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Characterization and biological activity of flavonoids from ripe fruit of an anthracnose-resistant blueberry cultivar

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ABSTRACT

Anthracnose fruit rot, caused by *Colletotrichum acutatum*, is among the most important diseases of blueberries. Most cultivars are susceptible but 'Elliott' is resistant. Our objective was to identify possible antifungal compounds that play a role in the resistance response. Chemical fractions from freeze-dried, ripe fruit of 'Elliott' and a susceptible cultivar (Jersey) were extracted with methanol and ethyl acetate. Extracts were screened on solid media for suppression of microconidiation of *C. acutatum*. The methanolic extract was fractionated and the soluble methanolic fraction from 'Elliott' was the most biologically active. This fraction was dried, dissolved in water, and screened *in vivo* by pre-treating ripe 'Jersey' fruit with 0.5, 1, 2, and 4% solutions (w/v) and subsequently inoculating the fruit with *C. acutatum*. An 88% reduction in infection incidence was observed after 12 days with the 4% solution. Anthocyanins and other flavonoids were then quantified in fruit of the two cultivars using HPLC-MS. 'Elliott' fruit contained more anthocyanins (4.87 mg/g of freeze-dried tissue) than 'Jersey' (3.27 mg/g of freeze-dried tissue); however, the same compounds were found in both cultivars. 'Elliott' fruit also contained more non-anthocyanin flavonoids (0.18 mg/g of freeze-dried tissue) than 'Jersey' (0.12 mg/g of freeze-dried tissue), including two distinctive compounds in 'Elliott'. The non-anthocyanin flavonoid fractions of both 'Elliott' and 'Jersey' significantly decreased the growth of *C. acutatum* in a liquid bioassay but the effect was more pronounced in the 'Elliott' fraction. The two distinctive compounds in 'Elliott' were further characterized by MS/MS and were identified as quercetin 3-O-rhamnoside and, putatively, syringetin rhamnoside. Additionally, we evaluated 'Elliott' and 'Jersey' methanolic extracts for their ability to inhibit lipid peroxidation, and the extract obtained from 'Elliott' was almost twice as active at inhibiting peroxidation. These results provide new insights into the role of antifungal compounds in the resistance response of ripe 'Elliott' blueberries to infection by *C. acutatum*.

1. Introduction

Anthracnose fruit rot caused by the fungus *Colletotrichum acutatum* J. H. Simmonds is a major postharvest disease of blueberries (*Vaccinium corymbosum* L.). Most blueberry cultivars are susceptible to anthracnose fruit rot but some resistant cultivars exist [1]. For instance, the cultivar Elliott has been consistently identified as resistant to *C. acutatum* in field as well as laboratory assays, whereas the cultivar Jersey has been found to be susceptible [1,2]. Additional research has shown that fruit rot resistance does not correlate with foliar infection [3] or the production of antimicrobial fruit volatiles [4].

Host-pathogen interactions have only been well characterized in a few *Colletotrichum*-fruit pathosystems, including avocado, citrus, and mango [5,6], and most of the information on resistance mechanisms in fruit comes from studies of *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. on avocado [7,8]. A microscopy study of infection of ripe blueberry fruit by *C. acutatum* showed different infection strategies depending on the cultivar being colonized [9]. In addition, an accumulation of amorphous phenolic globules was observed at the site of infection in the resistant cultivar [9]. More recent studies have identified an oxidative burst following fruit infection by *C. acutatum* in 'Elliott' blueberries [10] as well as a positive correlation between sugar content and fruit rot resistance in blueberry cultivars [11]. However, the role of antifungal compounds in the resistance response also needs to be investigated.

The biochemical composition, particularly anthocyanin content, of ripe blueberry fruit has been studied from a nutraceutical

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perspective [12–15]. Demand for blueberries and other fruit crops continues to rise as the beneficial effects on cardiovascular and neurodegenerative diseases in humans are becoming increasingly apparent [16–19]. Blueberries are rich in antioxidants in the form of anthocyanins and other phenolic compounds [17]. In fact, anthocyanins (the predominant class of flavonoids in blueberries) have been shown to aid in obesity prevention [20], cardiovascular health [21], act as an anti-inflammatory [22] and have anti-cancer effects [23–25].

Blueberries contain many different flavonoids, including five main classes of anthocyanins [26], three classes of flavonols [27], and one class of flavan-3-ols [28]. While not much is known about the role of chemical constituents in host plant defense to plant pathogens in blueberries, several studies have been carried out on the antifungal properties of ripe fruit extracts as they relate to fruit decay and herbivore preference in five Ericaceous species: *V. corymbosum*, *Vaccinium vacillans* Kalm ex Torr., *Gaylussacia frondosa* (L.) Torrey & A. Gray ex Torrey, *Vaccinium macrocarpon* Ait., and *Gaultheria procumbens* L. [29–31]. Although these studies did not identify individual compounds, they indicated that the main antifungal compounds present in ripe blueberry fruit were water-soluble phenolics and acids. They also proposed that resistance to fungal decay in ripe blueberries may be due to an interaction between simple phenolic compounds and organic acids and not necessarily to individual fungitoxic compounds.

Chemical compounds have been implicated in host plant resistance to *Colletotrichum* spp. in various crops. For instance, in avocado, antifungal dienes [32] and the flavonoid epicatechin [33] play a role in ontogenic resistance of unripe fruit to infection by *C. gloeosporioides*. A reduction in these compounds as fruit ripens is correlated with increased susceptibility to fungal infection. Researchers have also identified constitutive alk(en)ylresorcinols in fruit of *Colletotrichum*-resistant mango varieties [34]. In sorghum, the phytoalexin 3-deoxyanthocyanidin accumulates in response to infection by *Colletotrichum sublineolum* Henn. [35]. Based on microscopic observations in blueberry and chemical resistance mechanisms in other fruit crops mentioned above, the potential role of chemical constituents in the defense against anthracnose fruit rot in blueberry merits investigation as well. Therefore, the objectives of this study were to: (1) assess chemical extracts of blueberry fruit for biological activity against *C. acutatum*, and (2) identify and quantify specific compounds in the active fractions.

