Bioactive Properties of Wild Blueberry Fruits
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Abstract: Bioactive extracts from wild blueberries were isolated, fractionated, and analyzed. Antioxidant activity, cardioprotective capacity, and ability to inhibit the initiation stage of chemically-induced carcinogenesis were evaluated. Many fractions had antioxidant activity, especially those rich in anthocyanins and proanthocyanidins. The ease of isolation of bioactive compounds and the ability to obtain accurate bioassays depended strongly on the source material used. Extracts from spray-dried formulations containing magnesium citrate and tri-calcium phosphate provided easier materials for bioactivity assessment and demonstrated antioxidant, cancer preventive and cardioprotective activity. Extracts with potent antioxidant activity degraded readily, underscoring the need for rapid alternative fractionation tactics and rigorously controlled storage conditions.

Key words: blueberries, antioxidants, cancer preventives, cardioprotective compounds, Vaccinium angustifolium, fractionation, phenolics

Introduction

Natural plant-produced anthocyanin pigments, the substances that are largely responsible for intense red to blue colors in many foods, and related flavonoid phytochemicals are considered to be responsible for a range of unique and broad-spectrum health benefits (Serafini and others 1998). Although the reasons behind these disease-preventive properties are not fully understood, ample evidence suggests that these polyphenolic compounds are readily absorbed in vivo, are involved in antioxidant defenses, and may play similar roles in combating both heart disease and various forms of cancer (Argiles and others 1998; De Groot and Rauen 1998; Parthasarathy 1998). Blueberries are among the fruits that are best recognized for their anthocyanin and flavonoid content, and for their potential health benefits.

The complete phytochemical richness of wild blueberries has not been fully characterized, although much is known about the small to medium molecular weight constituents. Higher molecular weight components, such as proanthocyanidins, have been little explored because of their complex nature. Blueberries are noted for their high ascorbic acid content (Kalt and Dufour 1997), but the major source of the antioxidant properties of these fruits is not from their inherent ascorbic acid content (Prior and others 1998). Rather, the remarkably high antioxidant capacities have been directly attributed to their intense anthocyanin pigment content (Kalt and Dufour 1997; Prior and others 1998).

Many of the health-giving properties of blueberries are thought to be attributable to phenolic acids and to various types of flavonoids that the fruits contain. One of these compounds, quercetin, appears to play a role as an cardioprotective component of red wines (Formica and Regelson 1995). This flavonol has also been reported to have potential chemopreventive effects against certain types of tumors (Verma and others 1988). Anthocyanins are among the most obvious and most abundant flavonoids in blueberries (Kalt and McDonald 1996). Wild blueberries also contain condensed tannins, mostly consisting of proanthocyanidins. Recent studies have targeted the proanthocyanidin class of flavonoid phytochemicals from wild blueberries as the active agents inhibiting the promotion stage of chemically-induced carcinogenesis (Bomser and others 1996), and providing anti-adherence inhibition against the bacteria responsible for urinary tract infections (Howell and others 1998; Ofek and others 1996).

The juice from fresh berries is often treated with pectinases in the juice-making process, decreasing the yields of flavan-3-ols and anthocyanins (Heinonen and others 1998b). Many pectinases contain β-glucosidases and other enzymes; treatment with these substances may result in modification of many phytochemicals.

We have explored the bioactive properties of flavonoid-rich extracts from the wild blueberry, providing supporting evidence for bioactivity. The importance of the source material used to isolate fractions for bioactivity assays has been considered, as well as the need for careful handling to preserve phytochemical integrity during the extraction and fractionation steps in order to preserve sensitive bioactive compounds for evaluation of biological activity.

Results and Discussion

Source materials

During both the extraction procedures and initial bioassays, difficulties inherent with the use of frozen fruits, liquid extracts, and freeze-dried powdered fruit as starting material became apparent. Despite the fact that extracts from most sources were active, the extremely hygroscopic and gum-like nature of the extracts led to the formation of intractable residues that were difficult to remove from sample vials. These conditions made uniform delivery of samples to individual assay wells problematic. Although each of these sources of wild blueberry extract do contain significant amounts of metabolically active constituents, the intractable nature of the extracts prevented quantitative bioassay measurements. Heinonen and others (1998b) also noted significant influences of the source of flavonoid-rich extracts on results of antioxidant testing. In the present case, this appears to be due to the presence of large quantities of saccharides and polysaccharides, including pectins, in these extracts.

