7.41 (d, *J* = 1.6 Hz, H-2′).

2.4.12. *1-p-Coumaroyl-β-D-glucopyranoside (**12**)*

HPLC-Rt: 9.4 min, on-line UV λ_{max}: 314, 232 nm. ^{13}C NMR (CD$_3$OD): δ 62.07 (C-6′), 70.81 (C-4′), 73.70 (C-2′), 77.39 (C-3′), 78.41 (C-5′), 95.69 (C-1′), 114.28 (C-8), 117.10 (C-3,5), 127.30 (C-1), 131.81 (C-2,6), 148.81 (C7), 160.71 (C-4), 168.66 (C-9).

2.4.13. *1-Cinnamoyl-β-D-glucopyranoside (**13**)*

HPLC-Rt: 14.7 min, on-line UV λ_{max}: 278 nm. ^{13}C NMR CD$_3$OD): δ 62.77 (C-6′), 71.54 (C-4′), 74.46 (C-2′), 78.44 (C-3′), 79.27 (C-5′), 96.36 (C-1′), 118.69 (C-8), 129.78 (C-2,6), 130.48 (C-3,5), 132.21 (C-4), 135.38 (C-1), 148.06 (C-7), 167.39 (C-9).

3. Results and discussion

HPLC analysis of the acetone/water (7:3) extract of blackcurrant seed residue showed the presence of many polyphenols dominated by anthocyanins with characteristic absorption at ca 520 nm. Flavonoid glycosides, detected by their characteristic absorption at ca 350 nm, were present at moderate levels. Separation of the anthocyanins from other phenolics was successfully accomplished using a Polyamide column.

3.1. *Anthocyanins*

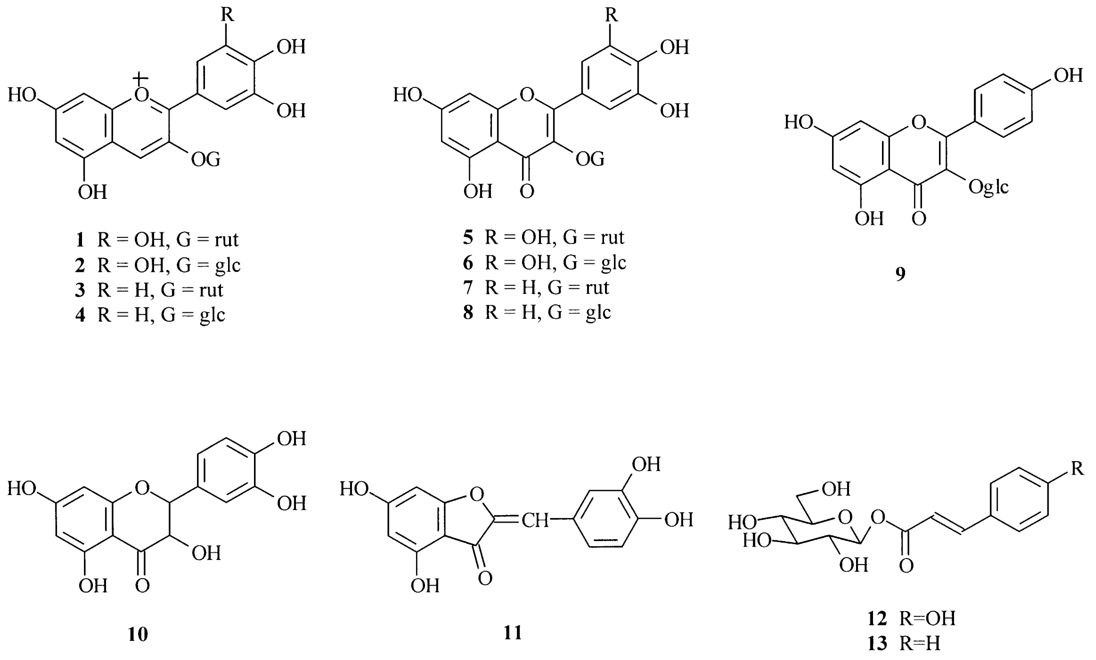
Compounds **1**–**4** were isolated from the anthocyanin fraction by column chromatography on Polyamide and MCI-HP20. The ^{1}H NMR spectra of **1**–**4** all showed a broad singlet at δ 8.9, characteristic for H-4 of the pyrylium C-ring and two mutually *meta*-coupled doublets at δ 6.8 and 6.6 for H-8 and H-6 of the phloroglucinol A-ring. Compounds **1** and **2** both showed a two-proton singlet at δ 7.7 indicative of a pyrogallol B-ring in contrast to **3** and **4** where an ABX resonance system for a catechol B-ring was observed. The ^{13}C NMR spectra of **1**–**4** (see Table 1) were also consistent with delphinidin and cyanidin as the respective aglycones as deduced from ^{1}H NMR. The sugar moieties in **1**/**3** were identified as rutinose (6-α-L-rhamnopyranosyl-β-D-glucopyranose) and, in **2**/**4** glucose, based on their characteristic 12 or 6 sugar carbon chemical shifts (see Table 1), together with proton–proton coupling patterns, namely two doublets at δ 4.7 (*J* = 1.2 Hz) and 5.3 (*J* = 7.6 Hz) in **1**/**3** and one doublet at δ 5.3 (*J* = 7.7 Hz) in **2**/**4**, for the anomeric proton(s), which were consistent with those reported for delphinidin- or cyanidin-3-glycosides (Andersen, 1988). The identification of these delphinidin-3-rutinoside (**1**) and -3-glucoside (**2**) and cyanidin-3-rutinoside (**3**) and -3-glucoside (**4**) were consistent with a number of literature reports on their presence in blackcurrant berries (Demina, 1974; Goiffon, Mouly, & Gaydou, 1999; Le Lous et al., 1975), but without detailed NMR characterisation.

