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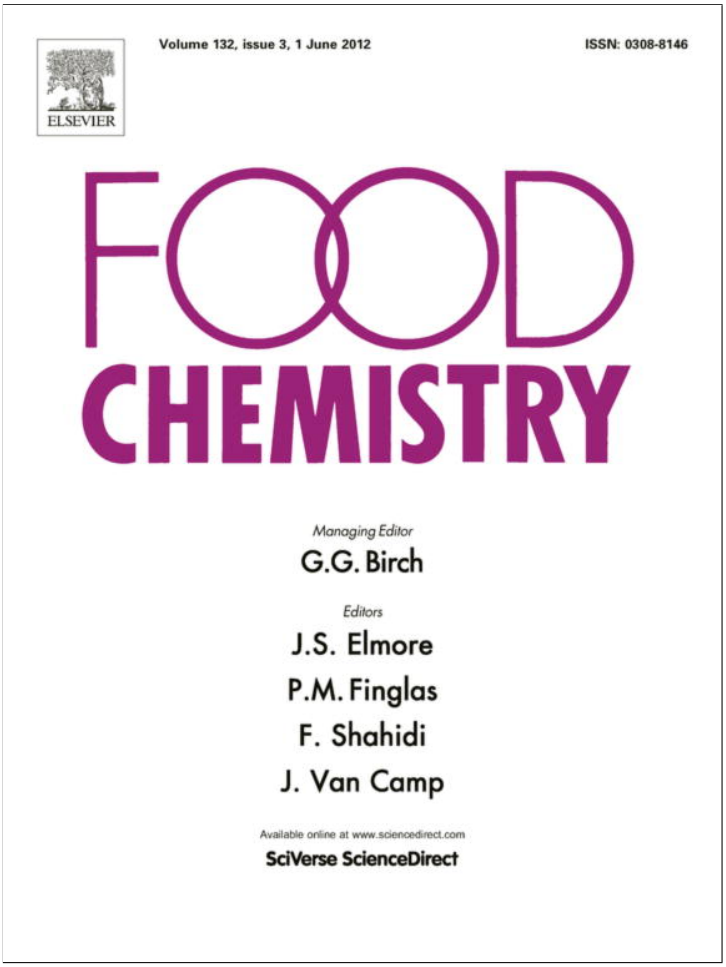

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Bioactivity and chemical composition of blackcurrant (*Ribes nigrum*) cultivars with and without pesticide treatment

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ABSTRACT

Eleven blackcurrant cultivars grown with pesticide (PT) and without pesticide treatment (PF) were evaluated to compare the differences in plant growth and physical condition, total anthocyanin content, ascorbic acid content, total antioxidant capacity, effect on prostaglandin E2 (PGE2) production and anticancer cell proliferation activities. Results showed that the yield and growth of PT blackcurrants were higher. However, PF blackcurrants contained a higher amount of ascorbic acid, and displayed an increased inhibition against cancer cells compared to PT blackcurrants, indicating that PF blackcurrants have an increased potential to deliver health-promoting benefit for consumers. Significant differences were observed between blackcurrant cultivars in relation to plant growth and physical condition, total anthocyanin content and PGE2 assay, highlighting the importance of cultivar selection.

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1. Introduction

Synthetic agrochemicals are extensively used in agricultural systems, mainly as pesticides and fertilisers. Agrochemical residues are a major source of environmental contamination in soil and ground water; the residues found in crops may lead to accumulation in humans and animals, causing public health risk problems, including an increasing number of different types of oncogenic risk and cancer incidences in communities (Gilden, Huffling, & Sattler, 2010; Weichenthal, Moase, & Chan, 2010). Thus, restriction of agrochemical application and increased governmental support for sustainable agricultural production systems have encouraged the growth of the organic sector in Denmark (Blumenthal, 2010; Phillips, 2009).