These problems from whole fruit were largely avoided when spray dried. Dextinized wild blueberry powder was used. Depectinization is routinely done in industry to improve recovery of desired fruit components in a final product (Heinonen and others 1998b). Accordingly, all subsequent trials were performed with spray-dried, depectinized wild blueberry powder (Jasper Wyman and Sons, Product Code BJS-1), which con-
tained wild blueberries, magnesium citrate, and tri-calcium phosphate. This depenicized material, typically used as a natural wild blueberry food additive for flavoring or color, contained 580 mg anthocyanins per 100 g dry weight and 3% water. However, BJS-1 was prepared from a juice extract, and bioactive polyphenolic compounds associated with the pulp and seeds may not be available in BJS-1. The absence of pectin and possibly the presence of magnesium citrate and tri-calcium phosphate made the BJS-1 product more amenable to our extraction and fractionation routines, and permitted us to make quantitative measurements in the bioassays described (Materials and Methods). It may be possible to alleviate some of these problems by depenicizing 70% acetone extracts immediately after initial extraction and removal of acetone. This is currently being pursued in our laboratory, in order to evaluate further potential bioactive constituents found only in whole blueberry fruit components, and that may not be found in spray dried powders.

**Fractionation of extracts**

The phytochemical content and resultant biological activity of blueberries could be strongly influenced by the methods used to preserve and extract the fruits. Frozen fruits that have been stored at −20 °C or lower appear to possess a relatively complete complement of the phenolic and flavonoid compound found in fresh fruit, until they are thawed. Freeze dried whole fruits also appear to retain most of the fruit phytochemical spectrum. However, when juice is removed by expression of the fruits and is concentrated by spray drying, the effects of oxidation and temperature may be significant. In many instances, other compounds such as magnesium hydroxide, magnesium citrate, and tri-calcium phosphate, are added to stabilize or to improve the flow of the product, a necessity for commercial markets.

For the isolation and characterization of phytochemicals and for identification of bioactive compounds, extraction with organic solvents is usually necessary. Aqueous acetone (70%) is an especially effective solvent for phenolic materials, especially for proanthocyanidins (Heinonen and others 1998b).

Freeze dried extracts were fractionated by means of vacuum chromatography (Millar 1998) with silica gel (Sigma, S-6503, Type G). This vacuum chromatography technique permits fractionation of relatively large amounts of extract (10 to 20 g), in a relatively short time span (2 to 3 hours), and uses relatively small amounts of solvents. Because of the shorter time that sensitive compounds are exposed to chromatographic supports and to air, this procedure often results in reduced modification of these substances. Separations were made with gradients ranging from pure EtOAc to EtOAc with increasing proportions of a mixture of MeOH and H2O. The results of separations can be conveniently monitored by applying aliquots of the fractions to thin layer plates. The solvent gradient can be modified in the course of a run, or individual, or combined fractions can be combined and rechromatographed, as necessary, to effect the desired separation. In most cases, fractions or combined fractions were bioassayed before additional chromatographic fractionation, and only fractions with the desired activity were pursued.

Analysis of extracts and fractions by HPLC and thin-layer chromatography (TLC) gave sufficient information for assignment of the compound-type, that is, whether the compound was an anthocyanin, flavonol, phenolic acid or proanthocyanidin. The final identification and quantification of each component has not yet been made. Quantification of compounds determined by UV-visible spectrophotometry requires isolation of individual compounds in order to determine extinction coefficients or to permit use of values from the literature. Approximate values obtained by comparison with internal standards are given in Table 1.

**Antioxidant activity**

The material extracted with 70% aqueous acetone from blueberry powder and fractions from that extract prepared by vacuum chromatography were tested for antioxidant activity at 5, 50 and 500 μg/mL.

**Galvinoxyl free radical quenching assay**

Galvinoxyl is a stable free radical that has been used as a probe to study reactions of antioxidants (Smith and Hargis 1985). Vitamin E and ascorbic acid, 2 of the best antioxidants that have been described, are extremely efficient at combining with free radicals to disrupt the initiation of oxidation and to protect cellular constituents. In tests with galvinoxyl, both of these antioxidants have reacted quickly and many investigators have used the galvinoxyl assay for comparison when evaluating new potential antioxidants (for example, see Shi and Niki 1998). We have adopted this assay to help separate the material from the fruit extracts and their subsequent fractionation, so that further testing of the more active fractions would proceed, choosing to focus on the fractions with the highest antioxidant activity.

