

Occurrence of Rutin and Chlorogenic Acid in Elderberry Leaf, Flower, and Stem in Response to Genotype, Environment, and Season

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Abstract

The American elderberry (*Sambucus nigra* subsp. *canadensis*) is being increasingly consumed as a nutraceutical. In Europe, tea made from flowers of the elderberry subspecies *nigra* is a popular herbal treatment for respiratory infections such as colds and influenza, but little is known about the medicinal attributes of the subspecies *canadensis*. Potentially-active compounds in elderberry include phenolics such as rutin, chlorogenic acid, and quercetin. Genetic, environmental and seasonal influences on the concentration of such metabolites in the fruit and non-fruit tissues of American elderberry are unknown. The objective of this study was to quantify the production of medicinal compounds in various non-fruit elderberry tissues in response to genotype, environment and season. In 2003, an experiment was established at Mt. Vernon and Mountain Grove, Missouri, and Corvallis, Oregon (USA) to evaluate the performance of 10 new elderberry selections compared with two older standard cultivars. In 2005, tissue samples were collected from each site in early June (at peak anthesis) and late July (at initiation of fruit ripening). Representative samples of leaf, flower, new green stem, and year-old woody stem were collected from all plots. Dried samples were analyzed by HPLC for the phenolic compounds chlorogenic acid and rutin. Mean levels of chlorogenic acid were 3,367 mg kg⁻¹ in leaf, 2,064 mg kg⁻¹ in flower, 584 mg kg⁻¹ in green stem, and 1,111 mg kg⁻¹ in woody stem, whereas mean levels of rutin were 6,746 mg kg⁻¹ in leaf, 5,546 mg kg⁻¹ in flower, 187 mg kg⁻¹ in green stem, and 44 mg kg⁻¹ in woody stem. Levels of chlorogenic acid and rutin generally varied among the 12 cultivars in leaf tissues only, with the cultivar 'Johns' showing very high levels of both compounds. Significant, but somewhat inconclusive, differences in rutin and chlorogenic acid levels were detected among the three locations; the two Missouri sites tended to have higher levels of rutin. In both leaf and green stem tissues, higher phytochemical levels were detected within tissues harvested earlier in the season. Such genotypic, environmental, and seasonal variations in occurrence of these two representative compounds point to the possibility of focused agricultural production of specific phytochemicals.

INTRODUCTION

The American elderberry (*Sambucus nigra* L. subsp. *canadensis* R. Bolli) is an attractive shrub native to central and eastern North America that produces edible flowers and berries. The fruit and flowers have traditionally been used to make jams, jellies, syrups and wines on a non-commercial scale. Commercial production of high-quality wines in the USA is now rapidly developing, but probably the fastest-growing market for elderberry products is fruit taken as a nutraceutical in the form of a juice concentrate or a processed dietary supplement. A Kansas (USA) winery now sells over \$500,000 worth of non-fermented juice concentrate as a health tonic annually (J. Brewer, pers. comm.).

American elderberry is one of six subspecies of *S. nigra* (Bolli, 1994); the European subspecies *nigra* is a very popular herbal treatment for upper respiratory infections such as colds and influenza. In European herbal tradition, elder flower tea is most commonly used; however, elderberry fruit syrups such as Sambucol[®] have become popular in recent years and have demonstrated efficacy against influenza in clinical trials (Zakay-Rones et al., 1995, 2004). The medicinal properties of elderberry products may be attributed to direct antiviral activity (Zakay-Rones et al., 1995) and to immunostimulant activity resulting in increased production of inflammatory and anti-inflammatory cytokines (Yesilada et al., 1997; Barak et al., 2002). Potential active compounds include several anthocyanins, some novel (Johansen et al., 1991; Nakatani et al., 1995), as well as common flavonoids including quercetin and rutin (Kolodynska and Pasieczna, 1967). However, elderberry is chemically complex and other compound classes, including sterols, lectins and triterpenoids, might be partially responsible for bioactivity (e.g., Gray et al., 2000). In investigating the plant's traditional use as a treatment for diabetes, Gray et al. (2000) discovered that insulin-like and insulin-releasing bioactivities could not be attributed to a known constituent and were associated with two solvent fractions, therefore resulting from the activities of multiple compounds.