Blackcurrant (*Ribes nigrum*), a species native to central and northern Europe and northern Asia is recognised as one of the major edible berries consumed in processed form. The plant is highly tolerant to leaf damage caused by *Sphaerotheca mors-uvae* (Pedersen, Toldam-Andersen, Funke, Hann, & Wünsche, 2002) and therefore it is suited for pesticide-free or organic farming (Kahu, Jänes, Luik, & Klaas, 2009). Blackcurrants contain high levels of ascorbic acid and are a rich source of phenolic compounds. Ascorbic acid is an antioxidant and acts as a cofactor for various enzymes in diverse metabolic pathways. Phenolic compounds are strong antioxidants and display a diverse range of biological activities. Blackcurrant phenolic extracts displayed effective protection against oxidative-stress-induced neuronal damage in human cells (Ghosh, McGhie, Zhang, Adaim, & Skinner, 2006). Blackcurrant anthocyanins increased blood flow and enhanced peripheral circulation as well as reducing muscle fatigue in humans (Matsumoto et al., 2005). Another study showed that blackcurrant juice induced peripheral vasodilatation, increased blood flow and decreased blood pressure in healthy women (Yonei et al., 2009). Oral intake of blackcurrant anthocyanins relaxed the bovine ciliary smooth muscles and improved the visual function (Matsumoto, Kamm, Stull, & Azuma, 2005). Crude extract of blackcurrants inhibited influenza virus type A and B in a high temperature environment (Knox, Suzutani, Yosida, & Azuma, 2003) and inhibited herpes simplex virus type-1 and type-2 by inhibiting protein synthesis of infected cells (Suzutani, Ogasawara, Yoshida, Azuma, & Knox, 2003). An investigation on different berry juices against cancer cell proliferation activity found out that blackcurrant juice extracts inhibited different cancer cells, including Caco-2, MCF-7, AGS, MDA-MB-231 and PC-3 (Boivin, Blanchette, Barette, Moghrabi, & Beliveau, 2007). Another research on berry phenolic extracts pointed out that berry juices inhibited HT-29 cancer cell growth by modulating the $p21^{WAF1}$ and Bax expression (Wu, Koponen, Mykkanen, & Törrönen, 2007).

Due to increased public awareness on pesticide contamination in foods, an increasing number of research studies are emerging

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on the nutritional quality and phytochemical content in organic compared to conventional food crops (Crinnion, 2010; Worthington, 2001). In addition, no investigation has been performed on the anticancer or anti-inflammatory activity of different blackcurrant cultivars. Most of the studies on berry bioactivities employed either solvent or acidic extraction methods, or purified compound. However these extractions might not fully represent the part for human consumption. Thus we have chosen to analyse the whole juice for the bioassays. The present study is the first report on the comparison of blackcurrants grown with pesticide (PT) and without pesticide (PF) treatment. Furthermore, the differences between individual cultivars have also been compared. The impact on yield, berry size, vegetative growth, damage caused by diseases and aphids, total anthocyanin content, ascorbic acid content, total antioxidant capacity, prostaglandin E2 (PGE2) assay and anticancer cell proliferation activities were investigated.

2. Materials and methods

2.1. Plant materials

Eleven cultivars of blackcurrants were used in this study: 'Baldwin', 'Ben Alder', 'Ben Dorain', 'Ben Gairn', 'Ben Hope', 'Ben Lomond', 'Narve Viking', 'Tiben', 'Titania', plus the breeding selections 8944-4 and 8944-13. In 2003 at Department of Horticulture, Aarhus University, Aarslev, Denmark, the blackcurrant plants were planted as one-year-old plants at a planting distance of 3.5×0.5 m. Plots consisted of six bushes planted in four blocks per cultivar: one block of plant with pesticide treatment (PT) and three blocks of plants without pesticide treatments (PF). All plants were randomised within each block and were grown in Mypex™ within the bush row. Additional weeds were removed mechanically or by hand. Organic poultry manure pellets were used as fertilisers in both PT and PF plants. No pesticides were used on the PF plants. For PT plants, the pesticide treatment consisted of mancozeb, boscalid + pyraclostrobin and kresoxim-methyl for the control of American gooseberry mildew (mildew) caused by *S. mors-uvae* (Schweinitz), white pine blister rust (rust) caused by *Cronartium ribicola* (J.C. Fischer) and leaf spot caused by *Gloeosporidiella ribis* (Libert). Propiconazol was used for the control of grey mould caused by *Botrytis cinerea* in the flowers and pirimicarb was used for the control of aphids. All blackcurrants were harvested mechanically within the period of July–August 2009, and samples were stored at −24 °C immediately after harvested.

2.2. Plant growth and physical condition

Vegetative growth was assessed in June by giving a score from 1 to 9, where 1 = no growth. Yield was recorded during harvest and berry size was calculated from the total weight of 100 berries from each cultivar. Damage caused by diseases and aphids was estimated visually and assessed by giving a score from 1 to 9, where 1 = no damage. Damage caused by mildew and aphids was observed in June 2009 and damage caused by leaf spot and rust was observed in August 2009.