By this method, we were also able to measure loss of antioxidant activity associated with degradation of the isolated components as the testing continued. Further, we observed that the presence of syrupy or pectinous material greatly retarded the antioxidant activity of the extracts.

The 70% aqueous acetone extract of the blueberry powder and the corresponding prepared fractions were tested for antioxidant activity with the galvinoxyl free radical quenching assay. At higher concentrations, the active blueberry constituents rapidly quenched the galvinoxyl radical, but the reaction was too fast to give acceptable kinetic data; at low concentrations, the rate was too slow to measure. At 50 μg/mL, linear data were obtained permitting comparisons between the various fractions (see Fig. 1). One fraction from vacuum chromatography (fraction 14) had the highest free radical-quenching activity. This fraction contained acylated anthocyanins, as well as proanthocyanidins and other flavonoids (Table 1).

Continued antioxidant testing of the fractions was difficult because significant loss of activity occurred, even though samples for evaluation were stored in the dark at −20 °C. The most active blueberry fraction (fraction 14), lost 50% of its activity in ten days as measured with the galvinoxyl free radical quenching assay. Upon dissolution of dry material from the fraction, the solution was obviously less pigmented (purple) than the initial sample. HPLC analysis confirmed a decrease of approximately 50% of the anthocyanin components. Although novel extraction and fractionation procedures were used in this study to isolate bioactive flavonoids, these compounds still degraded significantly in routine storage. Compounds that are good antioxidants are easily oxidized and often are converted to less active or inactive forms (Larson 1997). Anthocyanins are especially subject to degradation, especially when separated from stabilizing co-factors, such as other phenolic constituents, in particular, flavonols.
Wild Blueberry Bioactivity . . .

(Mazza and Miniati 1993). Possibly the best strategy is to store the relatively crude extracts in the dark at low temperature, remove only the amount needed for fractionation, fractionate quickly, and evaluate bioactivity as soon as possible after fractionation. At each step, oxygen, light, and heating of the samples should be avoided when possible. Loss of activity in crude extracts does not appear to occur as rapidly as in purified fractions.

Tyrosine oxidation

For this study, simple model compounds were chosen to further evaluate the antioxidant activity of the blueberry material. Tyrosine was chosen as a model for protein oxidation. When oxidized by free radical initiators, tyrosine loses an electron and forms dityrosine, a fluorescent molecule easily detected by simple fluorimeters. Antioxidants that are good electron scavengers inhibit this reaction, quenching the fluorescence, as demonstrated (Fig. 2) by the results of the addition of small amounts (50 µg/mL) of either the whole extract from the blueberry powder or material from the late-middle fractions (fractions 11 to 15).

Lipid oxidation assay

Linoleic acid was chosen as a model for lipids. Again, a free radical initiator was used to oxidize the fatty acid. The decrease in concentration of LA is proportional to the oxidative conversion to peroxidized products; antioxidants will prevent this decrease. At 5 µg/mL, the whole extract effectively slowed the initiation phase of oxidation, typical of chain-breaking antioxidants. At 50 and 500 µg/mL, both the initiation phase and propagation phases of oxidation were suppressed, indicating the contributions of stoichiometric antioxidants (Fig. 3).

Of note, is the ability of blueberry antioxidants to inhibit the oxidation, not only of hydrophilic cellular constituents such as amino acids, but also the hydrophobic lipids.

Anti-initiation cancer chemoprevention activity

The crude 70% acetone extract from wild blueberry (Jasper Wyman and Sons, BJS-1) exhibited clear, significant induction of QR activity (Fig. 4) (Prochaska 1994). There was no significant cy-

![Fig. 1](image1.png)

![Fig. 2](image2.png)

![Fig. 3](image3.png)

![Fig. 4](image4.png)
totoxicity evident at any of the CD<sub>x</sub> values, which indicates that the extract has the potential to be a potent inhibitor of the initiation stage of carcinogenesis.