2. Materials and methods

2.1. Plant and fungal material

Ripe fruit of blueberry (*V. corymbosum*) cultivars Elliott and Jersey were harvested in August 2010 from mature bushes at the Michigan State University Southwest Michigan Research and Extension Center in Benton Harbor, MI, USA and a commercial field in Traverse City, MI, USA, respectively. From each cultivar, a composite sample of approximately 10 kg of blueberries was harvested and transported to the laboratory. For analysis, a representative 0.5-kg subsample of fruit was stored overnight at −20 °C and freeze-dried. Freeze-dried material was stored at −20 °C until chemical extraction.

A single-conidium isolate of *C. acutatum* (isolate #0001) from blueberry fruit collected in Grand Junction, MI, USA in August of 2006 was used for all experiments. The isolate was stored as conidia in a glycerol stock, and cultured in accordance with previous studies [9,10]. For inoculum production, sporulating cultures were flooded with 3 ml of sterile deionized water (SDW), and conidia were dislodged using a sterilized L-shaped glass rod. Conidia were counted using a hemacytometer, and a concentration of 1×10^6 conidia/ml was achieved via dilution with SDW and used for all experiments.

2.2. Chemical extraction and fractionation

Five-gram fruit samples of 'Elliott' and 'Jersey' (prior to inoculation and 4 days post inoculation) were homogenized separately for 5 min with a Sorvall tissue homogenizer (Thermo Fisher Scientific, Waltham, MA, USA) in 100 ml of 80% acidified methanol (pH = 2.0), incubated for 30 min and centrifuged at 5000× *g* for 10 min. The extract was concentrated using a rotary evaporator, and the remaining water portion was lyophilized. The lyophilized material was dissolved in 100% acidified methanol (pH = 2.0) to a specific concentration (5 g fresh wt/ml). Methanol-insoluble material was filtered then further extracted with ethyl acetate and centrifuged at 5000× *g*. This extract was then dried down and dissolved in acidified methanol as described above. Methanolic extracts from ripe fruits of 'Elliott' and 'Jersey' (5 g of fresh weight per ml) before and 4 days after inoculation with *C. acutatum* conidia were loaded onto a 250-μm cellulose thin layer chromatography (TLC) plate (Analtech Inc., Newark, DE, USA) (50 μl per sample) and compounds were separated with butanol:acetic acid:water (4:1:5 top phase). Plates were allowed to dry overnight and then sprayed with an aqueous conidial suspension of *C. acutatum* (10^6 conidia/ml). Plates were incubated at room temperature (22–24 °C) for 60 h in a 100% relative humidity chamber and subsequently stained using the gas phase created from iodine crystals inside a TLC chamber. Areas of inhibition of fungal growth were measured. On a replicate plate, those areas were removed by scraping, dissolved in methanol, and scanned with a UV/Vis spectrophotometer at wavelengths between 200 and 600 nm (Fig. S1).

Since no significant differences were observed in antifungal activity between extracts from inoculated and non-inoculated 'Elliott' fruit, all further extractions were conducted on fresh or lyophilized, non-inoculated fruit. In fact, UV/Vis experiments showed degradation of flavonoids (particularly anthocyanins) in fruits at more than 6 days after inoculation (data not shown). Initially, an exhaustive extraction of fresh whole fruits with water, methanol, and ethyl acetate was used (Fig. S2). As described above, fresh tissue was homogenized for 5 min with a Sorvall tissue homogenizer in 150 ml of water and centrifuged at 5000× *g* for 10 min. The macerated fruit residue was then extracted in 150 ml of methanol and centrifuged at 5000× *g* for 10 min. The residue was further extracted with ethyl acetate and centrifuged at 5000× *g* for 10 min. All fractions were dried using a rotary evaporator or lyophilized and stored at −20 °C for bioassays. Initial screenings on PDA plates (described below) indicated that the methanolic extract had the most biological activity. Therefore, to increase yield, a second extraction with methanol was used on lyophilized ripe fruits of 'Elliott' and 'Jersey' (Fig. S3). Residue from the second extraction was further extracted with ethyl acetate. In order to remove sugars present, the dried methanolic extract was stirred in 20 ml of methanol for 1 min and separated into a methanol-soluble and -insoluble fraction.

2.3. Antimicrobial screening of extracts and in vivo activity of the methanol-soluble fractions

For the bioassay, 400-μl aliquots of an aqueous suspension of *C. acutatum* (1×10^6 conidia/ml) were applied to potato dextrose agar (Difco Laboratories, Detroit, MI, USA) in Petri plates (100 mm diam × 15 mm high), spread out over the surface using a sterile L-shaped glass rod and allowed to dry in a laminar flow hood. Chemical extracts and fractions were screened for antimicrobial activity by applying extracts dissolved in dimethyl sulfoxide (DMSO) at various concentrations (1000, 500, 250, 125, 63, 31, and 16 μg dried extract per 10-μl DMSO droplet) as droplets directly onto the spore-covered agar surface. Plates were covered with lids

and incubated for 48 h at 25 °C in the dark, then monitored for the inhibition of *C. acutatum* microconidiation (production of secondary conidia directly on conidia). Extracts or fractions were considered biologically active if microconidiation was absent. Each extract or fraction was tested at least twice and all experiments had at least two replicate droplets. For *in vivo* inhibition, the dried methanol-soluble fraction was dissolved in water at varying concentrations (0.5, 1, 2, 4%), and 'Jersey' fruits were pretreated by immersing fruit in the extract prior to inoculation with *C. acutatum* using five replicates and 10 fruits per replicate. These concentrations were chosen because whole blueberries contain approximately 0.1–0.2% anthocyanins by fresh weight [36]; however, these compounds are concentrated within the peel so higher concentrations were chosen. Fruits were incubated at 100% relative humidity, and disease incidence was rated visually 12 days post inoculation.

2.4. Identification and quantification of flavonoids

Initial UV/Vis experiments and previous research [15,37] suggested that the methanol-soluble fraction was rich in anthocyanins and other flavonoids. To quantify anthocyanins and non-anthocyanin flavonoids in accordance with previous studies [15], 5 g of freeze-dried blueberries per cultivar were extracted separately in 10 ml of non-acidified methanol following the protocols described above. Due to the fact that anthocyanins were the most abundant chemical, they were quantified directly from the extract. However for non-anthocyanin flavonoids, the anthocyanins were removed prior to quantification using a SepPak C18 cartridge (Waters Corporation) according to standard protocols [38].

