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# Phytochemical Composition and Metabolic Performance-Enhancing Activity of Dietary Berries Traditionally Used by Native North Americans

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Four wild berry species, *Amelanchier alnifolia*, *Viburnum trilobum*, *Prunus virginiana*, and *Shepherdia argentea*, all integral to the traditional subsistence diet of Native American tribal communities, were evaluated to elucidate phytochemical composition and bioactive properties related to performance and human health. Biological activity was screened using a range of bioassays that assessed the potential for these little-known dietary berries to affect diabetic microvascular complications, hyperglycemia, pro-inflammatory gene expression, and metabolic syndrome symptoms. Nonpolar constituents from berries, including carotenoids, were potent inhibitors of aldose reductase (an enzyme involved in the etiology of diabetic microvascular complications), whereas the polar constituents, mainly phenolic acids, anthocyanins, and proanthocyanidins, were hypoglycemic agents and strong inhibitors of IL-1$\beta$ and COX-2 gene expression. Berry samples also showed the ability to modulate lipid metabolism and energy expenditure in a manner consistent with improving metabolic syndrome. The results demonstrate that these berries traditionally consumed by tribal cultures contain a rich array of phytochemicals that have the capacity to promote health and protect against chronic diseases, such as diabetes.

**KEYWORDS:** ***Amelanchier alnifolia*; *Viburnum trilobum*; *Prunus virginiana*; *Shepherdia argentea*; diabetes; inflammation; energy expenditure**

## INTRODUCTION

Native Americans historically used a wide variety of wild berries, including serviceberry (*Amelanchier alnifolia*), highbush cranberry (*Viburnum trilobum*), chokecherry (*Prunus virginiana*), and silver buffaloberry (*Shepherdia argentea*), year round for food and medicine. Wild berries were picked when ripe and stored for use throughout the year by drying them and/or incorporating the berries into dried meat and animal fat, thus enabling tribal peoples to use them even when the fruits were not in season (*1, 2*).

Wild berries provide an ideal resource for investigating phytochemicals that influence human health because they contain natural levels of compounds which have not been influenced by commercial breeding and selection (*3*). Previous papers cited lower levels of bioactive plant secondary metabolites in conventionally grown crops compared to their organically grown counterparts (crops grown with fewer inputs) (*4, 5*). In addition, many crops grown using modern agribusiness technology may have a lower nutrient content now than they did three decades ago (*6*). Furthermore, several studies have demonstrated increased antioxidant activity and phenolic composition of wild berries compared to their domesticated (and genetically distinct) relatives (*7–10*).

*A. alnifolia* (Saskatoon berry, Juneberry, or serviceberry) is a deciduous shrub that is native to the western United States and Canada. It was extensively used by native cultures both medicinally (as a disinfectant and to prevent miscarriages) and

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as a food source, often in forms such as pemmican (a mixture of dried meat, dried berries, and fat) (*11*). More recently, Wang and Mazza (*12*) found that a concentrated crude extract of *A. alnifolia* berries inhibited nitric oxide production in activated macrophages, indicating a potential protective role against cardiovascular disease and chronic inflammation. Hu et al. (*13*) found the berries of *A. alnifolia* to be strong scavengers of free radicals without reducing cell viability. Fruit from the related species, *A. canadensis* and *A. arborea*, inhibit cyclooxygenase-1 and -2 in vitro, indicating a role in moderating inflammation (*14*). Previous cursory investigations of anthocyanin, phenolic acid, and flavonoid contents of *A. alnifolia* fruit revealed an anthocyanin content of 86–125 mg of anthocyanins/100 g of fresh berries with the 3-galactoside and 3-glucoside conjugates of cyanidin predominating (*15*). Other anthocyanins include cyanidin 3-xyloside, pelargonin 3-glucoside, and malvidin derivatives (*16*). Berries of *A. alnifolia* contain phenolic acids including 3-feruloylquinic, chlorogenic, and 5-feruloylquinic acids (*17*). Flavonoid compounds from the fruit include rutin, hyperoside, avicularin, and quercetin (*18*).

*V. trilobum* (highbush cranberry) is native to the northern United States and southern Canada from New York to Oregon (*19*). Native Americans ate the berries fresh, in jelly, and in pemmican (*20*). Few studies have investigated the chemical composition of the berries. Cyanidin 3-arabinosylsambubioside and cyanidin 3-arabinoglucoside are two anthocyanins that have been previously identified in the fruit (*21, 22*).