While the demand for elderberry products is thus increasing, very few elderberries are presently under managed cultivation in North America. Only a handful of minor studies (Ritter, 1958; Ritter and McKee, 1964; Hill, 1969; Skirvin and Otterbacher, 1977; Craig, 1978; Way, 1981) provide horticultural information to potential producers of American elderberry. Even less is known about genetic, environmental or seasonal influences on the production or concentration of putatively active elderberry metabolites, particularly in the non-fruit tissues. While some elderberry anthocyanins have been studied and quantified in multiple samples (e.g., Kaack and Austed, 1998; Chandra et al., 2001; Wu et al., 2004), none has been sampled from a true replicated genotype by environment planting. The objective of this study, therefore, was to quantify the occurrence of two representative medicinal compounds, rutin and chlorogenic acid, in various elderberry tissues in response to genotype, environment and season of harvest. Both of these compounds, which are present in a wide variety of distantly-related angiosperm lineages, have antioxidant and antimicrobial activities (e.g., Basile et al., 2000; Grace and Logan, 2000; van der Watt and Pretorius, 2001; Zhu et al., 2004; Cushnie and Lamb, 2005). Suites of flavonoids act synergistically; rutin in particular has been found to potentiate the activities of other related and unrelated compounds, even in assays in which it is not itself particularly potent (e.g., Arima et al., 2002; Noldner and Schotz, 2002; Grassmann, 2005). Chlorogenic acid has antiviral activity, notably against adenoviruses, which are among the causes of the common cold (Chiang et al., 2002), and has additionally shown some cancer-preventive activity in rodent studies (Conney et al., 1991; Mori et al., 2000).

MATERIALS AND METHODS

Field

In 2003, an experiment was established at two locations in southern Missouri, USA and a third location in northwest Oregon, USA to evaluate the horticultural and phenological performance of 12 elderberry cultivars growing in different environments.

The sites were at the University of Missouri-Columbia's Southwest Research Center at Mt. Vernon (lat. 37°4'N, long. 93°53'W, alt. 378 m), Missouri State University's State Fruit Experiment Station at Mountain Grove (lat. 37°13'N, long. 92°26'W, alt. 434 m), and the USDA-ARS North Farm, in the Willamette Valley near Corvallis, OR (lat. 44°30'N, long. 123°28'W, alt. 72 m). The two Missouri sites are 140 km apart, whereas the Oregon site is approximately 2,600 km northwest of the Missouri sites. Annual precipitation averages 1,103 mm at Mt. Vernon, 1,148 mm at Mountain Grove and 1,041 mm at Corvallis, with most precipitation at Corvallis falling between November and May. The soil at Mt. Vernon was a Hoberg silt loam (fine-loamy, siliceous, mesic Mollic Fragiudalfs) that is upland, deep, gently sloping and moderately-well-drained with a fragipan at 40 to 90 cm (Hughes, 1982). Soil tests at Mt. Vernon revealed pH 5.7, organic matter 3.6%, cation exchange capacity 13.5 meq/100g, low levels of P, and adequate levels of K, Ca and Mg. The soil at Mountain Grove was a Viraton silt loam (fine-loamy, siliceous, mesic Typic Fragiudalfs) with very similar properties (Robertson, 1981). A soil test indicated pH 6.3, organic matter 2.4%, cation exchange capacity 9.1 meq/100g, low levels of P, and high levels of K, Ca and Mg. The chief difference between the two Missouri soils is that the Hoberg generally has a darker, thicker, softer surface horizon with more organic matter compared with the Viraton. The soil at Corvallis was a Blachly-Kilowan sandy loam complex (fine, isotic, mesic Typic Dystrudepts), a fine-textured, deep to very deep, well-drained soil on mountain footslopes, with very high organic matter and very high to low water-holding capacity (Knezevich, 1975). No soil test was conducted in Oregon although pH had previously been tested at 5.6.