Anthocyanins and non-anthocyanin flavonoids were separated on a 150 × 4.6-mm Symmetry Shield C18 column with a 5-μm particle size (Waters Corporation, Milford, MA, USA) at a flow rate of 0.5 ml/min and a column temperature of 25 °C using a 2695 Separator Module, with a photodiode array (PDA) detector (#2996) and a Micromass ZQ mass spectrometer (Waters Corporation). For anthocyanins, compounds were eluted with a gradient of 10% formate and 1% formic acid in acetonitrile at a flow rate of 0.5 ml/min over a 55-min period with a gradient from 5% to 35% acetonitrile. The MS (mass spectrometer) and PDA detectors scanned ions (250–750 *m/z* in positive ion mode) at 200- to 550-nm wavelengths in order to identify individual compounds. Non-anthocyanin flavonoid compounds were eluted with a gradient of 1% formate and 1% formic acid in acetonitrile at a flow rate of 0.5 ml/min over a 40-min period with a gradient from 10% to 35% acetonitrile. The PDA detector scanned ions (250–750 *m/z* in positive ion mode with a scan time of 0.3 s, an interscan delay of 0.1 s and a source temperature of 140 °C) at 200- to 550-nm wavelengths. In order to identify and quantify compounds, quercetin 3-O-rhamnoside (Sigma–Aldrich, St Louis, MO, USA) and quercetin 3-O-glucoside (Sigma–Aldrich) were included as standards because they are inexpensive and abundant in blueberries. Published research on retention times, masses, and absorption spectra was used to identify many of the compounds (Tables 1 and 2) [15,37].

Each extract was analyzed with duplicate HPLC injections. The mean peak areas from the duplicate HPLC analyses were used to determine the concentration of anthocyanin and non-anthocyanin flavonoids from the standard curves. Data were collected for five replicate samples (extracted from different fruit samples) per cultivar and averaged to determine the quantity of different compounds. For anthocyanins, a wavelength of 520 nm and a standard curve plot of cyanidin 3-O-glucoside (Polyphenols Laboratories, Sandnes, Norway) were used. For non-anthocyanin flavonoids, a wavelength of 255 nm and a standard curve plot of quercetin 3-O-rhamnoside (Polyphenols Laboratories) were used.

The identities of all of the non-anthocyanin flavonoid compounds were further confirmed by MS/MS (Table 2), focusing on 09F and 10F because they appeared to be specific to 'Elliott' fruit (Fig. 4). Analysis was accomplished using a QTRAP 3200 mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA, USA) coupled to a UFLC LC-20AD system (Shimadzu Corporation, Kyoto, Japan). The mass spectrometer was operated in the positive ion mode with a TurboIon Spray source. A daughter ion scan was conducted using the parent ions 449 (compound 09F) and 493 (compound 10F) with an initial ionization of 20 V and a collision voltage of 30 V. The other ionization parameters were as follows: curtain gas (psi), 10; ion source gas 1 12; ion source gas 2 30; source temperature 400 °C; entrance potential 10 V; collision-activated dissociation high; ion spray voltage 5500 V. The mass spectrometer and the HPLC system were controlled by Analyst 1.4.2 software (Applied Biosystems/MDS Sciex).

2.5. Liquid bioassay of specific extracts

Minimal medium consisting of 0.7% KH_2PO_4, 0.4% KNO_3, 0.3% Na_2HPO_4, 0.1% $MgSO_4$, 0.03% $CaCl_2$, 1.5% BACTO-agar, and 4% D-glucose and 4% D-fructose [39] was used to grow *C. acutatum* in the presence of various extracts and fractions (using 50 μg of dried material per 100 μl of liquid medium). This concentration was chosen because blueberries have been reported to contain 39 μg of non-anthocyanin flavonoids and 1126 μg of anthocyanins per gram of fresh weight [36,40]. Fractions from which anthocyanins or flavonols had been removed, were prescreened using HPLC-MS as described above in order to confirm that the removal was successful and that the compounds were in their glycosylated state. Fractions were dissolved in methanol and applied to wells in 96-well culture plates and allowed to dry. A control consisted of dried methanol only. Minimal medium (90 μl) was then applied followed by an addition of 10 μl of an aqueous inoculum suspension containing 1×10^6 washed conidia/ml. Conidia were washed to remove the polysaccharide matrix by centrifuging at 500× *g* for 5 min and re-suspending the pellet with an equal volume of water three times. Cultures were incubated at 25 °C in the dark and non-inoculated controls were included for each treatment. Fungal growth was quantified daily by measuring the optical density (λ 590 nm). Light absorbance was read with an EL 800 Universal Microplate Reader (BioTek Instruments, Winooski, VT, USA). There were three replicate wells per treatment and experiments were conducted twice. Optical density readings of the non-inoculated controls were substracted from those of the inoculated treatments to account for background light absorbance of the various extracts. The relative area-under-the-growth curve (RAUGC) was calculated using the change in optical density (OD) values over time (days) according to the following equation: [((2 + 1)/2)*(day 2 OD-day 1 OD)]+[((4 + 2)/2)*(day 4 OD-day 2 OD)]+[((5 + 4)/2)*(day 5 OD-day 4 OD)].

2.6. Lipid peroxidation assay

To investigate the antioxidant potential of 'Elliott' and 'Jersey' methanolic extracts, we conducted a lipid peroxidation assay, evaluating model liposome oxidation using fluorescence spectroscopy according to a procedure reported previously [41]. Methanolic extraction was performed as described above, using non-acidified methanol on freeze-dried fruit tissue of 'Elliott and 'Jersey'. The lipid substrate, 1-stearoyl-2-linoleoyl-sn-glycero-3-phosphocholine (SLPC) was obtained from Avanti Polar Lipids (Alabaster, AL, USA). The fluorescent probe, DPH-PA, was obtained from Molecular Probes (Eugene, OR, USA). Positive controls, terbutylhydroquinone (TBHQ), butylated hydroxyanisole (BHA) and butylated hydroxytoluene

Table 1
Anthocyanins from the anthracnose fruit rot-resistant blueberry cultivar Elliott and susceptible cultivar Jersey identified by HPLC/MS analysis and quantified using HPLC/PDA. Compounds were putatively identified based on previous research, available spectra, standards, retention times and *m/z* ratios. Anthocyanins were quantified at a wavelength of 520 nm using a standard curve plot of cyanidin 3-O-glucoside.