In an earlier report (Bomser and others 1996), neither crude extracts nor flavonoid-rich fractions from wild blueberry were shown to induce QR activity. Instead, a hexane-chloroform subfraction containing lipids, sterols, carotenoids, and chlorophylls proved to have high quinone reductase inducing activity. In the present study, in contrast, the flavonoid-rich acetone extract of BJS-1 proved to be quite potent as a QR inducer (Fig. 4). It is possible that the bioactivity of the flavonoids in the previous study was degraded during extraction and column chromatography steps, whereas in the present study, the vacuum chromatographic method used to prepare the samples is faster and results in less degradation of sensitive compounds. It is also possible that the elimination of pectins in the BJS-1 product led to the detection of the QR induction capacity, which may be masked under the interference from pectins. We have previously demonstrated that fractions from wild blueberry rich in proanthocyanidins are also effective inhibitors of the promotion stage of chemically-induced carcinogenesis (Bomser and others 1996).

**Conclusion**

Once interferences from certain compounds present in wild blueberry fruits or whole fruit products were removed, wild blueberries exhibited significant antioxidant activities in both the lipid and aqueous environment. Further fractionation determined that these properties were found in fractions rich in flavonoids and anthocyanidins. Furthermore, the flavonoids from wild blueberry exhibited the ability to inhibit the initiation stage of chemically-induced carcinogenesis by inducing the activity of the quinone reductase enzyme, an assay used for detection of cancer preventive action. This study illustrates the necessity of isolating bioactive compounds for quantification of biological effects and the need for careful handling to avoid artificial loss of activity under laboratory storage conditions.

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**Materials and Methods**

**Plant materials**

Wild blueberries (*Vaccinium angustifolium* Ait.) were obtained from Jasper Wyman and Son (Milbridge, Me., U.S.A.) in the following formulations: Individually quick-frozen (IQF) wild blueberry fruits, concentrated wild blueberry juice liquid extracts, wild blueberry powder (freeze-dried), and depectinized wild blueberry powder (spray-dried). The freeze-dried wild blueberry product [20-2107] was prepared in collaboration with Oregon Freeze Dry, Inc. (Albany, Ore., U.S.A.), and consisted of whole blueberries freeze dried and powdered. The spray-dried wild blueberry product [BJS-1] was prepared by spray drying wild blueberry juice solids in a magnesium citrate carrier to retain maximum anthocyanin content, with tri-calcium phosphate added as a flow aid. All samples were immediately stored at ~80 °C on receipt, until analysis, which in all cases occurred within a three month period.

**Extraction/fractionation procedures**

Extractions were performed with a mixture of acetone-water (7:3) (Karchesy and others 1989) and the resultant extracts concentrated under vacuum (rotary evaporator) until most of the acetone was removed, then frozen and further concentrated on a lyophilizer.

Freeze dried extracts (10 to 20 g) were fractionated by means of vacuum chromatography (Millar 1998) with silica gel (Sigma, S-6503, Type G). Separations were made with gradients ranging from pure EtOAc to EtOAc with increasing proportions of MeOH and H<sub>2</sub>O (1:1), to only the mixture of MeOH and H<sub>2</sub>O (1:1). As fractionations were carried out, the results were monitored by applying aliquots (usually 20 to 40 μl) of the fractions to thin layer plates (Silica Gel 60, 0.25 mm) and developing the plates in a mixture of EtOAc:MeOH:H<sub>2</sub>O (79:11:10).

Samples of each extract of fraction (10 mg/mL) were analyzed by high performance liquid chromatography (HPLC). One mL of the solution to be examined was combined with 0.1 mL of an external standard (apigenin), prior to injection of 250 μL samples into the HPLC. The signal from the single wavelength recorder was connected to a Spectra-Physics integrator that recorded the peak areas and with a photodiode array (PDA) detection, as adapted from Hong and Wrolstad (1990), using a linear gradient from 100% A to 40% A in 50 min at 1 mL/min on a PRP-1 column (250 × 4.1 mm, Hamilton Co., Reno, Nev., U.S.A.). Solvent A was [HCl (5 mM):acetone (95:5)] and solvent B (acetone).

**Bioactivity assays**

Galvinoxyl, linoleic acid, and tyrosine were obtained from Sigma, St. Louis, Mo., U.S.A. The free radical initiators, 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH, water soluble) and 2,2'-azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeO-AMVN) were from Wako Chemicals, Richmond, Va., U.S.A. All solvents, including water, were glass distilled.