At Mountain Grove, the soil was moved into 20-cm raised soil ridges prior to planting, whereas flat, undisturbed ground was used at Mt. Vernon and Corvallis. All three planting sites were further prepared by killing existing vegetation in the planting rows with glyphosate herbicide prior to planting. Hardwood and softwood cuttings from our own mother plants were collected in early spring, 2003, rooted, then transplanted at all three sites in May, 2003. The 12 American elderberry cultivars included two long-established cultivars ('Adams 2' and 'Johns') and ten new cultivars from the Midwest, USA that were under evaluation for their promising horticultural potential, ('Competition #5', 'Eridu #1', 'Gordon B', 'Gordon E', 'Harris #4', 'Highway O', 'Netzer', 'Votra', 'Walleye' and 'Wyldeewood #1'). The origin and morphology of these new elderberry cultivars were described in Thomas and Byers (2000).

The experiment was established in a completely randomized design with four randomized, replicated plots of the same 12 cultivars at both Missouri sites. Each of the 48 plots per site contained 4 plants, with a total of 192 plants per site. Plants were 1.2 m apart within plots, which were separated by 2.4 m within and 3.3 m between rows. At Corvallis, the planting was also established as a completely randomized design, but the plots consisted of single plants in three replications, with plot / plant spacing 1.8 x 3 m. All plantings were fertilized each spring with 56 kg ha⁻¹ N and irrigated via drip-lines (Missouri) or overhead sprinklers (Oregon) as needed. At all sites, weeds were managed with mulch and glyphosate herbicide, and no insecticides or fungicides were used.

A variety of other horticultural, phenological, and post-harvest fruit studies were underway at the time this additional study was imposed. The first fruit harvest was conducted the year after planting (2004) and the plants entered their most vigorous and productive year when the present study was conducted in 2005. During the 2005 growing season, representative flower, leaf, new green stem, and year-old woody stem samples were collected from all plots at peak anthesis (22 June at Mt. Vernon, 21 June at Mountain Grove, 23 June at Corvallis), with a second sampling of leaf and green stem tissues collected at initiation of fruit ripening (28 July at Mt. Vernon, 27 July at Mountain Grove and Corvallis). Tissue samples were frozen, freeze-dried, then ground to a fine powder in preparation for laboratory analysis. A total of 483 tissue samples were used in the final analysis.

Laboratory

Elderberry flower, leaf, and stem tissues were analyzed for the medicinal compounds chlorogenic acid, rutin, quercetin, quercitrin and isoquercitrin. Procedures were adapted from Gray et al. (2003a). Freeze-dried, finely-ground tissue samples (500 mg) were extracted with 25 ml 80% methanol overnight. An aliquot of the extract was transferred to a microcentrifuge tube and centrifuged at 13,000 rpm for 5 min. One ml of the supernatant was removed and diluted with 2 ml distilled water. Prior to HPLC analysis, samples were filtered through a 0.45 μm membrane filter (Supelco, Bellefonte, PA) if they were cloudy. Stock solutions of chlorogenic acid, rutin and quercetin (Sigma-Aldrich, St. Louis, MO) were prepared in methanol at a concentration of 1.0 mg/ml. The solutions were diluted with 80% methanol to achieve working standards of 100/20/20, 50/10/10 and 25/5/5 ng/ml for rutin/chlorogenic acid/quercetin. Methanol and acetonitrile used were HPLC grade, whereas all other chemicals and reagents used in this study were analytical grade (Fischer Scientific, Pittsburgh, PA).

