A Comparative Evaluation of the Anticancer Properties of European and American Elderberry Fruits

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ABSTRACT—European elderberry (Sambucus nigra), recognized in Europe for its health-promoting properties for many generations, is known to contain a range of anthocyanins, flavonoids, and other polyphenolics that contribute to the high antioxidant capacity of its berries. American elderberry (Sambucus canadensis), on the other hand, has not been cultivated, bred, and promoted as a medicinal plant like its better-characterized European counterpart. In this study, aqueous acetone extracts of the berries from these two species were fractionated and tested in a range of assays that gauge anticarcinogenic potential. Both cultivated S. nigra and wild S. canadensis fruits demonstrated significant chemopreventive potential through strong induction of quinone reductase and inhibition of cyclooxygenase-2, which is indicative of anti-initiation and antipromotion properties, respectively. In addition, fractions of S. canadensis extract showed inhibition of ornithine decarboxylase, an enzyme marker related to the promotion stage of carcinogenesis. Analysis of active fractions using mass spectrometry and liquid chromatography-mass spectrometry revealed, in addition to flavonoids, the presence of more lipophilic compounds such as sesquiterpenes, iridoid monoterpenes glycosides, and phytosterols.

KEY WORDS: • bioactivity • flavonoids • iridoid monoterpenes • phenolics • Sambucus spp.

INTRODUCTION

It is now widely known that many fruits and vegetables contain natural phytochemical compounds with antioxidant, antimicrobial, anti-inflammatory, cardioprotective, and cancer-chemopreventive properties. Berry fruits are particularly rich in flavonoid compounds (including anthocyanins and proanthocyanidins), although other secondary compounds (iridoid glycosides, sesquiterpenes, triterpenes, and phytosterols) from berries also have demonstrated ability to counteract or interfere with the progress of many chronic disease conditions.1–3

European black elderberry (Sambucus nigra), frequently consumed in preserves, wine, and juice, has long been recognized in Europe for its nutraceutical value.5,5 Anthocyanins make up as much as 1% (dry weight) of S. nigra fruits. The four primary anthocyanins in S. nigra have been identified as cyanidin-3-O-sambubioside-5-O-glucoside, cyanidin-3-O-sambubioside, cyanidin-3-O-glucoside, and cyanidin-3,5-O-diglucoside.5–8 Cyanidin-3-O-rhamnoglucoside and cyanidin-3-O-xylloglucoside are also present. Other flavonoids reported in S. nigra fruits include hyperoside, isoquercetin, and rutinoside.9 These polyphenolic flavonoids are of particular interest due to their putative health-beneficial (anticarcinogenic and antioxidant) properties, as reported also for other types of berry fruits.1

S. nigra fruits have higher antioxidant capacity than vitamin C or E, are capable of enhancing immune system response through elevated production of cytokines, and have been used in European folk medicine to circumvent the ravages of colds, asthma, arthritis, and even constipation for thousands of years.10,11 A product made from S. nigra extracts called Sambucol® (manufactured by Razei Bar Industries, Ltd., Jerusalem) functions as an effective anti-inflammatory treatment by increasing the production of cytokines in vivo. Components of the extract have been cited as therapeutic for patients suffering from a compromised immune system, due to AIDS or chemotherapy.12 The Sambucol product has been reported to inhibit numerous strains of influenza virus.13 S. nigra extracts have demonstrated powerful antioxidant activity in vitro.14 Youdim et al.15 demonstrated that anthocyanins from black elderberry could
be taken up by endothelial cells, which were subsequently effectively protected against oxidative stress. Recent studies have demonstrated that the anthocyanins from elderberries are absorbed unchanged in their glycosylated forms in humans.  

Based on this diverse base of anecdotal, epidemiological, and laboratory-based experimental evidence, the black elderberry has been well established as a medicinal species in Europe and has been the subject of intensive breeding research (to amplify anthocyanin content) and controlled cultivation in plantations harvested for both the pigment and purported health benefits of the berries.  

The native American species of elderberry, *Sambucus canadensis*, has not been cultivated, bred, and promoted as a medicinal plant like its better-characterized European cousin. Most native elderberry plants currently exist in the wild, although they have been incorporated to some extent into landscape settings as a plant that attracts birds and other wildlife, and the fruits of this species have also been used in wines, jams, and pastries. In Brazilian folk medicine, *S. canadensis* leaves, flowers, and berries have been used historically to treat respiratory and pulmonary disorders. In addition, extracts from the plant provide moderate antibacterial and antifungal activity.  