Compound ID[a]	Compound name[b]	RT (min)[c]	(M+H) *m/z* (total, aglycone)[d]	Elliott ± SE[e] (μg/g lyophilized fruit)	Jersey ± SE[e] (μg/g lyophilized fruit)
01A	Delphinidin 3-O-galactoside[f,g]	15.5	465, 303	583.45 ± 83.83	346.10 ± 64.29
02A	Delphinidin 3-O-glucoside[f,g]	18.2	465, 303	31.13 ± 27.27	201.67 ± 45.08
03A	Cyanidin 3-O-galactoside[f,g]	20.1	449, 287	106.77 ± 32.64	68.68 ± 28.46
04A	Delphinidin 3-O-arabinoside[f,g]	22.0	435, 303	406.36 ± 66.87	300.88 ± 56.78
05A	Cyanidin 3-O-glucoside[f,g]	23.6	449, 287	24.56 ± 24.56	42.63 ± 27.61
06A	Petunidin 3-O-galactoside[f,g]	24.4	479, 317	443.85 ± 111.96	209.08 ± 44.86
07A	Cyanidin 3-O-arabinoside[f,g,h]	27.5	419, 287	65.94 ± 28.87	198.36 ± 40.08
08A	Petunidin 3-O-glucoside[f,g,h]	27.5	479, 317	++[h]	++[h]
09A	Peonidin 3-O-galactoside[f,g]	29.8	463, 301	64.54 ± 29.19	37.05 ± 26.34
10A	Petunidin 3-O-arabinoside[f,g]	32.2	449, 317	256.31 ± 47.46	163.53 ± 40.61
11A	Malvidin 3-O-galactoside[f,g,i]	33.5	493, 331	1330.57 ± 153.76	452.26 ± 68.51
12A	Peonidin 3-O-glucoside[f,g,i]	33.5	463, 301	++[i]	++[i]
13A	Malvidin 3-O-glucoside[f,g]	37.0	493, 331	71.90 ± 30.64	467.01 ± 67.37
14A	Peonidin 3-O-arabinoside[f,g]	38.3	433, 301	28.87 ± 28.87	24.56 ± 24.56
15A	Malvidin 3-O-arabinoside[f,g]	39.5	463, 301	1454.57 ± 240.91	763.07 ± 99.66
Total				**4868.82 ± 102.6**	**3274.87 ± 150.2**

[a] Compounds are listed in elution order, and A denotes an anthocyanin.
[b] All compounds were identified based on UV/Vis spectra and *m/z* ratios. Cyanidin 3-O-glucoside was confirmed with a standard.
[c] RT = retention time in minutes.
[d] Mass to charge ratio (*m/z*) (positive ion mode, (M+H)), total and aglycone refers to the parent and daughter ion mass.
[e] Data are expressed as μg per gram of lyophilized fruit tissue. Blueberry fruit contained 79–82% water.
[f] Previous research from Harborne et al. (1975) was used for identification.
[g] Previous research from Prior et al. (2001) was used for identification.
[h] Cyanidin 3-O-arabinoside (419/287) coelutes with petunidin 3-O-glucoside (479/317) (27.5 min). Petunidin 3-O-glucoside predominates.
[i] Malvidin 3-O-galactoside (493/331) coelutes with peonidin 3-O-glucoside (463/301) (33.5 min). Malvidin 3-O-galactoside predominates.

(BHT) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). The lipid and fluorescent probe, DPH-PA, were combined in dimethylformamide (DMF) and dried at room temperature under vacuum. Large unilamellar liposomes (LUVs) were produced by resuspension of the lipid-probe mixture (0.15 M NaCl, 0.1 mM EDTA and 0.01 M MOPS maintained over chelating resin Chelex 100) followed by ten freeze–thaw cycles in a dry ice-ethanol bath and extrusion (29 times) through a 100-nm pore size membrane (Avestin Inc., Ottawa, Canada). The final assay volume was 2 ml consisting of 100 ml HEPES buffer (50 mM HEPES and 50 mM Tris), 200 ml 1 M NaCl, 1.64 ml N_2 sparged water, 20 ml of test sample or dimethyl sulfoxide (control) and a 20-ml aliquot of liposome suspension. The peroxidation was initiated by the addition of 20 ml $FeCl_2.4H_2O$ (0.5 mM) for the positive control and test samples at 50 ppm. Fluorescence was measured at 384 nm and monitored at 0, 1, and 3 min, and every 3 min thereafter up to 21 min using a Turner model 450 digital fluorometer (Barnstead Thermolyne, Dubuque, IA, USA). The decrease of relative fluorescence intensity with time indicated the rate of peroxidation, and the data are reported for 21 min after the initiation of peroxidation. The relative fluorescence (*Ft*/*F*0) was calculated by dividing the fluorescence values by the concentrations. The inhibition of lipid peroxidation by anthocyanins has been reported previously [22,42] and hence purified blueberry anthocyanins were not assayed in this study.

Table 2
Non-anthocyanin flavonoids from the resistant blueberry cultivar Elliott and susceptible blueberry cultivar Jersey identified by HPLC/MS analysis and quantified using HPLC/PDA. Compounds were putatively identified based on previous research, available spectra, standards, retention times and *m/z* ratios. Non-anthocyanin flavonoids were quantified at a wavelength of 255 nm using a standard curve plot of quercetin 3-O-rhamnoside. HPLC-MS/MS was also used to confirm the *m/z* ratio for the total molecule and the daughter ions.