2.3. Chemicals

6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), sodium dihydrophosphate, acetonitrile, methanol, sulphuric acid, trifluoroacetic acid (TFA), D,L-homocysteine, oxalic acid, *N*-ethylmaleimide, fluorescein, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), ammonium persulphate, 2,6-dichlorophenol-indophenols and deuterium oxide 99.9 atom% D containing 0.05% (w/v) 3-(trimethylsilyl) propionic 2,2,3,3-d_4 acid sodium salt (D_2O containing 0.05% TSP) were purchased from Sigma–Aldrich (St. Louis, MO). Dulbecco's Modified Eagle Medium-Glutamax™ (DMEM) was from Invitrogen (Paisley, UK). Trypsin–ethylenediaminetetraacetic acid (Trypsin–EDTA) and phosphate buffer saline–ethylenediaminetetraacetic acid (PBS–EDTA) were from Lonza (Braine, Belgium). Penicillin G-potassium salt and streptomycin sulphate were from Serva (Heidelberg, Germany). Foetal calf serum (FCS) was from PAA Laboratories (Pasching, Austria). WST-1 cell proliferation reagent was from Roche Diagnostics (Mannheim, Germany). Lipopolysaccharide (LPS) was from Calbiochem, Merck (Damstadt, Germany). 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) and monoclonal prostaglandin E2 enzyme immunoassay (PGE2 EIA) kit were purchased from Cayman Europe (Tallinn, Estonia).

2.4. Sample preparation

Frozen blackcurrants were removed from the freezer and approximately 200 g of frozen blackcurrants were homogenised separately with deionised water (2 g of fruit with 1 mL water), using a food blender. Thereafter the homogenates were centrifuged at 12,000 rpm for 30 min at 4 °C. The supernatants were collected and filtered with MinisartCA 0.45-μm filters (Sartorius, Göttingen, Germany) and the filtrates were kept at −80 °C until analysis.

2.5. Total anthocyanin content

Total anthocyanin content of blackcurrants was measured spectrophotometrically (Wrolstad, 1976). The homogenates were extracted with 50 mL acidified methanol (96% methanol:3% sulphuric acid, w/w) and the ultraviolet absorption was measured at a wavelength of 526 nm with an MPS-2000 Shimadzu multipurpose recording spectrophotometer (Shimadzu, Kyoto, Japan). Each sample was measured in two replicates. Total anthocyanin content was calculated as malvin equivalents per 100 g (ME/100 g) blackcurrants (extinction coefficient, $\varepsilon = 37{,}700\ M^{-1}\ cm^{-1}$). The mean value and standard deviation were calculated.

2.6. Ascorbic acid content

The homogenates were extracted with 0.1% aqueous oxalic acid. The mixtures were purged with nitrogen during extraction (Kaack & Austed, 1998). After that, the mixtures were filtered and the aliquot was treated with D,L-homocysteine to reduce dehydroascorbic acid to ascorbic acid. The excess homocysteine was eliminated with *N*-ethylmaleimide and the extracts were titrated with 2,6-dichlorophenol-indophenols to determine the ascorbic acid content (Lento, Daugherty, & Denton, 1963). Each sample was measured in duplicate. The mean values and standard deviations were calculated.

2.7. Total antioxidant capacity

Total antioxidant capacity of blackcurrants was measured using the Trolox equivalent antioxidant capacity (TEAC) assay and oxygen radical absorbance capacity (ORAC) assay.

For TEAC assay, ABTS (19.4 mM) was dissolved in water and mixed with ammonium persulphate (8.8 mM). The mixture was left overnight at room temperature to allow the formation of $ABTS^{\cdot+}$. The mixture was then diluted 100× with phosphate buffer (75 mM, pH 7.42) prior to use. The experiment was performed in triplicate in a Nunc transparent 96-well plate (Thermo Fisher Scientific, Loughborough, UK) with two independent tests. Trolox was used for standard curve calibration. A 50-μL subsample of standard or filtered samples was mixed with 200 μL of $ABTS^{\cdot+}$ working solution and the absorbance was read at a wavelength

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of 734 nm using BioTek Synergy 2 multi-mode microplate reader (BioTek, Winooski, VT). Solutions without samples were used as blank. Mean value and standard deviation were calculated. The results were expressed as Trolox equivalents per gram (TE/g) blackcurrants.

ORAC assay was performed as described by Huang with minor modification (Huang, Ou, Hampsch-Woodill, Flanagan, & Prior, 2002). Fluorescein solution was prepared (1.2×10^{-8} mM) in phosphate buffer (75 mM, pH 7.42) and AAPH was dissolved in phosphate buffer at a final concentration of 15 mM. The assay was performed in triplicate in a Nunc black 96-well plate (Thermo Fisher Scientific) with two independent tests. Trolox was used for standard curve calibration. A 25-μL subsample of standard or filtered samples was mixed with 150 μL of fluorescein solution and incubated at 37 °C for 30 min. The assay was initiated by adding 25 μL AAPH solution. Fluorescence was measured at minute intervals for 60 min with an excitation wavelength of 485 nm and an emission wavelength of 515 nm using BioTek Synergy 2 multi-mode microplate reader. Mean values and standard deviations were calculated. The results were expressed as Trolox equivalents per gram (TE/g) blackcurrants.