The HPLC system consisted of a Hitachi L-7100 pump (Naperville, IL) equipped with a Hitachi L-7400 UV detector operated at 284 nm wavelength and a Hitachi L-7200 autosampler (20 μl injection). The reverse-phase chromatographic separations were carried out on a Hypersil[®] BDS C18 analytical column (250 x 4.6 mm, 5 μm) fitted with a SecurityGuard ODS C18 guard column (4.0 x 3.0 mm, 5 μm) (Phenomenex, Torrance, CA). The mobile phase consisted of a solvent A (water with 1% acetic acid) and solvent B (acetonitrile with 1% acetic acid) gradient, pumped at a flow rate of 1 ml/min. The gradient started with 100% mobile phase A at min 0 and was decreased to 50% mobile phase A and 50% mobile phase B at 25 min. Mobile phase A was then brought back to 100% at 30 min. Chromatographic peak areas were measured and data processed by a Hitachi D-7000 data acquisition package with ConcertChrom software on a micro-computer.

Statistical Analysis

Levels of rutin and chlorogenic acid detected in the various plant tissues were statistically analyzed using the GLM procedure (SAS Institute, Cary, SC). After a general analysis evaluating differences among tissues, cultivars, locations, harvest seasons, and interactions thereof, we sorted the data by tissue for a more focused analysis. Duncan's multiple range test was used to separate means at the $P \leq 0.05$ level.

RESULTS AND DISCUSSION

The elderberry plants remained healthy and vigorous at all three locations during this study; however, plant growth, insect/disease pressure, and fruit yields varied significantly among cultivars and sites (unpublished data). The results in the present study, therefore, should be indicative of a valid genotype by environment analysis.

The compounds quercitrin and isoquercitrin were not detected in elderberry leaf, flower, or stem tissues. Quercetin was detected but at insignificant levels relative to rutin and chlorogenic acid (data not shown); therefore we did not pursue the study of quercetin. The four vegetative tissues analyzed contained significantly differing quantities of rutin and chlorogenic acid (Table 1). Across all experimental parameters (genotype, environment and season), leaf tissues contained significantly more rutin and chlorogenic acid compared with flower tissues, which in turn contained more of both compounds than either green or woody stem tissues (Table 1). Mean leaf levels of rutin were 6,746 mg kg⁻¹, whereas mean leaf levels of chlorogenic acid were 3,367 mg kg⁻¹. Table 1 also compares phytochemical levels among the 12 elderberry cultivars within the four plant tissues. In general, little or no difference in phytochemical content among cultivars was detected within flower, green stem, or woody stem tissues, whereas significant differences in leaf phytochemical content were detected among the 12 cultivars. However, easily-discernable patterns are not apparent, except that, in general, the cultivars that produced higher or lower levels of rutin tended to produce similarly higher or lower levels of chlorogenic acid. The one stand-out cultivar is 'Johns' which had the highest leaf levels of rutin

(9,708 mg kg⁻¹) and among the highest of chlorogenic acid (4,697 mg kg⁻¹). This cultivar tended to perform more poorly and yield less than most other cultivars in the experimental plots (unpublished data). 'Johns' originated as a wild selection from Ontario that was released in Nova Scotia in 1954 (Craig, 1978) and therefore might not be well-adapted to either Missouri or Oregon. This scenario could possibly suggest that higher rutin and chlorogenic levels may be a manifestation of environmental stress, at least in the cultivar 'Johns.' Stressors such as drought, herbivory, cold stress, and competition have been observed to increase, or occasionally decrease, levels of certain potent secondary metabolites in other species (e.g., Gray et al., 2003b; Roitto et al., 2003; Almeida-Cortez et al., 2004; Dumay et al., 2004; Kirakosyan et al., 2004; Mumm et al., 2004; Banchio et al., 2005a, b; Hernandez et al., 2006). Gray et al. (2003a) found that the content of eight of ten selected phytochemicals in St. John's wort (*Hypericum perforatum* L.) was increased by drought stress, including, notably, chlorogenic acid and rutin, the latter of which increased by 36%, the greatest proportionate increase observed.