Compound ID[a]	Compound[b]	RT (min)[c]	(M+H) *m/z* (total, aglycone)[d]	Elliott ± SE[e] (μg/g lyophilized fruit)	Jersey ± SE[e] (μg/g lyophilized fruit)
01F	Myricetin rutinoside[f]	23.9	627, 319	3.93 ± 0.29	9.72 ± 0.34
02F	Quercetin rutinoside[f]	26.1	611, 303	10.52 ± 0.41	5.38 ± 0.73
03F	Myricetin pentoside[f,g]	27.0	451, 319	0.29 ± 0.69	0.27 ± 0.88
04F	Myricetin methyl pentoside[f]	27.6	465, 319	10.61 ± 0.87	5.39 ± 0.64
05F	Quercetin hexoside[f,g]	28.1	465, 303	16.19 ± 2.29	56.32 ± 10.39
06F	Quercetin 3-O-glucoside[f,g]	28.3	465, 303	5.18 ± 0.10	0.45 ± 0.74
07F	Quercetin pentoside[f,g]	30.9	435, 303	14.53 ± 0.90	28.28 ± 0.01
08F	Quercetin pentoside[f,g]	31.8	435, 303	30.78 ± 3.25	12.28 ± 0.31
09F	Quercetin 3-O-rhamnoside[f]	32.7	449, 303	61.28 ± 7.70	2.11 ± 1.50
10F	Syringetin methyl pentoside[f]	36.5	493, 347	26.54 ± 2.79	1.06 ± 0.74
Total				**179.86 ± 12.61**	**123.28 ± 14.55**

[a] Compounds are listed in elution order, and F denotes a non-anthocyanin flavonoid.
[b] All compounds were identified based on UV/Vis spectra and *m/z* ratios. Quercetin 3-O-glucoside and quercetin 3-O-rhamnoside were confirmed with standards.
[c] RT = retention time in minutes.
[d] Mass to charge ratio (*m/z*) (positive ion mode, (M+H)), total and aglycone refers to the parent and daughter ion mass.
[e] Data are expressed as μg per gram of lyophilized fruit tissue. Blueberry fruit contained 79–82% water.
[f] Previous research from Harborne et al. (1975) was used for identification.
[g] Previous research from Latti et al. (2009) was used for identification.

2.7. Statistical analysis

Data were subjected to an analysis of variance and mean separation by Fisher's Protected LSD ($\alpha = 0.05$) using Statgraphics Centurion (StatPoint Technologies, Warrenton, VA, USA) and SIGMAPLOT version 11 (SYSTAT Software, San Jose, CA, USA).

3. Results and discussion

3.1. Extracts and fractions from the resistant cultivar Elliott suppress growth, microconidiation and blueberry fruit infection by C. acutatum

The TLC plate assay showed evidence of growth inhibition of *C. acutatum* when methanolic extracts from ripe fruit of the anthracnose-resistant blueberry cultivar Elliott were separated on a cellulose plate (Fig. S1). Since no inhibition was observed using 'Jersey' extracts, this led us to believe that 'Elliott' contained preformed antimicrobial compounds. The regions of the TLC plate where inhibition was observed also exhibited a characteristic UV/Vis spectrum common to flavonoids. All flavonoids, including anthocyanins, flavonols, isoflavonols and flavones, absorb light between 250 nm and 280 nm; typically this absorption pattern is referred to as Band II. Dependent upon specific pH conditions, anthocyanins also absorb light between 460 and 550 nm and have an absorption maximum of 520 nm. Flavonols exhibit an absorption pattern from 350 to 385 nm [43].

Extracts and fractions from fruit of the resistant blueberry cultivar Elliott were more effective at inhibiting microconidiation of *C. acutatum* on agar plates than those from the susceptible cultivar Jersey. In particular, the methanol, methanol-soluble, and non-anthocyanin flavonoid fractions were the most biologically active (Table S1). The non-anthocyanin flavonoid fraction of 'Elliott' was the most effective at inhibiting microconidiation (activity observed when $\geq$ 125 μg per 10-μl DMSO droplet were applied directly to a microconidiating *C. acutatum* culture). In the 'Elliott' anthocyanin fraction, activity was only observed at 1000 μg/10-μl DMSO droplet, and no activity was observed in the 'Jersey' anthocyanin fraction. No activity was observed in the ethyl acetate extract of either cultivar (Table S1). Since, the most active fraction was the methanolic extract of 'Elliott', and higher activity was observed in the non-anthocyanin flavonoid fraction, this indicated that antifungal compounds were soluble in methanol and were able to be eluted from a SepPak column with ethyl acetate and therefore considered more hydrophobic in nature.

Non-anthocyanin flavonoids appear to be the primary antimicrobial compounds because the most active fractions always contained non-anthocyanin flavonoids. Previous research has demonstrated the antimicrobial activity of non-anthocyanin flavonoids against *Colletotrichum* species, including methoxylated flavones [44], isoflavonoids [45], and 3-deoxyanthocyanidins [35].

An 88% reduction in anthracnose infection incidence in 'Jersey' fruits was observed when they were pretreated with a 4% solution of the methanol-soluble fraction from 'Elliott' fruit (containing mainly anthocyanins and non-anthocyanin flavonoids). A dose response was evident and statistically significant ($P = 0.011$), indicating that the 'Elliott' methanol-soluble fraction was more effective as the concentration increased (Fig. 1). Activity was also observed using 'Jersey' fractions but it was not as distinct as that of the 'Elliott' fractions and the dose response was not as clear. This showed that the extracted compounds also had antifungal activity *in planta*.