2.8. Anticancer cell proliferation activities

Two human colon cancer cell lines, HT-29 and Caco-2 (European Collection of Cell Cultures, Salisbury, UK) were used in the experiment. Cells were grown in DMEM medium supplemented with 10% FCS, 100 IU/mL penicillin and 100 μg/mL streptomycin. The medium was changed every second day and cells were passaged every fourth day. Trypsin–EDTA was used for detachment of cells from the culture flask. Cells were incubated in an 8000 WJ CO_2 incubator (Thermo Fisher Scientific) at 37 °C with 5% humidified CO_2.

Cells were seeded into Nunc sterile transparent 96-well plate (Thermo Fisher Scientific) at a density of 1×10^4 cells per well and incubated for 24 h. Filtered samples were added into the 96-well plate to a final concentration of 50 μL/mL and the cells were incubated for another 72 h. Proliferation assay was determined using WST-1, based on the cleavage of tetrazolium salt to formazan by mitochondrial dehydrogenase. After 3 h of incubation with WST-1, the absorbance was detected at a wavelength of 450 nm and a reference wavelength of 630 nm using BioTek Synergy 2 multi-mode microplate reader. The absorbance was corrected by subtracting the value at 630 nm from the value at 450 nm. All samples were tested in triplicate in two independent experiments. Wells without sample were used as control and inhibition was calculated relative to the control for each sample.

2.9. PGE2 assay

PGE2 assay was used to determine the cyclooxygenase-2 inhibitory activity. In brief, a total of 1×10^4 Caco-2 cells were seeded into each well of Nunc sterile transparent 96-well plate and incubated for 48 h. Cells were then incubated with 500 μM aspirin for 3 h to inactivate the endogenous cyclooxygenase-1 (Bang et al., 2002). Cells were washed twice with PBS–EDTA, and 200 ng/mL LPS was added with or without filtered samples. After incubation for 4 h, media were collected and centrifuged. All media were tested according to the given protocol for the PGE2 EIA kit set.

2.10. HPLC analysis

Filtered samples were diluted 10× and separated on a Kinetex 2.6 μm C18 100 Å, 100 × 4.6 mm column (Phenomenex, Torrance, CA) using a Dionex Ultimate 3000 HPLC system (Dionex, Sunnyville, CA) equipped with Chromeleon software program. All samples were measured twice using an isocratic elution with a mobile phase consisting of 0.5% TFA aqueous (solvent A, 95%) and acetonitrile (solvent B, 5%) at a flow rate of 1.0 mL/min for 13 min, followed by 1 min column wash with 75% solvent A and 25% solvent B. Measurement of anthocyanins were determined at wavelengths of 365 nm and 520 nm.

2.11. NMR experiments

A 500-μL aliquot of filtered sample was mixed with 100 μL D_2O containing 0.05% TSP. The ^{1}H NMR spectra were recorded at 25 °C on a Bruker Avance 600 spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at a proton frequency of 600.13 MHz, equipped with a 5 mm ^{1}H TXI probe (Bruker Biospin). The spectra were acquired using a single 90° pulse experiment with relaxation decay of 5 s. Water suppression was achieved by irradiating the water peak during relaxation decay. A total of 32 K data points spanning a spectral width of 12.1 ppm were collected. All spectra were referenced to TSP at 0 ppm. The spectra were manually phased and baseline corrected using Topspin 2.1 (Bruker Biospin).

2.12. Data processing and statistical analysis

^{1}H NMR spectra were subdivided into 0.015 ppm integral regions and integrated, reducing each spectrum into 819 independent variables. Further analysis was performed using SIMCA P + 12.0.1.0 (Umetrics, Umeå, Sweden). NMR data above 5.0 ppm is considered as aromatic region and below 5.0 ppm is aliphatic region. Between 3.0 and 5.0 ppm represents the sugar group and was removed from the data set to reduce the noise while performing principal component analysis (PCA) and partial least squares (PLS) regression. HPLC and NMR data were set as *X* variables; yield, berry size, vegetative growth, damage caused by diseases and aphids, total anthocyanin content, ascorbic acid content, total antioxidant capacity, PGE2 assay and anticancer cell proliferation activities were set as *Y* variables. PCA was applied to data to observe clustering behaviour of the samples and PLS regression with full cross validation was used to investigate the correlation between NMR spectra or HPLC data and *Y* variables. NMR data was scaled with pareto scaling; HPLC data was scaled with centering scaling; other data used in SIMCA analysis was scaled with unit variance scaling.