*P. virginiana* (chokecherry) is native to North America and commonly found in most of the United States and southern Canada. It was widely used by Native Americans both as a food, such as wine, juice, and *wasna* (a mixture of dried berries and dried meat) (*23*), and as a medicine (*11*). Recently, Acuña et al. (*24*) showed that methanol extracts of the fruit had antioxidant capacity. No studies to date have focused on the chemical composition of the fruits or other parts of the plant.

*S. argentea* (silver buffaloberry) berries were traditionally used by Native Americans to treat stomach troubles and ceremonially to honor females entering puberty (*11, 23*). They were typically eaten fresh, dried, or in jelly (*11*). The berries contain $\beta$-carotene, vitamin C, leucoanthocyanins, catechols, and flavonols (*25, 26*). Tannins from the leaves of *S. argentea* have demonstrated the capacity to inhibit HIV-1 reverse transcriptase (*27*). Recently, Ritch-Krc et al. (*28*) found that extracts of *S. canadensis* (Canadian buffaloberry), a closely related species, inhibited the growth of mouse mastocytoma cells.

The objectives of this study were to characterize the major phytochemical constituents of four tribal wild berry genotypes (*A. alnifolia*, *V. trilobum*, *P. virginiana*, and *S. argentea*) and to evaluate their ability to protect against diabetes (by improving glucose utilization and inhibiting aldose reductase), suppress the expressions of pro-inflammatory genes (cyclooxygenase-2 and interleukin-6), and improve other conditions associated with metabolic syndrome (by measuring changes in lipid metabolism and energy expenditure). These attributes were likely to have contributed to the health protective value of berry fruits in the traditional tribal diets.

## MATERIALS AND METHODS

**Berry Sources.** *V. trilobum* (highbush cranberry), *P. virginiana* (chokecherry), and *S. argentea* (silver buffaloberry) berries were all collected in late summer and fall harvest seasons 2005 and 2006 on tribal lands in North Dakota by members of the five tribes served by United Tribes Technical College (Bismarck, ND). Due to drought conditions and consequent poor serviceberry yield on tribal lands during these collection seasons, *A. alnifolia* fruits were purchased from Room 2 Grow (Pincher Creek, AB, Canada) and were a mixture of 'Smoky', 'Theissen', and 'Northline' cultivars. All berries were frozen after harvest and stored at −80 °C.

**Extraction and Fractionation of Berries.** Frozen berries were slightly thawed, mashed, refrozen, and then lyophilized until completely dry. The dried berry mass ranged from 37% (*S. argentea*) to 18% (*A. alnifolia*) of the fresh mass. Freeze-dried berries of all species were extracted in 80% aqueous ethanol (1:10, grams of berries/milliliter of solvent) by stirring for 1 h and then filtered using several layers of cheesecloth. This process was repeated three additional times. The filtrate (crude extract) was filtered through no. 4 Whatman filter paper and then concentrated under vacuum in a 40 °C water bath to a thick, aqueous slurry. The percent recovery of crude extract from fresh mass ranged from 11% (*V. trilobum*) to 25% (*S. argentea*).

A liquid–liquid extraction of the crude extract was made by adding equal portions of water and ethyl acetate to the concentrated crude extract followed by shaking the mixture and then separating the ethyl acetate phase (top) from the water phase (bottom). The water phase was mixed with fresh ethyl acetate four times. Both phases were concentrated under vacuum in a 40 °C water bath and lyophilized until dry.

For each species, a portion of the concentrated water phase from the liquid–liquid extraction was separated into six fractions using vacuum liquid chromatography (VLC) to facilitate the identification of compounds in the water phase. A HW-40F Toyopearl (TP; Tosoh, Bioscience LLC, Montgomeryville, PA) column (7 × 5 cm, w × l) was used and supported by a medium-pore-size fritted glass filter. Fractions were eluted using water (850 mL), 50% aqueous methanol (MeOH, 1 L), 100% MeOH (1 L), 100% acetone (1 L), 50% aqueous acetone (1 L), and 35% aqueous ethanol (500 mL), yielding fractions TP1−TP6, respectively. These fractions were concentrated under vacuum in a 40 °C water bath and then lyophilized until dry.

**Mass Spectrometry.** Electrospray ionization (ESI) mass spectral analysis was used to determine the phytochemical content of berry extracts and fractions in a manner similar to that of Kraft et al. (*29*). Spectra were recorded over the range $m/z$ of 100−2000. All samples were run in positive mode and dissolved in MeOH.