Extracted fruit materials were fractionated and tested within ten days of preparation. Samples were stored in a ~20 °C freezer during that time. Immediately prior to a particular assay, aliquots were removed from the freezer, allowed to warm to room temperature and then prepared at initial concentrations of 10 mg/mL in methanol or water. Dilutions of 1 : 10 and 1 : 100 of the initial material were prepared and all assays replicated (at least 3 times) at each of 3 different concentrations. Data points for each assay below and CD<sub>x</sub> values were determined graphically. Whereas absolute rate constants had a variance of ± 15%, the measured activity always correlated in a dose-responsive manner.

**Galvinoxyl free radical quenching assay**. A solution of galvinoxyl in methanol was prepared that had an initial absorbance of 1.8–2.0 O.D. at 429 nm (Marley and others in preparation; adapted from Smith and Hargis, 1985). An aliquot (0.1 mL) of the dissolved test substance (concentrations ranging from 0.1–10 mg/mL) was added to galvinoxyl solution (2 mL), so that the final concentration of the extract/fraction was 5 to 500 μg/mL. At least 2 or 3 concentrations of each fraction were measured, in order to obtain regression coefficients that confirmed first order rate constants. The quenching of the galvinoxyl radical was recorded for five minutes at 30 sec intervals using a Beckman DU 7400 spectrophotometer with a scan rate of 0.5 s. Data were then plotted as a function of ln [Abs t/Abs 0] vs time to obtain rate plots, the slope of which yielded k, the rate constant. Rate constants were calculated using first-order kinetics based on decrease of absorbance vs time.

**Lipid oxidation assay**. Oxidation of linoleic acid (LA) was initiated by MeO-AMVN (Noguchi and others 1998). A solution of LA (100 mL, 350 μM) containing MeO-AMVN (1750 μM) in oxygen-saturated acetonitrile was heated to 37 °C with vigorous shaking. The extract/fraction to be tested was added so that the final test concentration varied from 5-500
µg/mL. Aliquots (1 ml) were removed at specified intervals. Loss of LA was monitored by HPLC with UV detection at 210 nm using a PRP-1 column (250 × 4.1 mm). Peroxidized products were separated from the starting material by gradient elution: 5 min at 40-60 water-acetonitrile followed by a linear increase to 100% acetonitrile in 25 min at 1 ml/min.

**Tyrosine oxidation assay.** Oxidation of tyrosine was initiated by AAPH using an adapted method of Holler and Hopkins (1989). A solution of tyrosine (100 µM, 150 µM) including AAPH (900 µM) in phosphate buffer (0.05 M, pH 9) was heated to 37°C with vigorous shaking. The final concentration of the material to be tested was 5-500 µg/mL. Aliquots (3 ml) were removed at specified intervals and the shaking intensity of the resulting tyrosine dimer was measured at 405 nm (λ exc = 325 nm) with a Perkin Elmer Model MPF 44B fluorescence spectrophotometer.

**Quinone reductase assay.** An in vitro NAD(P)H (quinone-acceptor) oxidoreductase [QR] assay was used to assess the potential anti-initiator activity of phytochemical extracts from wild blueberry. Prior to use in the assays, extracts (1 ml aliquots) were placed under nitrogen gas for 30 min until solvent was completely evaporated. The dry samples were resuspended in DMSO-1% HCl (v:v, 100 µl) and sonicated for 5 min. Samples were stored at −4°C under nitrogen. The QR-inducing activity of extracts was normalized based on the dry weight of each sample.

Hepa 1c1c7 cells (ATCC CRL 2026) used for the QR assay were grown at 37°C in humidified incubators containing 5% CO2 in alpha-minimal essential medium (without deoxyribonucleosides or ribonucleosides) supplemented with 10% fetal bovine serum (FBS). Growth medium was replaced three times per week. Cultured cells were free from mycoplasma contamination. The QR assay and evaluation of cytotoxicity were conducted as described by Prochaska (1994). Cytotoxicity was evaluated by examining relative cell density through crystal violet staining after 48 h exposure of cells to the extract samples. The concentration of the extracts, expressed as µg DW present in 150 µl medium per microtiter plate well, required to double QR specific activity (CD50) was used as an indicator of induction potency.

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**References**


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