The statistical differences between pesticide treatment and individual cultivars were calculated with general linear model in SAS 9.1 software package (SAS Institute, Cary, NC). Linear regressions between total anthocyanin content, ascorbic acid content, total antioxidant capacity, PGE2 assay and anticancer cell proliferation activities were investigated, to determine the correlation significance.

3. Results and discussion

3.1. Plant growth and physical condition

Table 1 shows the average of yield, berry size, scores of vegetative growth and scores of damage caused by diseases and aphids of PT and PF plants and individual cultivars. Significant differences in yield and vegetative growth were found between PT and PF plants; PT plants had a stronger growth than PF plants and there was approximately a 100% increase in yield of PT blackcurrants compared to PF blackcurrants. This is most likely attributed to exposure of the plants to diseases because of the absence of pesticide protection. No significant difference was observed in berry size between two treatments but there were significant differences between cultivars, indicating that berry size depends on individual

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Table 1
Average of yield, berry size, scores (1–9, where 1 = no growth or no infections) for vegetative growth and damage caused by diseases and aphids for blackcurrant with pesticide treatment (PT) and without pesticide treatment (PF) and average of individual cultivars.

Pesticide treatment	Yield, (kg)	Berry size, (g/100)	Vegetative growth	Aphids	Mildew	Leaf Spot	Rust
PT	2.03	67	6.6	2.5	1.4	3.8	1.7
PF	1.05	62	5.4	2.5	1.7	5.5	2
LSD	0.56***	N.S.	0.55***	N.S.	N.S.	1.05*	N.S.
Cultivar							
Baldwin	1.22 ABCD	48 CD	5.1 CD	1.9 B	3.1 A	8.5 A	1.5 DE
Ben Alder	1.98 ABC	65 AB	4.8 D	2.8 A	1.4 C	5.3 CD	2.4 BC
Ben Dorain	0.92 BCD	59 BC	5.0 CD	2.4 AB	1.3 C	4.3 DE	3.1 A
Ben Gairn	1.43 CD	67 AB	5.3 CD	3.0 A	1.0 C	2.9 E	1.4 DE
Ben Hope	2.09 ABC	75 A	6.6 AB	1.9 B	1.0 C	4.1 DE	3.1 A
Ben Lomond	2.29 AB	75 A	6.0 ABC	2.4 AB	3.5 A	6.9 B	1.9 CD
Narve Viking	2.31 A	74 A	7.0 A	2.3 AB	1.0 C	1.2 F	2.7 AB
Tiben	1.48 ABCD	72 A	6.7 AB	2.8 A	1.3 C	5.8 BC	1.7 DE
Titania	0.35 C	71 AB	6.8 AB	2.8 A	1.0 C	3.7 E	1.0 E
8944-4	0.46 D	45 D	5.0 CD	2.6 A	1.0 C	6.4 BC	1.1 E
8944-13	1.41 ABCD	50 CD	5.8 BCD	2.4 AB	2.0 B	5.5 BCD	1.3 DE
p	*	***	***	***	***	***	***

Level of significance: N.S. = no significance, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Pesticide treatment: LSD test; cultivar: values within a column with the same letter show no significant difference between cultivars.

cultivars and not pesticide treatment. The incidence of leaf spot was significantly less on PT plants than PF plants but no significant differences were observed in the incidences of mildew and rust between the two treatments. There was a similar level of damage by aphids on both PT and PF plants. Significant differences were found between individual cultivars in all plant growth and physical condition measurements, indicating that yield and vegetative growth not only depend on pesticide treatment, but on individual cultivars too. In addition, different cultivars had different levels of resistance to diseases and aphids.

3.2. Total anthocyanin content

Anthocyanins are the major group of phenolics in blackcurrants, accounting for approximately 80% of total phenolics; the four main pigments delphinidin 3-*O*-glucoside, delphinidin 3-*O*-rutinoside, cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside contribute up to 97% of the total anthocyanin in blackcurrants (Anttonen & Karjalainen, 2006; Karjalainen et al., 2009). 'Baldwin', 'Ben Alder', 'Ben Lomond', 'Ben Dorain' and 'Titania' have previously been investigated for their total anthocyanin or total phenolic content (Bordonaba & Terry, 2008; Wu, Gu, Prior, & McKay, 2004; Moyer, Hummer, Finn, Frei, & Wrolstad, 2002). In this study we furthermore included 'Ben Gairn', 'Ben Hope', 'Narve Viking', 'Tiben' and '8944-4' and '8944-13'. Blackcurrant cultivars grown in Denmark contain 168–613 mg ME/100 g, depending on cultivars and harvest year (Pedersen, 2008).