**Bioactivity Assays.** *Aldose Reductase Assay.* Aldose reductase (alditol:$NADP^+$ 1-oxidoreductase, EC 1.1.1.21) catalyzes the first reaction in the polyol pathway, which is the reduction of glucose to sorbitol. Even though aldose reductase has a low affinity for hexoses, such as glucose, there is increased sorbitol production in a diabetic hyperglycemic state because the substrate level is elevated. This increased formation of sorbitol has been linked to diabetic microvascular complications such as retinopathy, neuropathy, and nephropathy. Therefore, inhibition of aldose reductase is a recognized antidiabetic mechanism of plant extracts (*30*). Aldose reductase (Wako Chemicals, Richmond, VA) activity was measured by monitoring the change in $\beta$-nicotinamide adenine dinucleotide phosphate (NADPH; Sigma, St. Louis, MO) absorbance at 340 nm over a 5 min interval (*30*). Berry samples were dissolved in dimethyl sulfoxide (DMSO) and tested at a concentration of 5 $\mu$g/mL. 3,3-Tetramethyleneglutaric acid was used as a positive control. Seventy percent inhibition and greater was considered to be strong activity, and inhibition between 40 and 70% was considered to be moderate activity. Berry samples were run in quadruplicate and expressed as average percent inhibition ± standard error.

*Quantitative Real-Time RT-PCR.* Inflammation is associated with obesity, problems with lipid metabolism, and development of diabetes (*31*). Inhibition of pro-inflammatory gene expression was measured using methods similar to those of Ma et al. (*32*). Briefly, mouse monocyte/macrophage cell line RAW 264.7 (American Type Culture Collection, Manassas, VA) was plated at a density of $0.4 \times 10^6$ in 24-well plates 12 h before treatment. Cells were treated with the berry samples (100 $\mu$g/mL, dissolved in 95% aqueous EtOH) 2 h before elicitation with 1 $\mu$g/mL lipopolysaccharide (LPS; Sigma). RNA was harvested from cells using TRIzol reagent (Invitrogen, Carlsbad, CA) 6 h after LPS elicitation. Expression of COX-2, IL-1$\beta$, and $\beta$-actin (control) genes were quantified using Stratagene Mx 3000*p* RT-PCR System (Stratagene, La Jolla, CA) and a Brilliant SYBR Green PCR master mix kit (Stratagene). The amplification program was the same

**Table 1.** Partial Composition of *A. alnifolia*, *V. trilobum*, *P. virginiana*, and *S. argentea* Berries As Determined by ESI-MS[a]

| *A. alnifolia* | *V. trilobum* | *P. virginiana* | *S. argentea* |
|---|---|---|---|
| | Phenolic Acids | | |
| chlorogenic acid | chlorogenic acid[b] | chlorogenic acid[b] | chlorogenic acid |
| caffeic acid[b] | caffeic acid[b] | caffeic acid[b] | |
| hydroxybenzoic acid[b] | hydroxybenzoic acid[b] | ferulic acid[b] | |
| | | sinapic acid[b] | |
| | Anthocyanins | | |
| cyanidin 3-glu/gla | cyanidin 3-glu/gal | cyanidin 3-glu/gal[b] | |
| petunidin 3-glu/gla[b] | petunidin 3-glu/gal[b] | delphinidin 3-glu/gal[b] | |
| cyanidin 3,5-diglu[b] | cyanidin 3,5-diglu[b] | cyanidin 3,5-diglu[b] | |
| cyanidin 3-xyloside | | delphinidin 3,5-diglu[b] | |
| | | petunidin 3-acetylglu[b] | |
| | | cyanidin 3-*p*-coumarolylglu[b] | |
| | Proanthocyanidins | | |
| dimer[b] | dimer[b] | | |
| trimer[b] | trimer[b] | | |
| tetramer[b] | tetramer[b] | | |
| pentamer[b] | pentamer[b] | | |
| hexamer[b] | hexamer[b] | | |
| | Flavanols | | |
| catechin/epicatechin[b] | catechin/epicatechin[b] | | |
| epicatechin gallate[b] | | | |
| | Carotenoids | | |
| | $\beta$-carotene/lycopene[b] | $\beta$-carotene/lycopene[b] | $\beta$-carotene/lycopene |
| | | $\beta$-cryptoxanthin[b] | $\beta$-cryptoxanthin[b] |
| | | | zeaxanthin[b] |

[a] The listed compounds have molecular weights consistent with ESI-mass spectral (positive mode) analysis. Anthocyanins may be glucosides (glu), galactosides (gal), or both unless otherwise indicated. [b] Indicates first time compound reported in the tribal berry.

as that used by Ma et al. (*32*). Samples were run in duplicate. Inhibition greater than or equal to 75% was considered to be high activity, 50–75% inhibition was moderate activity, and less than 50% inhibition was low activity.