While the European cropped species contains primarily four anthocyanins, *S. canadensis* contains seven. In addition to the four present in *S. nigra*, *S. canadensis* fruits also accumulate cyanidin 3-O-(6-O-E-p-coumaroyl-2-O-β-D-xylopyranosyl)-β-D-glucopyranoside-5-O-β-D-glucopyranoside, cyanidin 3-O-(6-O-Z-p-coumaroyl-2-O-β-D-xylopyranosyl)-β-D-glucopyranoside-5-O-β-D-glucopyranoside, and cyanidin 3-O-(6-O-E-p-coumaroyl-2-O-β-D-xylopyranosyl)-β-D-glucopyranoside. Although anthocyanins are only one of the phytochemical groups purported to be involved in phytomedicinal bioactivity, the presence of a more diversified range in the native American elderberry introduces the possibility that different nutraceutical potential may be realized in this species.  

In this report, we compare the phytochemical content and biological activity of extracts from *S. nigra* and *S. canadensis*, using a range of chemoprevention bioassays to gauge activity against the initiation and promotion stages of carcinogenesis. Our objective was to determine whether the native American elderberry has potential for development as an alternative medicinal crop. To our knowledge, neither species of elderberry has been previously screened specifically in terms of cancer-chemopreventive properties.

**MATERIALS AND METHODS**

**Plant materials**  
*S. nigra* fruits plantation-cultivated in Austria were obtained from Artemis International, Inc. (Fort Wayne, IN). Ripe fruits of *S. canadensis* were collected in the wild from Piatt and Lee Counties, Illinois. Fruits were stored at −80°C until use.

**General materials**  
Cellulose-type Toyopearl polymer HW-40F (TP; Tosohaas, Bioseparation Specialists, Montgomeryville, PA) and silica gel 60 (Sigma Chemical, St. Louis, MO) were used for liquid vacuum chromatography (LVC). Thin-layer chromatography (TLC) was carried out on aluminum plates pre-coated with silica gel 60 (0.2 mm thickness, particle size 2–25 μm, pore size 60 Å Sigma). All solvents (Fisher Scientific, Pittsburgh, PA) were of reagent grade.

**Extraction and fractionation of elderberry compounds**  
Frozen fruit was extracted by blending 300 g aliquots of fruit in 70% aqueous acetone (1 L) at room temperature and filtering the extract with cheesecloth to remove seeds and other solid material; this process was repeated three times. Acetone was removed under vacuum at 40°C, and the resulting liquid was lyophilized to obtain a dry crude extract (1 kg of fruit from each species yielded 160 g of dry crude extract). A portion of the dry crude extract (50 g) was dissolved in water and fractionated using LVC by passing it over a TP column (70 mm column diameter with a medium-pore-size fritted glass filter) to yield five fractions (TP1–TP5). Fractions (1 L) were eluted with water (TP1), 50% aqueous methanol (TP2), 100% methanol (TP3), 100% acetone (TP4), and 50% aqueous acetone (TP5). Methanol and acetone were removed under vacuum at 40°C, and the remaining aqueous portions were lyophilized (Figs. 1 and 2). Fractions were then analyzed by silica gel TLC (ethyl acetate:methanol:water, 79:11:10 by volume) and two spray reagents (vanillin-HCl and dichromate reagent); after spraying, plates were heated at 100°C until color developed. These sprays allow for visual monitoring of proanthocyanidins (which turn pink when exposed to the vanillin-HCl reagent and heat) and organic compounds (such as sugars, which react with the dichromate reagent and turn black). All fractions were then tested for anticancer potential using cyclooxygenase (COX)-1, COX-2, quinone reductase (QR), and ornithine decarboxylase (ODC) assays, as described below.

Fractions that demonstrated positive activity (TP3 and TP4 for both *S. nigra* and *S. canadensis*) were combined (parent fraction) and further fractionated with LVC on silica gel 60 (SGLVC). Approximately 1 g of the parent fraction was dissolved in methanol and mixed with 25 g of silica gel 60 using a mortar and pestle, and the methanol was evaporated. The mixture was added to a column containing 75 g of silica gel 60 and washed with petroleum ether. A total of 22 fractions (75 mL each; SGLVC1–SGLVC22) were eluted with solvents in the following order: 100% petroleum ether; 1:1 petroleum ether:ethyl acetate; 100% ethyl acetate; ethyl acetate mixed with increasing amounts of 1:1 methanol:water (1, 2, 5, 7, 9, 12, 15, 20, 30, 35, 40, 50, 60, 70, 80, 90, 100%); 100% methanol; and 100% water (Figs. 1 and 2). Solvents were removed under vacuum at 40°C, analyzed using TLC, and tested in assays. The same procedure
of TP LVC followed by SGLVC was used separately for both *S. nigra* and *S. canadensis* fruit.