As shown in Table 2, 'Ben Gairn' and 'Ben Alder' were the cultivars containing the highest concentration of total anthocyanin; 'Baldwin' and '8944-4' were the lowest. Research comparing organic and conventional grown blackcurrants reported no significant differences in total phenolic content (Anttonen & Karjalainen, 2006), which is in agreement with our study, showing no significant differences between PT and PF berries. However, significant differences were observed between individual cultivars. This suggested that anthocyanin production in blackcurrants is genetically predetermined and pesticide treatments on plants have little or no effect on the biosynthesis of anthocyanins. Nevertheless, significant differences in total phenolics were found between conventional, organic and sustainably grown marionberries and strawberries (Asami, Hong, Barrett, & Mitchell, 2003).

3.3. Ascorbic acid content

Blackcurrants are excellent sources of ascorbic acid, with an average content of 125–151 mg/100 g fresh weight (Szajdek & Borowska, 2008). Ascorbic acid content in different blackcurrant cultivars harvested from 2001 to 2005 in Denmark ranged from 96 to 219 mg/100 g (Pedersen, 2008). As shown in Table 2, the cultivar 'Baldwin' had the highest concentration of ascorbic acid, followed by 'Ben Dorain'. 'Titania' and 'Ben Alder' had the lowest concentration of ascorbic acid among the blackcurrant cultivars. However, no significant difference was observed between individual cultivars. In this study we found out that pesticide treatment had significant impact on ascorbic acid content (Table 2). Previous reports determined higher levels of ascorbic acid in organically-grown crops than conventional crops (Worthington, 2001). A three-year study showed that blackcurrants from an organic production system had significantly higher content of ascorbic acid than fruit from a conventional system (Kahu et al., 2009).

3.4. Total antioxidant capacity

Epidemiological studies have indicated that regular dietary intake of food rich in antioxidants will help to reduce the risk of cancer and cardiovascular diseases (Dragovic-Uzelac et al., 2007; Wu et al., 2004). Blackcurrants possess a high content of ascorbic acid, contributing together with phenolics to the high level of antioxidant potential (Cho et al., 2009; Halvorsen et al., 2002). Previous research showed that ORAC values of blackcurrant cultivars were between 37 and 93 μmol TE/g fresh-frozen fruit (Moyer et al., 2002). In this study, TEAC values ranged from 44 to 55 μmol TE/g. 'Ben Gairn' and 'Ben Dorain' had the highest TEAC values. 'Narve Viking', 'Titania' and 'Ben Hope' displayed the lowest TEAC values. In contrast to the results obtained by TEAC assay, cultivars with the highest ORAC value were 'Baldwin' and '8944-13'. 'Ben Hope' and 'Ben Dorain' had the lowest ORAC values among all cultivars. Neither TEAC assay nor ORAC assay showed significant differences in relation to cultivation methods or between individual cultivars (Table 2).

3.5. Bioassay activities

PGE2 is one of the major metabolites in the inflammatory process stimulated by cyclooxygenase-2 (COX-2). COX-2 is a key enzyme for the inflammatory process; it is a mediator enhancing

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Table 2
Average of total anthocyanin content (TA), ascorbic acid content (AA), TEAC assay, ORAC assay, PGE2 assay and anticancer cell proliferation (HT-29 and Caco-2) activities for blackcurrant with pesticide treatment (PT) and without pesticide treatment (PF) and average of individual cultivars.

Pesticide treatment	TA, (mg ME/100 g)	AA, (mg/100 g)	TEA, (μmol TE/g)	ORAC, (μmol TE/g)	PGE2 production, (%)	HT-29 inhibition, (%)	Caco-2 inhibition, (%)
PT	321	100	48	84	46	11	17
PF	350	139	51	82	47	33	34
LSD	N.S.	26.7**	N.S.	N.S.	N.S.	16.3**	14.3*
Cultivar							
Baldwin	203 F	176 A	53 ABC	90 A	45 BC	51 A	56 A
Ben Alder	408 AB	90 B	49 ABC	81 A	54 A	16 AB	29 AB
Ben Dorain	369 BC	152 AB	54 AB	77 A	50 AB	27 AB	30 AB
Ben Gairn	445 A	102 B	55 A	89 A	52 AB	23 AB	10 AB
Ben Hope	313 CDE	114 AB	46 C	74 A	50 AB	33 AB	20 AB
Ben Lomond	274 DE	111 AB	46 BC	84 A	45 BC	−9 B	22 AB
Narve Viking	359 BC	117 B	44 C	86 A	52 AB	29 AB	22 AB
Tiben	411 AB	105 AB	52 ABC	84 A	47 AB	7 AB	23 AB
Titania	329 CD	87 B	46 C	78 A	35 D	12 AB	21 AB
8944-4	268 E	125 AB	49 ABC	82 A	38 CD	20 AB	15 AB
8944-13	314 CDE	131 AB	52 ABC	89 A	45 BC	34 AB	33 B
p	***	N.S.	N.S.	N.S.	**	N.S.	N.S.