*Culture Systems.* L6 cells, which are widely used as a cell model for studying insulin action (*33*), were purchased from American Type Culture Collection. L6 culture was performed as described by Mandel and Pearson (*34*). Specifically, the cells were seeded at the density of 10000/cm$^2$ in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 100 units/mL penicillin, 100 $\mu$g/mL streptomycin, and 10% heat-inactivated fetal bovine serum (FBS). When the cells reached 70% confluence, serum-free medium consisting of DMEM supplemented with 1% (wt/vol) fatty acid-free bovine serum albumin (BSA; Sigma) or 1% FBS-containing DMEM (differentiation medium) was added for 5 days. After differentiation, the botanical extracts were added and, after overnight incubation, processed.

*Basal and Insulin-Stimulated Glycogen Accumulation.* Decreasing hyperglycemia, the cornerstone of diabetes treatment, is primarily accomplished by enhancing the uptake of glucose from the blood into muscle tissue, the primary site of glucose uptake. Muscle cells use blood glucose to produce glycogen. Changes in glycogen accumulation in muscle cells are therefore a means of measuring potential hypoglycemic activity. Glycogen contents of L6 cells were measured as described by Gomez-Lechon et al. (*35*) with some modifications. Briefly, berry sample-treated L6 myotubes were deprived of serum for 2 h. Cells were incubated with or without insulin for 2 h (100 nM insulin plus 30 mM glucose) and were then washed three times with phosphate buffer solution (PBS, pH 7.4). Two hundred microliters of 0.2 M sodium acetate buffer (pH 4.8) was added to each well, and the plate was sonicated. Fifty microliter aliquots of these berry samples were taken for protein assay. Amyloglucosidase was added, and plates were shaken for 2 h at 40 °C. After centrifugation, 50 $\mu$L of supernatant was transferred to a 96-well plate, and 150 $\mu$L of assay solution {(0.4 unit/mL glucose oxidase, 0.8 unit/mL peroxidase, and 1 mg/mL ABST [2,2′-azinobis(3-ethylbenzothialine-6-sulfonic acid) in 100 mM phosphate buffer (pH 6)} was added. The berry samples were incubated at room temperature in the dark for 30 min. The intensity of the color reaction was measured at 405 nm using a microplate reader. A blank of the reaction was performed using incubated cell homogenate without the addition of amyloglucosidate; this value represents the free glucose and was subtracted from the total glucose obtained after enzymatic hydrolysis. A standard curve was constructed with a known amount of rabbit liver glycogen and processed as test samples. The glycogen content was expressed as nanomolar glucose equivalent/ per well after correction for protein concentration.

*Fatty Acid Oxidation.* A common manifestation of metabolic syndrome is defective fat metabolism leading to enhanced levels of circulating free fatty acids and fat accumulation. Marker assays for fat metabolism were used to detect berry samples that influence the metabolism of fat in vitro as a model for how fat is metabolized in vivo. Fatty acid oxidation was measured as described by Muoio et al. (*36*). Briefly, L6 myotubes were incubated with 20 $\mu$g/mL compounds as indicated overnight without 10% FBS. Then, cells were incubated at 37 °C in sealed 12-well plates containing 1 mL of medium plus 12.5 mmol/L HEPES, 0.2% BSA, 1.0 mmol/L carnitine, 100 $\mu$mol of oleate, 50 $\mu$g/mL gentamicin, and 1.0 $\mu$Ci/mL [$^{14}$C]palmitic acid (PerkinElmer Life Science, Boston, MA). After 3 h, the incubation medium was assayed for labeled $CO_2$ and acid-soluble metabolites as previously reported (*37, 38*). The cells were placed on ice, washed twice with PBS, and scraped into a 1.5 mL Eppendorf tube in two additions of 0.30 mL of 0.05% SDS lysis buffer. Cell lysates were assayed for protein, and results are expressed as percent change relative to controls. Assays were performed in triplicate.

*Mitochondrial Number.* Mitochondria are the cellular organelles responsible for the energy status of the cell and are the site of fatty acid oxidation. Mitochondria are impaired in function and number as a result of insulin resistance and metabolic syndrome, especially in diabetic patients. Therefore, measurement of mitochondrial number provides a means of evaluating the effect of berry samples on the energy status of animal cells. The effect of berry samples on mitochondrial quantity was determined by washing L6 cells with PBS followed by staining them with the mitochondrial stains MitoTracker Green FM (MTG, 100 nM, Invitrogen, Carlsbad, CA). MTG stains lipids characteristic of mitochondrial membranes (*39*), and the assay was performed according the manufacturer's protocol. Samples were measured in triplicate. Data were expressed as percent change compared to control cells.