In general, rutin levels were higher at the Missouri sites compared with Oregon, but no pattern was discernable with chlorogenic acid even though there were statistical differences among sites (Table 2). In both compounds, a significant location by tissue interaction was detected. Suggesting specific environmental factors that truly affect production of these two compounds would be difficult and speculative, but could possibly be elucidated with additional study. These results do suggest, however, that focused production of both compounds, especially rutin, in elderberry leaf and flower could be viable in Missouri.

In all cases, significantly higher levels of rutin and chlorogenic acid were detected in leaf and green stem tissues harvested at anthesis (early June) compared with early fruit ripening (late July) (Table 3). This suggests that these two compounds are produced abundantly in spring or early summer for some purpose in the plant, and that they are later either broken down, consumed, or possibly translocated into fruit or roots during ripening. We (Thomas et al., 2007) found similar patterns in an unrelated medicinal species, black cohosh (*Actaea racemosa* L.), and similarly hypothesized that the plant may consume or translocate such compounds into reproductive or storage tissues as the growing season concludes. Seasonal variation in phytochemical content is frequent in plants (e.g., Vance et al., 1994; Agerbirk et al., 2001; Dumay et al., 2004). Often, although by no means always, the highest content of bioactive metabolites occurs in early summer or summer (e.g., Salminen et al., 2001; Southwell and Bourke, 2001; Roca-Perez et al., 2004), followed by a decrease as fall or winter approaches; plausibly, the function of this pattern is to maximize the content of defensive compounds at the season of greatest insect herbivory. Chlorogenic acid has direct antifeedant activity (e.g., Jassbi, 2003) and acts synergistically to increase the activity of antifeedant isoflavonoids (Simmonds and Stevenson, 2001). Rutin has rather unreliable antifeedant activity, actually being a phagostimulant in one grasshopper (Bernays et al., 1991), but it has been reported to slow insect growth (Hoffmann-Campo et al., 2001) and delay molting (Oberdorster et al., 2001); its presence may discourage egg-laying by butterflies (Haribal and Feeny, 2003).

Both rutin and chlorogenic acid are water soluble (especially in boiling water); therefore, an elderberry flower or leaf infusion may be a practical way of consuming these anti-oxidants. Tea from the flower of the subspecies *nigra* is commonly consumed in Europe, while the flowers of the American subspecies *canadensis* have traditionally been cooked and eaten as conventional food. However, leaves and stems (as well as raw, and especially unripe, fruits) have purgative effects and their consumption can sometimes cause severe gastrointestinal irritation, vomiting and diarrhea (Burrows and Tyrl, 2001). Consumption of crude leaf extracts should therefore generally be avoided, although leaves may prove to be valuable as sources of isolated individual bioactive compounds.

In reasonable growing conditions, well-established elderberry plants are fast-growing and resilient. A June harvest of leaves and flowers would normally cause little harm to the plants, which should quickly re-foliate with proper care. Of course, fruit harvest might be compromised, but if markets were established for the phytochemicals

contained in elderberry leaves and flowers, the plants could easily be grown specifically for that purpose, and additional horticultural research could likely optimize production of certain non-fruit organs that contain high levels of specific medicinal compounds. Simple genetics (genotype) also appear to play an important role in phytochemical production, and specific cultivars could likely be selected and further developed with high phytochemical production potential. These results should be useful to both producers and pharmacognosists as production and harvest of specific phytochemicals for the nutraceutical industry become more sophisticated and commonplace.

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Tables

Table 1. Mean concentrations (mg kg⁻¹) of rutin and chlorogenic acid in four non-fruit elderberry tissues within 12 cultivars at three locations, 2005.