Active fractions from silica gel LVC were then combined and further purified by medium performance liquid chromatography (MPLC). For *S. nigra*, SGLVC5–SGLVC13 (SGLVC fractions from the combination of TP3 and TP4) were combined, dissolved in petroleum ether and ethyl acetate, and purified using MPLC on a normal-phase silica gel column (Merck, grade 9385, 230–400 mesh, 60 Å, 1.5 H 50 cm; Aldrich, Milwaukee, WI) with a gradient of increasing proportions of methanol in water resulting in 138 fractions (Fig. 1). Based on similarity in TLC, these 138 fractions were grouped together in seven fractions for chemical analysis and testing in assays. For *S. canadensis*, SGLVC5–SGLVC13 (fractions from TP3 and TP4) were combined and purified using MPLC on a C-18 reverse-phase column (Bondesil, 40 μm particle size, 1.5 × 50 cm; Varian, Harbor City, CA) with a gradient of increasing proportions of methanol in water resulting in 153 fractions (Fig. 2). Based on similarity in TLC, these 153 fractions were grouped together into 12 fractions for chemical analysis and testing in assays. For both species, approximately 300 mg of sample was used for MPLC.

**Assays**

Samples for all bioassays were diluted to a concentration of 4 mg/mL in dimethyl sulfoxide.

**COX.** Both COX assays (COX-1 and COX-2) were done by measuring prostaglandin E$_2$ production from arachidonic acid using an enzyme-linked immunosorbent assay method as described by Cuendet and Pezzuto$^{19}$ and Waffo-Teguo et al.$^{20}$ and by measuring prostaglandin F$_2$α production from arachidonic acid using the COX Inhibitor Screening Assay (Cayman Chemicals, Ann Arbor, MI). Indomethacin was used as a positive control for the prostaglandin E$_2$ measurements, and SC-560 (COX-1 inhibitor) and DuP-697 (COX-2 inhibitor) were used for prostaglandin F$_2$α measurements (Cayman). Results were expressed as a percentage relative to control (solvent-treated) samples and are an average of two replicates.

**QR.** For the evaluation of samples as inducers of QR, cultured mouse Hepa 1c1c7 cells were used as described previously.$^{21,22}$ Bromoflavone was the positive control. Induc-
tion of QR activity was calculated from the ratio of specific enzyme activities of sample-treated cells in comparison with a solvent control, and CD values (\( g/mL \), the concentration of sample needed to double QR induction) were generated based on the average of two replicates.

**ODC.** An in vitro ODC assay was conducted. Methods for cell culture and for treating the cells with sample were described in Kandil et al., except that sulforaphane was used as a positive control, samples were tested at a concentration of 20 \( g/mL \), and the JB6 mouse epidermal cell line was used. The measurement of \( ^{14}CO_2 \) release from \( L-[1-^{14}C]ornithine \) was essentially done according to the procedure of Lichti and Gottesman, as described previously. All samples were run in duplicate.

**Chemical analysis**

**Mass spectrometry (MS).** Electron impact (EI) MS of the bioactive MPLC fractions was carried out on a 70-VSE (A) mass spectrometer (VG, Manchester, UK) in the Mass Spectrometry Laboratory of the School of Chemical Sciences, University of Illinois, Urbana, IL. The EI voltage was 70 eV, and continuous mass spectra were recorded over the range \( m/z \) 100–2,000 with a scan time of 9.9 s and an interscan delay of 0.1 s. Samples were run in positive mode.

**Liquid chromatography-MS (LC-MS).** Identification of some compounds from SGLCV fractions was accomplished using LC-MS [in the electrospray ionization (ESI) mode] following the methods of Yousef et al. The samples were filtered through surfactant-free cellulose acetate filters (pore size, 0.45 \( \mu m \); Corning Inc., Corning, NY) before injection.

**RESULTS**

**Activity of extracts from fruits**

**COX inhibition.** All fractions were screened for inhibition against both COX-1 and COX-2 at 10 \( g/mL \), and those samples that showed greater than 60% inhibition were considered active. The \( S. nigra \) crude extract produced from the 70% aqueous acetone extraction exhibited no significant activity in either COX assay. However, fractions TP3 and TP4 showed 87% and 86% inhibition in the COX-2 assay, respectively; neither significantly inhibited COX-1.
fractions (SGLVC5–SGLVC13) also demonstrated a range of activity in the COX-2 assay (63–98% inhibition). Activity of these same samples for COX-1 inhibition was minimal. Additionally, MPLC fractions 4 and 5 were active in the COX-2 assay (82% and 73% inhibition, respectively), but were not significantly active in the COX-1 assay (Fig. 1). It is of particular value to have COX-2 inhibitory activity with little or no COX-1 activity. COX-1 is a constitutively expressed enzyme (e.g., the gastric mucosa) and plays an important role in gastrointestinal maintenance. COX-2, on the other hand, is inducible at sites of cancer and inflammation.