## RESULTS

**Phytochemical Content.** *A. alnifolia* extract and fractions revealed the presence of phenolic acids, anthocyanins, and a series of proanthocyanidin polymers (**Table 1**). Chlorogenic acid

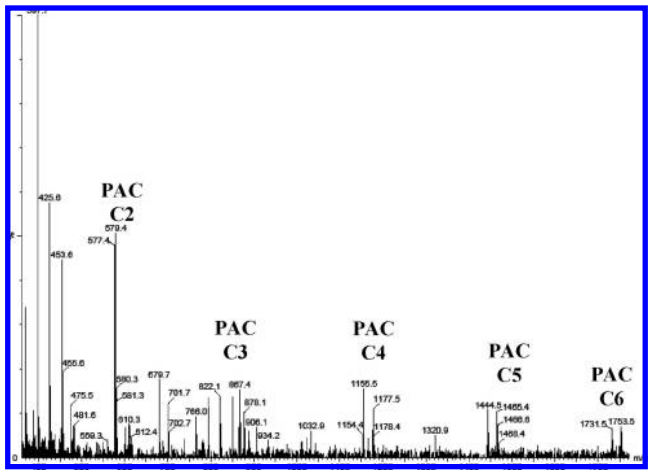


**Figure 1.** Representative ESI-MS of Toyopearl fraction 5 of *V. trilobum* showing a series of proanthocyanidin (PAC) oligomers and polymers.

(*m/z* 355.3), caffeic acid (*m/z* 181.1), and hydroxybenzoic acid (*m/z* 139.2) were indicated in the ESI-mass spectra. The anthocyanin compounds 3-glucosides and/or 3-galactosides of cyanidin (*m/z* 449.3) and petunidin (*m/z* 479.3), cyanidin 3-xyloside (*m/z* 419.4), and cyanidin 3,5-diglucoside (*m/z* 611.4) were detected. Additionally, flavanols, such as catechin/epicatechin (*m/z* 291.3) and epicatechin gallate (*m/z* 443.4), and a series of proanthocyanidin polymers ranging from dimers to hexamers (from *m/z* 579.4 to 1731.6) were consistent with the mass spectra. This analysis provides the first documentation of caffeic acid, hydroxybenzoic acid, petunidin 3-glucoside/galactoside, cyanidin 3,5-diglucoside, catechin/epicatechin, epicatechin gallate, and the proanthocyanidin series in this species.

Spectra of *V. trilobum* berry samples revealed phenolic acids, anthocyanins, flavanols, a series of proanthocyanidin polymers, and one carotenoid (**Table 1**). Caffeic acid (*m/z* 180.2), chlorogenic acid (*m/z* 355.3), and hydroxybenzoic acid (*m/z* 139.2) were phenolic acids consistent with the mass spectra of *V. trilobum* berry samples. Cyanidin 3,5-diglucoside (*m/z* 610.3), petunidin 3-glucoside/galactoside (*m/z* 479.5), and cyanidin 3-glucoside (*m/z* 449.3) were anthocyanins consistent with the mass spectra, and the latter appeared to be the dominant anthocyanin in the berry. In addition, the flavanols catechin and/or epicatechin (*m*/z 291.2) and a proanthocyanidin series of polymers ranging from dimers to hexamers (from *m/z* 579.4 to 1731.6) were prominent (**Figure 1**). $\beta$-Carotene/lycopene (*m/z* 536.2) was also identified. Other than cyanidin 3-glucoside, this is the first time these compounds have been reported for *V. trilobum*.

*P. virginiana* berry samples were found to contain phenolic acids, a diverse range of anthocyanins, and carotenoids (**Table 1**). Chlorogenic acid (*m/z* 355.3), ferulic acid (*m/z* 194.3), caffeic acid (*m/z* 181.1), and sinapic acid (*m/z* 225.2) were phenolic acids consistent with the mass spectra of *P. virginiana* berries. The anthocyanins that afford most of the color associated with the berry included 3-glucosides and/or 3-galactosides of cyanidin (*m/z* 449.3) and delphinidin (*m/z* 464.4), petunidin 3-acetylglucoside (*m/z* 520.5), delphinidin 3,5-diglucoside (*m/z* 668.6), cyanidin 3-*p*-coumaroylglucoside (*m/z* 595.4), and cyanidin 3,5-diglucoside (*m/z* 610.7). In addition, the berries contain the carotenoids $\beta$-carotene/lycopene (*m/z* 536.2) and $\beta$-cryptoxanthin (*m/z* 553.0). The chemical composition of this species has not been previously studied; hence, this chemical analysis represents the first time any compounds have been identified in its fruit.