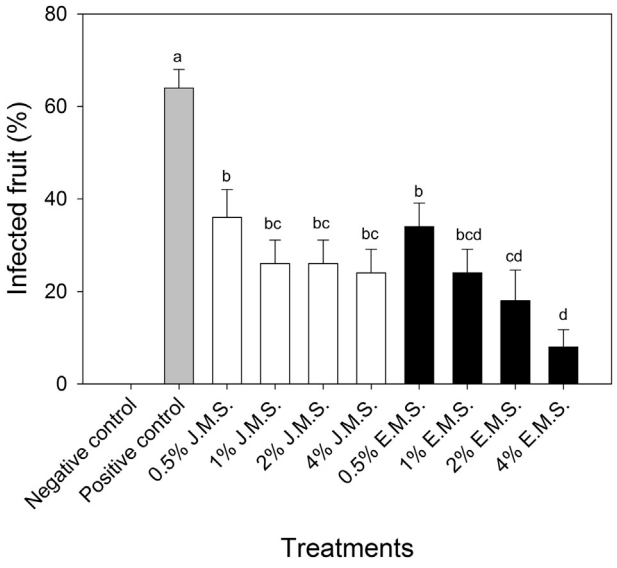

Fig. 1. Anthracnose infection incidence in fruit of the susceptible blueberry cultivar Jersey treated with methanol-soluble fractions obtained after methanol extraction of lyophilized fruit of 'Jersey' and the resistant cultivar Elliott prior to inoculation with *Colletotrichum acutatum*. Bars denote the standard error of the mean ($n = 5$). From left to right: Negative control = non-inoculated fruit with no extract applied, Positive control = inoculated fruit with no extract applied, J.M.S. = 'Jersey' methanol-soluble fraction, and E.M.S. = 'Elliott' methanol-soluble fraction. Bars sharing the same letter are not significantly different according to Fisher's Protected LSD test ($\alpha = 0.05$).

3.2. The resistant cultivar Elliott contains more anthocyanins and other flavonoids than the susceptible cultivar Jersey

Overall, 'Elliott' fruits contained 4.87 mg anthocyanins per gram of freeze-dried tissue and 'Jersey' fruits contained 3.27 mg of anthocyanins per gram of freeze-dried tissue. However, 'Jersey' had a more diverse anthocyanin profile than 'Elliott' as demonstrated by additional peaks (Fig. 2, Table 1). Furthermore, MS analysis showed that anthocyanidins in 'Elliott' tended to be glycosylated with arabinose and galactose, and anthocyanidins in 'Jersey' were more commonly glycosylated with glucose. The profile of anthocyanins in 'Jersey' was similar to that of other cultivars, including Coville [46], Blueray [12], and the rabbiteye (*Vaccinium ashei* Reade) cultivar Tifblue [15]. However, 57% of the anthocyanins in 'Elliott' comprised two compounds: malvidin-3-O-galactoside and malvidin-3-O-arabinoside. No acylated anthocyanins were observed in either cultivar.

In addition, 'Elliott' fruits contained more non-anthocyanin flavonoids (0.18 mg/g of freeze-dried tissue) than 'Jersey' (0.12 mg/g of freeze-dried tissue) (Table 2). 'Elliott' also had a more diverse chemical profile than 'Jersey'. The majority of non-anthocyanin flavonoids in 'Jersey' consisted of compound 05F, a putative quercetin hexoside (Fig. 3). Some specific non-anthocyanin flavonoids were almost exclusive to 'Elliott', including compound 09F (61 μg/g of freeze-dried tissue in 'Elliott' versus 2 μg/g of freeze-dried tissue in 'Jersey') and compound 10F (27 μg/g of freeze-dried tissue in 'Elliott' and 1 μg/g of freeze-dried tissue in 'Jersey').

These two compounds were further characterized using MS/MS. Daughter ions for compound 09F (parent M + H ion 449) included 303, 147 and 129, indicating an aglycone mass consistent with quercetin and glycosylated with a methyl pentose (Fig. 4A). Using a standard and additional UV/Vis information (peaks at 256 nm and a shoulder at 350 nm) this compound was identified as quercetin-3-O-rhamnoside (Fig. 4B). Daughter ions for compound 10F (parent M + H ion 493) included 347, 147 and 129,

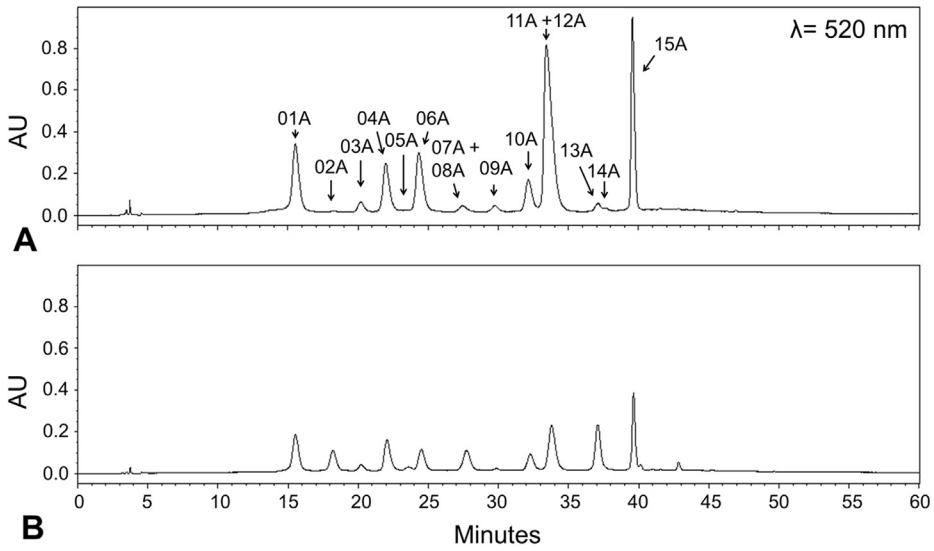

Fig. 2. HPLC chromatograms of anthocyanins extracted from ripe fruit of the anthracnose fruit rot-resistant blueberry cultivar Elliott (A) and susceptible cultivar Jersey (B) using 10-μl injections at $\lambda = 520$ nm. AU refers to absorbance units.

indicating an aglycone mass consistent with a syringetin and glycosylated with a methyl pentose (Fig. 4A). Additional UV/Vis information yielded peaks at 256 nm and a shoulder at 350 nm. These data indicated that this compound is a syringetin rhamnoside (Fig. 4B). Several glycosylated syringetin compounds have been reported in bog bilberries (*Vaccinium uliginosum* L.) [37]. Given that the two distinctive compounds in the resistant blueberry cultivar contain a methylated sugar and the latter compound is methoxylated twice, the chemical contribution to the resistance response in 'Elliott' fruit may be due to the fungitoxicity of these specific compounds. Researchers have found that methoxylated flavones can inhibit the growth of *C. gloeosporioides* from citrus fruits [44]. Methyoxylation may also be important in imparting a certain degree of lipophilicity to the flavonoid and may be required for the compounds to be biologically active [47].