Cultivar	Rutin (mg kg ⁻¹)							
	Leaf	n ¹	Flower	n	Green stem	n	Woody stem	n
Adams 2	6955 bc ²	20	4947 a	12	53 b	6	38 a	6
Competition #5	7372 b	18	6028 a	10	62 b	8	23 a	5
Eridu #1	6448 bc	18	5287 a	10	142 b	5	96 a	5
Gordon B	5799 bcd	18	6707 a	10	134 b	5	37 a	4
Gordon E	4310 d	17	8111 a	8	170 b	10	36 a	5
Harris #4	6923 bc	19	4827 a	10	542 a	8	63 a	5
Highway O	7323 bc	18	6303 a	9	185 b	5	22 a	4
Johns	9708 a	17	4749 a	9	227 b	7	43 a	2
Netzer	5389 cd	22	5763 a	11	122 b	7	61 a	5
Votra	6939 bc	18	4676 a	9	243 b	8	68 a	5
Walleye	7751 b	17	4637 a	8	138 b	6	14 a	2
Wyldeewood #1	6411 bc	19	4689 a	8	143 b	8	18 a	6
Overall mean ³	6746 a ³	221	5546 b	114	187 c	83	44 c	54

Cultivar	Chlorogenic acid (mg kg ⁻¹)							
	Leaf	n	Flower	n	Green stem	n	Woody stem	n
Adams 2	5156 a	19	3064 a	11	378 a	6	1237 a	6
Competition #5	3828 abc	18	2158 a	10	676 a	7	1326 a	5
Eridu #1	4698 a	18	1982 a	10	413 a	5	703 a	5
Gordon B	2353 cd	17	2012 a	11	441 a	5	748 a	4
Gordon E	2810 bcd	17	2700 a	9	618 a	10	1491 a	5
Harris #4	2439 bcd	19	1515 a	10	1167 a	7	1277 a	5
Highway O	2397 cd	19	1180 a	9	313 a	5	643 a	4
Johns	4697 a	17	1858 a	11	340 a	7	822 a	2
Netzer	3164 bcd	22	1936 a	11	478 a	8	1781 a	5
Votra	3114 bcd	18	2853 a	9	932 a	8	1241 a	5
Walleye	1788 d	17	1318 a	8	380 a	6	783 a	2
Wyldeewood #1	3947 ab	17	2040 a	9	597 a	8	784 a	6
Overall mean ³	3367 a	218	2064 b	118	584 d	82	1111 c	54

¹ n = number of samples in the previous column.

² Means within a column with the same letters are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).

³ Means within this row having the same letters are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).

Table 2. Mean concentrations (mg kg⁻¹) of rutin and chlorogenic acid in four elderberry tissues at three locations, 2005.

Location	Rutin (mg kg ⁻¹)							
	Leaf	n ¹	Flower	n	Green stem	n	Woody stem	n
Mountain Grove	7425 a ¹	92	5297 b	45	130 a	33	36 a	16
Mt. Vernon	6660 a	91	7278 a	42	252 a	40	48 a	31
Corvallis	5309 b	38	3268 c	27	116 a	10	42 a	7

Location	Chlorogenic acid (mg kg ⁻¹)							
	Leaf	n	Flower	n	Green stem	n	Woody stem	n
Mountain Grove	4126 a	89	1595 b	47	428 ab	33	767 b	16
Mt. Vernon	2540 b	90	2987 a	45	786 a	39	1192 ab	31
Corvallis	3546 a	39	1319 b	26	313 b	10	1539 a	7

¹ n = number of samples in the previous column.

² Means within a column with the same letters are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).

Table 3. Mean concentrations (mg kg⁻¹) of rutin and chlorogenic acid in leaf and green stem harvested in June and July at three locations, 2005.

Harvest time	Rutin (mg kg ⁻¹)				Chlorogenic acid (mg kg ⁻¹)			
	Leaf	n ¹	Green stem	n	Leaf	n	Green stem	n
June	7357 a ²	103	235 a	54	4107 a	101	681 a	54
July	6213 b	118	97 b	29	2729 b	117	396 b	28

¹ n = number of samples in the previous column.

² Means within a column with the same letters are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).