*S. argentea* berries contained several carotenoids, no detectable anthocyanins, and few phenolic acids (**Table 1**). Mass spectral analysis revealed the presence of $\beta$-carotene/lycopene (*m/z* 536.2), $\beta$-cryptoxanthin (*m/z* 553.0), and zeaxanthin (*m/z* 570.5), which are responsible for the yellow-orange pigmentation associated with the berries. Chlorogenic acid (*m/z* 355.5) was found in small quantities. With the exception of $\beta$-carotene, this is the first time the above compounds have been reported in the berry.

**Table 2.** Percent Inhibition of Aldose Reductase by Tribal Berry Samples

| berry sample | % inhibition[a] |
|---|---|
| *A. alnifolia* | |
| crude extract | NA |
| water | NA |
| ethyl acetate | 82 ± 0.73 |
| *V. trilobum* | |
| crude extract | 24 ± 2.31 |
| water | NA |
| ethyl acetate | 76 ± 1.57 |
| *P. virginiana* | |
| crude extract | 42 ± 0.76 |
| water | 43 ± 1.56 |
| ethyl acetate | 73 ± 0.98 |
| *S. argentea* | |
| crude extract | 30 ± 0.87 |
| water | 27 ± 1.68 |
| ethyl acetate | 64 ± 2.27 |

[a] NA, not active; samples tested at 5 $\mu$g/mL in quadruplicate. Expressed as percent inhibition ± SE.

**Table 3.** Percent Change in Glycogen Accumulation by Tribal Berry Samples in Non-Insulin-Stimulated Cells

| berry sample | % change in glycogen[a] |
|---|---|
| *A. alnifolia* | |
| crude extract | 76* |
| water | 92* |
| ethyl acetate | 23 |
| *V. trilobum* | |
| crude extract | 168** |
| water | 4 |
| ethyl acetate | −12 |
| *P. virginiana* | |
| crude extract | 8 |
| water | 12 |
| ethyl acetate | −9 |
| *S. argentea* | |
| crude extract | 54* |
| water | 5 |
| ethyl acetate | −29 |

[a] * denotes significant activity, $P < 0.05$; ** denotes very significant activity, $P < 0.001$. Samples run in triplicate and expressed as percent change of glycogen accumulation compared to control.

**Bioactivity.** *A. alnifolia* samples demonstrated the ability to inhibit aldose reductase, improve glucose uptake, and reduce expression of IL-1$\beta$ (one of the anti-inflammatory markers). The nonpolar fraction of *A. alnifolia* strongly inhibited aldose reductase (82% inhibition; **Table 2**). The crude extract and polar fraction, on the other hand, exhibited significant capacity to improve glycogen accumulation at a basal state (**Table 3**). The crude extract and the nonpolar fraction, but not the polar fraction, minimally inhibited IL-1$\beta$ gene expression (36 and 32%, respectively; **Table 4**). These results indicate that nonpolar compounds from *A. alnifolia* may provide protection against diabetic microvascular complications and may help mediate inflammation, whereas polar compounds may help reduce inflammation as well, but also improve glucose uptake via an insulin-like effect.

*V. trilobum* berries showed the capacity to inhibit aldose reductase, improve glucose uptake, reduce IL-1$\beta$ expression,

**Table 4.** Percent Inhibition of LPS-Induced Inflammatory Gene Expression By Tribal Berry Samples

| | inflammatory markers[a] | |
|---|---|---|
| berry sample | IL-1b | COX-2 |
| *A. alnifolia* | | |
| crude extract | 36 | NA |
| water | NA | NA |
| ethyl acetate | 32 | NA |
| *V. trilobum* | | |
| crude extract | 56 | NA |
| water | 45 | NA |
| ethyl acetate | NA | NA |
| *P. virginiana* | | |
| crude extract | 78 | 47 |
| water | 74 | 43 |
| ethyl acetate | NA | 24 |
| *S. argentea* | | |
| crude extract | 76 | 45 |
| water | 38 | 70 |
| ethyl acetate | NA | NA |

[a] Samples run in duplicate and tested at 100 $\mu$g/mL. IL-1b, interleukin 1b; COX-2, cyclooxygenase-2; NA, not active; <50% is low activity, 50–75% is moderate activity, and >75% is high activity. Samples run in duplicate and expressed as percent inhibition.