3.3. Non-anthocyanin flavonoids suppress the growth of *C. acutatum* in liquid medium

In the liquid assay, methanolic extracts of 'Elliott' and 'Jersey' inhibited the hyphal growth of *C. acutatum* at the concentrations tested (50 μg lyophilized extract per 100 μl of culture); however, the reduction in growth was more pronounced with the 'Elliott' than the 'Jersey' methanolic extract. The anthocyanin standard cyanidin-3-O-glucoside increased hyphal growth of *C. acutatum*. Additionally, anthocyanin fractions from which non-anthocyanin flavonoids were removed also increased the growth of *C. acutatum* (Table 3). This indicated that anthocyanins have little antifungal activity against *C. acutatum*. Thus, while they are considerably more abundant in both 'Elliott' and 'Jersey' fruit than other flavonoids, they do not seem to play a direct role in the resistance response. However, 'Elliott'

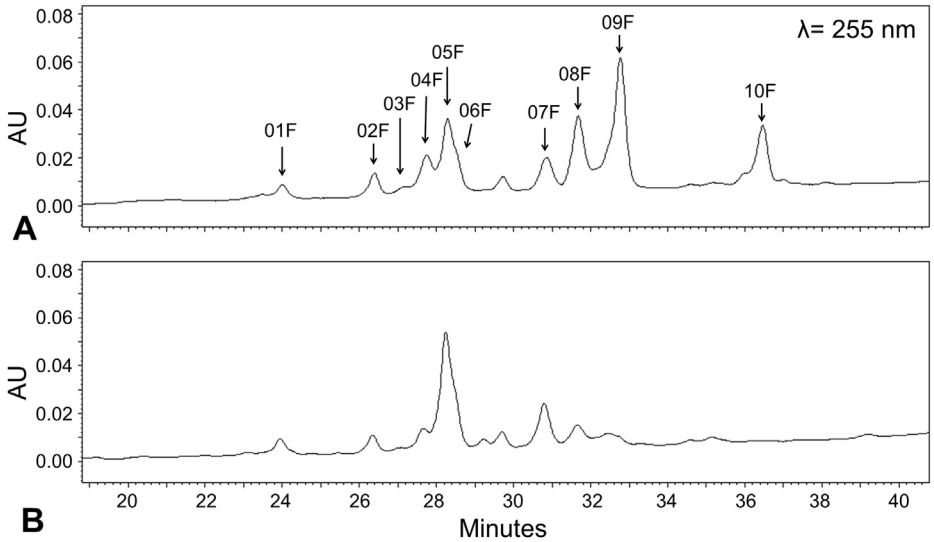

Fig. 3. HPLC chromatograms of non-anthocyanin flavonoids extracted from ripe fruit of the anthracnose fruit rot-resistant blueberry cultivar Elliott (A) and susceptible cultivar Jersey (B) using 10-μl injections at $\lambda = 255$ nm. Arrows denote compounds selected for further analysis. AU refers to absorbance units.

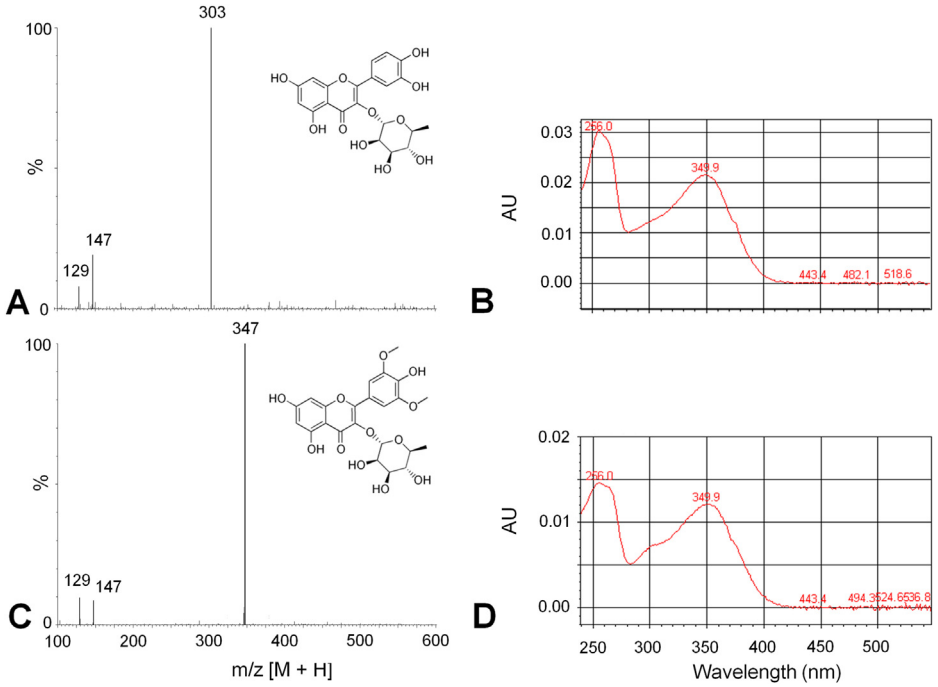

Fig. 4. MS/MS and UV/Vis spectra of two distinctive flavonoid compounds in ripe fruit of the anthracnose fruit rot-resistant blueberry cultivar Elliott. (A) Daughter ions detected when selecting for the parent ion of quercetin 3-O-rhamnoside (compound 09F in Table 2) and chemical structure of parent (quercetin 3-O-rhamnoside). (B) UV/Vis spectrum of quercetin 3-O-rhamnoside (compound 09F in Table 2). (C) Daughter ions detected when selecting for the parent ion of syringetin rhamnoside (compound 10F in Table 2) and chemical structure of putative parent (syringetin-3-O-rhamnoside). (D) UV/Vis spectrum of syringetin rhamnoside (compound 10F in Table 2). AU refers to absorbance units.

fruits are known to produce an oxidative burst of H_2O_2 following inoculation with *C. acutatum* that is more than two times greater than in 'Jersey' fruits [10]. Therefore, the main contribution of anthocyanins might be to scavenge reactive oxygen species following the oxidative burst in order to prevent damage to host tissues. Interestingly, we have also found that there is an increase in anthocyanin concentration in the resistant cultivar Elliott around the site of infection indicating an active response to an invading pathogen [48].