However, the presence of other anthocyanins, such as pelargonidin-3-rutinoside or the sophorosides of delphinidin and cyanidin reported by Le Lous et al. (1975), were not detected. Four novel pyranoanthocyanins from blackcurrant seed had been reported (Lu, Sun, & Foo, 2000) but they were subsequently shown to be artefacts formed by the reaction of the blackcurrant anthocyanins with acetone used as the extracting solvent (Lu & Foo, 2001).

The blackcurrant anthocyanins have attracted great interest due to their high concentrations in the berries and their antioxidant and pharmaceutical activities (Andersen, Helland, & Andersen, 1997; Costantino et al., 1992, 1993). New separation technologies have been explored, such as combined column chromatography (Froytlog, Slimestad, & Andersen, 1998), droplet counter-current chromatography (Francis & Andersen, 1984) and high speed counter-current chromatography (Degenhardt, Knapp, & Winterhalter, 2000) for their isolation on a large scale.

3.2. *Flavonoids*

Compounds **5**–**9** were identified as flavon(ol) glycosides from their characteristic on-line UV absorption at ca. 350 nm. Their isolation was achieved by chromatography on MCI-HP20 column and their identification as myricetin-3-rutinoside (**5**), myricetin-3-glucoside (**6**), quercetin-3-rutinoside (**7**), quercetin-3-glucoside (**8**) and kaempferol-3-glucoside (**9**) was achieved by NMR (see Table 1) spectral comparison with published data (Agrawal, 1989; Markham & Chari, 1982). Although this is the first report of their presence in blackcurrant seed, these flavonoids are widely distributed in the plant kingdom including blackcurrant berries (Hakkinen & Auriola, 1998; Koeppen and Herrmann, 1977; Le Lous et al., 1975). In addition to these glycosides the flavonoid



1 R = OH, G = rut
2 R = OH, G = glc
3 R = H, G = rut
4 R = H, G = glc

5 R = OH, G = rut
6 R = OH, G = glc
7 R = H, G = rut
8 R = H, G = glc

9

10

11

12 R=OH
13 R=H

Fig. 1. Chemical structures of polyphenols from blackcurrant (*Ribes nigrum*) seed.