**Table 5.** Ability of Tribal Berry Samples To Modulate Lipid Metabolism and Energy Expenditure

| | lipid metabolism markers[a] | | | |
|---|---|---|---|---|
| berry sample | acid soluble metabolite accumulation | fatty acid oxidation rate | $CO_2$ production | energy expenditure marker, mitochondria no.[a] |
| *A. alnifolia* | | | | |
| crude extract | −1.5 | −6.6 | −13.0 | 20.2* |
| water | −11.6 | −13.1 | −15.1 | −4.5 |
| ethyl acetate | 2.4 | −5.5 | −15.5 | 21.1 |
| *V. trilobum* | | | | |
| crude extract | 14.6 | 17.2 | 20.5* | −23.3 |
| water | 32.8* | 27.6* | 20.8* | 3.2 |
| ethyl acetate | 6.8 | 17.4 | 31.3* | −31.9 |
| *P. virginiana* | | | | |
| crude extract | 28.0* | 7.1 | −4.7 | −27.6 |
| water | −8.3 | −10.4 | −11.6 | −33.7 |
| ethyl acetate | 6.4 | −1.6 | −6.0 | 24.1* |
| *S. argentea* | | | | |
| crude extract | −11.8 | 12.2 | 29.5* | −25.1 |
| water | 85.1** | 43.8* | 14.0 | −26.4 |
| ethyl acetate | −46.0 | −15.9 | 5.8 | −31.5 |

[a] * denotes significant activity, $P < 0.05$; ** denotes very significant activity, $P < 0.001$. Samples run in triplicate and expressed as percent change compared to control.

and modulate energy expenditure. The nonpolar fraction of *V. trilobum* strongly inhibited aldose reductase (76% inhibition; **Table 2**). The crude extract also showed very significant ability to improve glycogen accumulation at a basal state (**Table 3**). Both the crude extract and the polar fraction moderately inhibited IL-1$\beta$ (56 and 45%, respectively; **Table 4**), and all three preparations of *V. trilobum* modulated energy expenditure via several mechanisms (**Table 5**). These results suggest that although not a powerful mediator of inflammation, compounds from *V. trilobum* can not only provide protection against diabetic microvascular complications but also improve glucose uptake via an insulin-like effect as well as change energy expenditure. Taken together, these indicate potential to partially modulate mechanisms that may play a role in the development of insulin resistance and metabolic syndrome.

*P. virginiana* samples exhibited potential to inhibit aldose reductase, reduce the expression of both IL-1$\beta$ and COX-2, and alter energy expenditure. The nonpolar fraction of *P. virginiana* strongly inhibited aldose reductase (73% inhibition), and the other two samples showed only moderate inhibition (**Table 2**). Both the crude extract and polar fractions strongly inhibited IL-1$\beta$ expression (78 and 74%, respectively; **Table 4**), and these two samples and the ethyl acetate fraction minimally inhibited COX-2 expression (47, 43, and 24% respectively; **Table 4**). Additionally, both the polar and nonpolar fractions demonstrated some capacity to modulate energy expenditure (**Table 5**). These results indicate that compounds from *P. virginiana* have the potential to reduce the development of diabetic microvascular complications and are strong inhibitors of inflammation.

*S. argentea* samples showed the capacity to inhibit aldose reductase, improve glycogen accumulation, reduce the expression of both IL-1$\beta$ and COX-2, and alter energy expenditure. All samples from *S. argentea* moderately inhibited aldose reductase; inhibition ranged from 27% (polar fraction) to 64% (nonpolar fraction) (**Table 2**). In addition, the crude extract demonstrated the ability to improve glycogen accumulation at a basal state (**Table 3**), and the polar fraction showed significant capacity to modulate energy expenditure via different mechanisms (**Table 5**). The crude extract strongly inhibited expression of IL-1$\beta$ (76% inhibition) but only minimally reduced expression of COX-2 (45% inhibition). The water fraction only minimally inhibited expression of IL-1$\beta$ and moderately inhibited expression of COX-2 (70%) (**Table 4**). These results indicate that compounds from *S. argentea* can moderately provide protection from diabetic microvascular complications and can also reduce inflammation. Furthermore, compounds from this species have the potential to counter the symptoms of metabolic syndrome via improving glucose uptake and energy expenditure.

## DISCUSSION

These four berry species, each components in traditional tribal diets, differ greatly in content. This range of phytochemicals among the different species implies that the traditional tribal diet consisted of a rich array of bioactive compounds that may have promoted health and provided protection from chronic diseases via a range of potential mechanisms. *A. alnifolia* and *V. trilobum* berries contain very few carotenoids but are rich in phenolic acids, anthocyanins, and proanthocyanidins. *S. argentea*, on the other hand, is rich in carotenoids but not phenolic acids or flavonoids. *P. virginiana* has a high content of phenolic acids, anthocyanins, and carotenoids but lacks proanthocyanidins. All four tribal berry species examined in this study contain biologically active chlorogenic acid.