LSD test, level of significance: N.S. = no significance, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Cultivar: values within a column with the same letter show no significant difference between cultivars.

pain, swelling, fever, vascular permeability and redness during inflammation (Smith, Dewitt, & Garavito, 2000). Thus COX-2 activity in cells can be estimated by measuring the PGE2 production. The proanthocyanidins in blackcurrants inhibit PGE2 synthesis *in vitro* but not in the whole blood assay (Christensen, 1990). In this study, we proved that all blackcurrant extracts inhibited PGE2 production. After treatment with blackcurrant extracts, PGE2 production was reduced to between 35% and 54%. The positive control, indomethacin, reduced PGE2 production to 29%. The cultivars 'Titania' and '8944-4' displayed the highest inhibitory activity against COX-2 enzyme. No significant difference on pesticide treatment was observed, however individual cultivars showed different inhibition against PGE2 production (Table 2).

Blackcurrant extracts have been shown to inhibit the growth of a number of cancer cell lines: Hela, Fem X, LS 174, MCF-7, PC-3 and MDA-MB-231 (Cho et al., 2009; Konic-Ristic et al., 2011). In this study, HT-29 cancer cell proliferation inhibition of blackcurrants was from 0% to 51%. Caco-2 cancer cell proliferation inhibition ranged from 10% to 56%. The cultivar 'Baldwin' and '8944-13' displayed the strongest inhibitory effect against both cancer cell lines. 'Ben Lomond' and 'Ben Gairn' showed the weakest inhibitory effect in HT-29 and Caco-2, respectively. PF blackcurrants showed significantly higher inhibitory effect against HT-29 and Caco-2 cancer cell proliferation compared to PT blackcurrants; however, no significant difference was observed between cultivars (Table 2).

3.6. Correlation analysis

Table 3 shows the linear correlation between total anthocyanin content, ascorbic acid content, total antioxidant capacity, PGE2 assay and anticancer cell proliferation activities. In this study we found that total antioxidant capacities were poorly correlated with total anthocyanin content and ascorbic acid content in blackcurrants, although they are both strong antioxidants. This is probably because the blackcurrant juice extracts contained both anthocyanin and ascorbic acid, in different proportion. Additionally we found no correlation between TEAC and ORAC assay. As reported previously, blackcurrant anthocyanins have a poor correlation to the total antioxidant capacity (Moyer et al., 2002). Dragovic-Uzelac et al. (2007) reported that different total antioxidant methods resulted in different values and might not have any correlation. Differences in the results from ORAC and TEAC in this study reflected the presence of several types of antioxidant in blackcurrants with different reactivity towards the radicals. However, we observed significant correlation between ascorbic acid content and anticancer cell proliferation activities. Ascorbic acid exerted antiproliferative effects by inhibiting the cell cycle of prostate carcinoma cells (Frömberg et al., 2011) and induced apoptosis in human breast cancer cells SK-BR3 and Hs578T (Hong et al., 2007). Although the effectiveness of ascorbic acid in cancer treatment remains controversial, the interest among scientists has not decreased, as a number of research results continue to point out that ascorbic acid therapy is effective depending on the protocol used (Ohno, Ohno, Suzuki, Soma, & Inoue, 2009; Padayatty & Levine, 2000; Tamayo & Richardson, 2003).

Table 3
Linear regression correlation between total anthocyanin content (TA), asocorbic acid content (AA), TEAC assay, ORAC assay, PGE2 assay and anticancer cell proliferation (HT-29 and Caco-2) activities of blackcurrants.

	TA	AA	TEAC	ORAC	HT-29
AA	0.090				
TEAC	0.064	0.227			
ORAC	0.006	0.028	0.074		
PGE2	0.265*	0.023	0.065	0.029	
HT-29	0.008	0.418***	0.138	<0.001	
Caco-2	0.070	0.374**	0.068	0.012	0.424***

Level of significance: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

From the results we suggest that in addition to anthocyanins and ascorbic acid, there may well be additional bioactive compounds in blackcurrants present in lower concentrations that are responsible for the biological performance, for example other flavonoids, phenolic acids and polysaccharides (Takata, Yamamoto, Yanai, Konno, & Okubo, 2005; Zadernowski, Naczk, & Nesterowicz, 2005). Moreover, synergistic effects of all phytochemicals should be taken into consideration (Liu, 2004).