The *S. canadensis* crude extract produced from the 70% aqueous acetone extraction also exhibited no significant activity in either COX assay. Fraction TP3 exhibited borderline bioactivity in the COX-2 assay (58% inhibition) but did not significantly inhibit COX-1. SGLVC fractions (SGLVC5–SGLVC13) also inhibited COX-2 (58–84% inhibition), but were not significantly active in the COX-1 assay (Fig. 2).

**QR induction.** All fruit fractions and subfractions were screened for activity in the QR assay at 10 and 20 μg/mL, and those samples that at least doubled QR activity at a concentration ≤10 μg/mL were considered significantly active. For *S. nigra* fruit, the crude extract was not active in the QR assay, but SGLVC5, SGLVC9–SGLVC12, and SGLVC14 exhibited significant activity in the QR assay by doubling QR activity when screened at 10 μg/mL (Fig. 1). The crude extract from *S. canadensis* was also not active, but fraction TP4 exhibited significant activity in the QR assay. In addition, MPLC fraction 2 from *S. canadensis* fruit exhibited significant QR induction by doubling activity at 2.5 μg/mL (Fig. 2).

**ODC inhibition.** All fruit fractions and subfractions were screened for activity in an *in vitro* ODC assay at 20 μg/mL. Samples that inhibited ODC activity by 50% were considered active. Significant ODC activity was only found in fractions of *S. canadensis* fruit. SGLVC fraction 12 (SGLVC12) and MPLC fractions 3 and 8 inhibited ODC activity more than 50% when tested at 20 μg/mL (Fig. 2).
Active fraction composition analysis

MS. EI-MS was used to analyze the COX-active MPLC fractions from S. nigra (fractions 4 and 5). The EI-MS of MPLC fraction 4 had major peaks at 248.2 m/z and 414.4 m/z, and the EI-MS of MPLC fraction 5 had a major peak at 248.3 m/z. The 414.4 m/z peak is consistent with compounds having an empirical formula of C_{29}H_{30} and in this case is most likely β-sitosterol. The 248.2 m/z peak is probably a sesquiterpene, which is consistent with an empirical formula of C_{16}H_{24}O_{2}. Therefore, MPLC fraction 4 from S. nigra probably contains a mixture of β-sitosterol and a sesquiterpene, and MPLC fraction 5 contains mostly a sesquiterpene.

LC-MS. LC-MS analysis revealed active fractions were composed not only of flavonoids but also lipophilic compounds such as iridoid monoterpene glycosides. Figure 3 is an example spectrum of S. nigra SGLCV11. LC-MS analysis of active vacuum chromatography fractions of S. nigra indicates that they contain quercetin (303.1 m/z), quercetin monoglucoside (464.9 m/z), gallocatechin (606.9 m/z), and a series of proanthocyanidins (dimer, 579.0 m/z; trimer, 866.9 m/z; and tetramer 1154.9 m/z), among other unidentified compounds. LC-MS of spectra of active S. canadensis vacuum chromatography fractions correlates with the presence of quercetin, epigallocatechin (306.8 m/z), quercetin monoglucoside, and monotropein (390.9 m/z), in addition to other unidentified components.

Several compounds were also eluted in the lipophilic portion of the chromatographic series from both S. nigra and S. canadensis that could not conclusively be identified. However, compounds with a molecular weight (300–400 m/z) correlate closely with iridoid monoterpene glycosides, which are known to exist in this genus.\textsuperscript{29,30} Examples of possible iridoid monoterpene glycosides include 7-dehydrologanin (388.0 m/z) and sweroside (358.0 m/z).

DISCUSSION

Bioactivity relevant to inhibition of both the initiation and promotion stages of carcinogenesis was detected in fruit extracts from S. nigra and S. canadensis. The medicinal value of elderberries in most previous applications has been linked to the concentrated anthocyanin pigments in the fruits. Recently, anthocyanins in elderberry extracts were found to spare the antioxidant power of vitamin E in laboratory rats, although there was no effect on cholesterol levels,\textsuperscript{31} and the anthocyanins in extracts were also considered responsible for protection against oxidative stress.\textsuperscript{15}

In this study, the anticancer bioactivity of elderberries, especially against the initiation and promotion stages of carcinogenesis, was found in fractions of fruit extract that contained not only phenolics (quercetin, quercetin monoglucoside, proanthocyanidins, and epigallocatechin) but also nonphenolic compounds (probably iridoid monoterpene glycosides, sesquiterpenes, and phytosterols). Furthermore, this range of compounds and bioactivity was found in both the wild American elderberry (S. canadensis) and the well-recognized, medicinally important European elderberry (S. nigra).

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