The standards quercetin 3-O-glucoside and quercetin 3-O-rhamnoside as well as the non-anthocyanin flavonoid fractions of 'Elliott' and 'Jersey' significantly decreased the growth of *C. acutatum* in liquid culture (Table 3); the effect was somewhat more pronounced in the 'Elliott' than the 'Jersey' fraction (Table 3). This result matches our initial biological activity screenings and demonstrates that the non-anthocyanin flavonoids have the most significant effect on the growth of *C. acutatum*. In other studies, quercetin 3-O-rhamnoside had more antimicrobial activity against *Pseudomonas maltophilia* Palleroni and Bradbury than other glycosides; interestingly, this compound is distinctive to 'Elliott' and may

Table 3
Change in the optical density ($\lambda = 590$ nm) of liquid minimal medium over time, indicating hyphal growth of *Colletotrichum acutatum* in the presence of various chemical fractions (50 μg of dried material per 100 μl of culture) obtained from ripe fruit of the anthracnose-resistant blueberry cultivar Elliott and susceptible cultivar Jersey. Methanolic extracts contained both anthocyanins and non-anthocyanin flavonoids. The standards cyanidin 3-O-glucoside, quercetin 3-O-rhamnoside and quercetin 3-O-glucoside (50 μg per 100 μl of culture) were included. Optical density values were calculated relative to parallel treatments without the fungus. The relative area-under-the-growth curve (RAUGC) was calculated using the change in optical density over time (days).

Treatment	Optical density (590 nm)				RAUGC[a]	
	Day 1	Day 2	Day 4	Day 5		
Medium with no amendments	0.01	0.06	0.21	0.33	1.06	c
'Elliott' methanolic extract	0.00	0.05	0.10	0.25	0.91	cd
'Jersey' methanolic extract	0.01	0.09	0.24	0.31	0.89	cd
Anthocyanins						
Cyanidin 3-O-glucoside	0.03	0.07	0.35	0.46	1.39	bc
'Elliott' anthocyanin fraction	0.00	0.08	0.34	0.56	1.90	ab
'Jersey' anthocyanin fraction	0.01	0.08	0.48	0.79	2.71	a
Non-anthocyanin flavonoids						
Quercitin 3-O-rhamnoside	0.01	0.07	0.14	0.20	0.56	e
Quercitin 3-O-glucoside	0.00	0.06	0.12	0.24	0.80	cd
'Elliott' non-anthocyanin flavonoid fraction	0.00	0.04	0.11	0.19	0.63	de
'Jersey' non-anthocyanin flavonoid fraction	0.01	0.07	0.13	0.26	0.85	cd

[a] RAUGC values followed by the same letter are not significantly different according to Fisher's Protected LSD test ($\alpha = 0.05$).

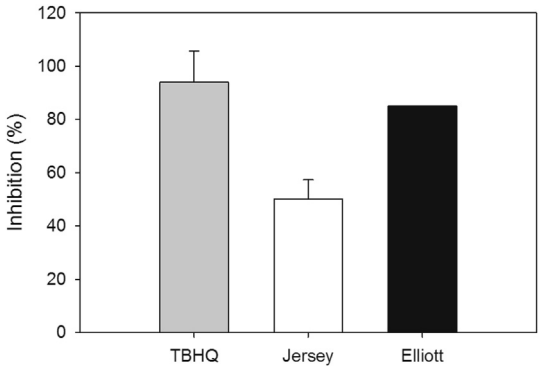

Fig. 5. The effect of methanolic extracts of ripe 'Elliott' and 'Jersey' blueberry fruit (50 ppm) on inhibition of lipid peroxidation initiated by exogenous application of $FeCl_2$, using terbutylhydroquinone (TBHQ) as a positive control. Inhibition was measured at 21 min using a fluorometer. Bars denote the standard error of the mean ($n = 2$).

be important in the resistance response [49]. Additionally, over time, flavonol glycosides can oxidize and become deglycosylated [50]. Deglycosylation tends to increase the biological activity of flavonoids [51], and further oxidation can lead to the creation of quinones [52], which are known to be antimicrobial against *Colletotrichum* species [53].

3.4. Methanolic extracts from ripe 'Elliott' fruit inhibited lipid peroxidation more than extracts of 'Jersey' fruit

The lipid peroxidation assay showed that methanolic extracts from ripe 'Elliott' fruit inhibited peroxidation by 86% compared to the TBHQ standard, whereas 'Jersey' extracts only inhibited peroxidation by 50% (Fig. 5). Since anthocyanins have been previously reported to be strong inhibitors of lipid peroxidation [22], such an activity difference between cultivars may be explained to a large extent by the higher anthocyanin concentrations found in the resistant cultivar Elliott vs. Jersey, the ratio of which between the cultivars (1.5) was slightly lower than that of peroxidation activity (1.7) (Table 1). The higher amounts of anthocyanins likely serve to protect host tissue from oxidative damage during the oxidative burst, which we have detected as higher concentrations of hydrogen peroxide in ripe 'Elliott' fruit 18 h after inoculation with *C. acutatum* [10].

4. Conclusion

The anthracnose fruit rot-resistant blueberry cultivar Elliott has more anthocyanins and other flavonoids, including two distinctive flavonol compounds, than the susceptible cultivar Jersey. Anthocyanins do not seem to play a direct role in the resistance response but may play an indirect role by protecting host tissues from oxidative damage, as also suggested by the lipid peroxidation assay. The non-anthocyanin flavonoid fraction from the resistant cultivar Elliott appears to play a key role in suppressing growth and development of *C. acutatum*. This fraction contains two distinctive compounds, namely quercetin 3-O-rhamnoside and another flavonol glycoside, putatively identified as syringetin rhamnoside, which may be important in the resistance response due to their demonstrated anti-fungal activity.

These pre-formed antifungal compounds likely complement other components of 'Elliott' fruit rot resistance that have been previously described, such as an oxidative burst in the early stages of infection [10] and a combination of relatively high sugar levels and low pH, which have been shown to suppress hyphal growth [11]. Further investigation into how this process is regulated at the molecular level might provide new insights into the host–pathogen interaction.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.pmpp.2013.02.004.

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