aglycones myricetin, quercetin and kaempferol were also detected by HPLC and confirmed by comparison of their retention times and UV spectral absorption with authentic samples. These aglycones are likely hydrolysis products of the respective glycosides. Flavonoids in blackcurrant, unlike other berries, are dominated by myricetin, followed by quercetin and kaempferol (Häkkinen, Kärenlampi, Heinonen, Mykkänen, & Törrönen, 1999; Mikkonen, Määttä, Hukkanen, Kokko, Törrönen, Kärenlampi et al., 2001; Vuorinen, Määttä & Törrönen, 2000). These flavonoids were also present in the leaves of blackcurrant (Calamita et al., 1983) or buds (Rolland et al., 1977).

Compound **10** was identified as dihydroquercetin (taxifolin) by HPLC and NMR spectroscopic (Markham & Chari, 1982) comparison with an authentic sample. Compound **11** had totally different UV absorption from the common flavon(ol)s and, with λ_{max} at 400 nm, an aurone structure was indicated (Markham, 1982). Due to the small sample size, compound **11** was subjected only to ^{1}H NMR spectroscopy, which showed two *meta*-coupled doublets at δ 6.09 and 6.20 ($J=1.5$ Hz), indicative of a phloroglucinol A-ring, an ABX resonance system at δ 6.83 (d, $J=8.2$ Hz), 7.41 (d, $J=1.6$ Hz) and 7.18 (dd) of a catechol B-ring and a singlet at δ 6.47 for the α-H, indicating **11** to be aureusidin. This deduction was corroborated by published data for the compound (Markham & Geiger, 1993). Aureusidin, like myricetin, quercetin or kaempferol, could also be a hydrolysis product. Aureusidin or its glycosides, although known natural products, are rare in nature (El-Habashy, Mansour, Zahram, El-Hadidi & Saleh, 1989) and this is the first report of its presence in blackcurrant.

3.3. Phenolic acids

The presence of caffeic acid, ferulic acid, *p*-coumaric acid, gallic acid, protocatechuic acid and *p*-hydroxybenzoic acid in blackcurrant seed extract was detected by HPLC and confirmed by comparison with authentic samples. *p*-Coumaric acid was the most abundant and these phenolic acids were also considered to be hydrolysis products of the corresponding glycosides (Stoehr & Herrmann, 1975). In addition, 1-*p*-coumaroyl-β-D-glucoside (**12**) and 1-cinnamoyl-β-D-glucoside (**13**) were also isolated and their identity was confirmed by NMR spectral comparison with published data (Strack, Heilemann, Wray, & Dirks, 1989; Latza, Ganβer, & Berger, 1996; Mouly, Gaydou, Faure, & Estienne, 1997). Such 1-*O*-acylated glycosides are common in fruits (Reschke & Herrmann, 1981; Macheix, Fleuriet, & Billot, 1990) and the presence of 1-*O*-caffeoyl-, 1-*O*-feruloyl and 1-*O*-*p*-coumaroyl-β-D-glucosides in blackcurrant has also been reported (Koeppen & Herrmann, 1977). However, to our knowledge, the 1-cinnamoyl-β-D-glucoside (**13**) is a newly discovered blackcurrant constituent.

4. Conclusion

Phytochemical study carried out in this work on the blackcurrant seed residue left after oil extraction revealed that the seed had a similar phenolic composition to that found in the berries. Anthocyanins were found to be the predominant components consisting of rutinosides (major) and glucosides (minor) of delphinidin and cyanidin. Other flavonoids present in moderate concentrations were myricetin and quercetin glucosides. Among phenolic acids identified, only *p*-coumaric acid was present at significant level. Aureusidin, a minor aurone, and 1-cinnamoyl-β-D-glucoside were identified as blackcurrant constituents for the first time. These polyphenols may have a role in protecting highly labile polyunsaturated fatty acids in the intact blackcurrant seeds.

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