Multivariate data analyses was applied to investigate correlations between yield, berry size, vegetative growth, damage caused by diseases and aphids, total anthocyanin content, ascorbic acid content, TEAC assay, ORAC assay, PGE2 assay, anticancer cell proliferation activities, NMR and HPLC data. Fig. 1(a) shows the corresponding PCA score plot. PCA could separate two main groups according to pesticide treatment, though not completely. Fig. 1(b) shows the corresponding loading plot. Pesticide treatment had a great impact on yield, vegetative growth, ascorbic acid, leaf spot but not PGE2 assay or aphids on the plants. Although the statistical analysis in Table 1 indicated that there was no significant difference for berry size between cultivation methods, the multivariate

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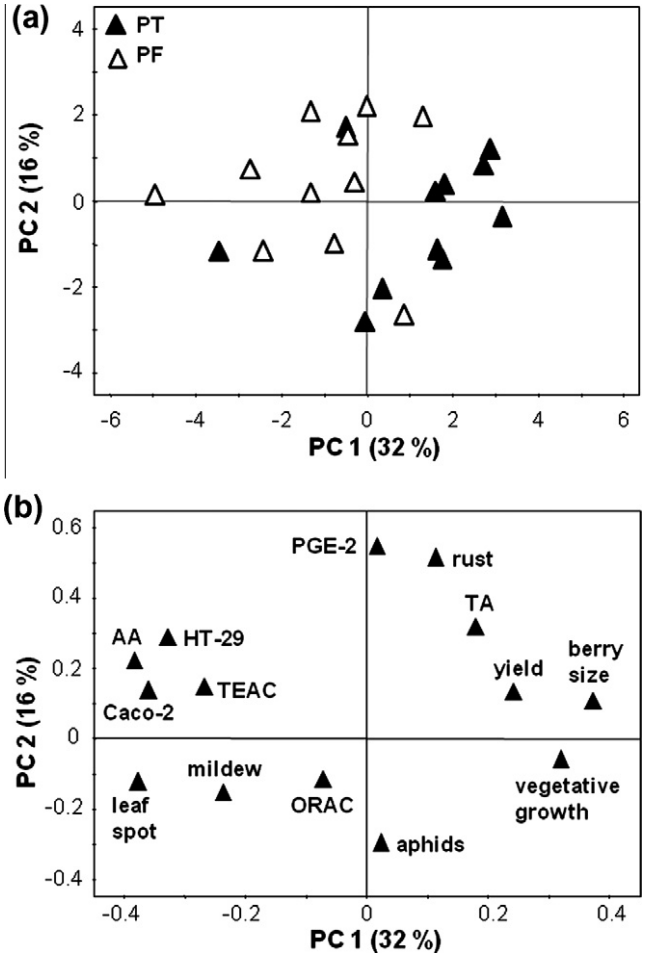

Fig. 1. (a) PCA score plot and (b) PCA loading plot for yield, berry size, vegetative growth, damage caused by diseases and aphids, total anthocyanin content, ascorbic acid content, TEAC assay, ORAC assay, PGE2 assay and anticancer cell proliferation activities.

data analysis showed that pesticide treatment has an impact on berry size. Correlation between ascorbic acid and anticancer cell (Caco-2 and HT-29) proliferation activities were confirmed by the close proximity of these three variables in the loading plot. Total anthocyanin content and ascorbic acid content were negatively correlated; however, further investigation is needed to confirm this relationship. PC1 and PC2 explained only 32% and 16% of the variation, respectively, indicating that PCA analysis was unable to describe the effect of pesticide treatment effectively.

In this study, neither PCA modelling of NMR nor HPLC data was able to distinguish the cultivation method used. The results showed that modelling of ^{1}H NMR data and HPLC data was unable to describe the majority of the *Y* variables. There was a good correlation between both data and total anthocyanin content, with a linear regression of 0.68 for NMR data and 0.72 for HPLC data, which is not surprising, since anthocyanins are the dominant compounds in blackcurrant fruits and the absorbance specific to phenolics was measured in HPLC analyses. Similarly, modelling of liquid chromatography–mass spectroscopy data of blackcurrants has been previously used to distinguish blackcurrants from different farms but not the cultivation methods (Anttonen & Karjalainen, 2006).

4. Conclusions

This study showed that pesticide treatment had a significant impact on yield, vegetative growth, leaf spots, ascorbic acid content and anticancer cell proliferation inhibition activities. PF blackcurrants contained higher concentration of ascorbic acid and displayed better inhibitory effects against cancer cell proliferation compared to PT blackcurrants, and thus blackcurrants from plants grown without pesticide treatment are potentially more health-promoting. However, anthocyanin content was dependent on individual cultivars not pesticide treatment.

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