Nonpolar botanical compounds, such as terpenes, have been shown by others to be strong aldose reductase inhibitors. Compounds in the ethyl acetate fraction of these tribal berries, such as carotenoids, inhibited aldose reductase, whereas polar constituents and crude extract did not inhibit enzymatic activity (**Table 4**), which indicates the potential of these nonpolar compounds to protect against diabetic complications as aldose reductase inhibitors. Flavonoids and phenolic acids (polar phytochemicals) have previously demonstrated aldose reductase inhibitory activity (*40*), but our flavonoid-rich water fractions did not demonstrate such activity at the tested concentration. This apparent lack of activity from the flavonoid-rich fractions may be due to interference or a dilution effect from other compounds.

The anti-inflammatory effects of the berries on in vitro gene expression were mostly observed in crude or polar fractions, except in the case of *A. alnifolia* (**Table 2**). The variation among different berries in terms of the activity levels (moderate to high)

could be due to the differences in the nature of their bioactive constituents and/or the variation in the amount of those constituents present in the samples. The anti-inflammatory properties of these wild berries augment the health beneficial attributes observed in the other assays. For example, the associations between obesity, impaired skeletal muscle fatty acid metabolism, and type 2 diabetes are well established (*31*). Inflammation due to obesity acts as a precursor to defects in skeletal muscle fatty acid oxidation and generates a vicious cycle exacerbating the development of insulin resistance. In the obese state, elevated circulating fatty acids set off a pro-inflammatory cascade by macrophage activation (*31*).

Both *V. trilobum* and *A. alnifolia* crude extracts and water fractions may provide benefit with regard to diabetes by improving blood glucose utilization. Samples from both of these berries improved glycogen accumulation at a basal state, whereas insulin-stimulated glycogen accumulation was not enhanced for any of the berries. Tabular data indicate only basal state glycogen accumulation. These were also the two berry species with a series of proanthocyanidin polymers, suggesting that higher molecular weigh proanthocyanidins may be integral to the improved glycogen accumulation. Proanthocyanidins have previously been reported to ameliorate hyperglycemia (*41*) in vivo in a rat model.

Lipid metabolism and energy expenditure are other important parameters associated with metabolic syndrome and related disorders. Acid-soluble metabolites and fatty acid oxidation were used to investigate possible activities of the berry samples in regard to lipid metabolism. The water-soluble fractions from *V. trilobum* and *S. argentea* as well as the crude extract of *P. virginiana* had notable activity for enhancing lipid metabolism. The positive enhancement of both biomarkers by *V. trilobum* and *S. argentea* suggest a robust effect, especially when coupled with the $CO_2$ production enhancement from the *V. trilobum* samples. Each of the *V. trilobum* extracts enhanced $CO_2$ production to a similar extent, suggesting multiple active compounds with different polarities. Because only the crude extract of *S. argentea* increased $CO_2$ production, the fractionation procedure must have somehow eliminated the activity. Changes in mitochondrial number associated with the berry extracts were used as an indicator for change in energy expenditure potential. High numbers of mitochondria are noted in tissues with high levels of energy expenditure. Only two of the extracts increased mitochondrial number, but they did not correlate with $CO_2$ production.

Spring drought conditions during the two seasons of this research precluded harvest of sufficient *Amelanchier* fruits in the wild; Juneberry has an earlier berry maturation and harvest season than the other berries in this study, which bear later in the season. Because of the importance of Juneberry in traditional tribal diets, we obtained fruit from a commercial grower with climatic conditions generally similar to those of North Dakota. The deposition of the key phytochemical metabolites important to bioactivity is likely to be less intense in a cultivated environment (characterized by irrigation, higher fertility, and pest control), and the berries larger, than expected in wildcrafted harvests. Thus, it is possible that the level of bioactivity gauged for Juneberry in this study will underestimate the potential for a wild berry sample.

Specific modes of action for the berries, which may have contributed to their value as medicinal and subsistence foods in traditional diets, cannot be conclusively determined from a screening study based solely on in vitro bioassays. In vitro bioassays can help to identify cellular mechanisms, but the results cannot be directly extrapolated to formulate health claims. Further experimentation in animal models is required to confirm in vivo efficacy of ingested berry compounds. The ability of these extracts to affect specific cellular targets has been demonstrated, however, which suggests that further activity-guided fractionation and characterization will lead to validation of the basis for their biological activity after consumption.

There is a growing interest within tribal communities for fostering a return to more traditional dietary choices, as well as an increasing interest in the broader U.S. population for discovery of new food choices that offer a proactive means of health maintenance. An opportunity for small-scale production on tribal lands to serve this niche market is emerging; however, given the impact of cultivation practices on phytochemical composition and bioactive potency of berries, it is critical that cultivated berry plots be designed to mimic, to the extent possible, conditions that are inherent in the wild.

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