POLISH JOURNAL OF FOOD AND NUTRITION SCIENCES
Pol. J. Food Nutr. Sci. 2003, Vol. 12/53, SI 1, pp. 166–169

ANTIOXIDANT ACTIVITY OF CRUDE PHENOLIC EXTRACTS FROM WILD BLUEBERRY LEAVES

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Key words: blueberry leaves, phenolics, antioxidant properties, free-radical scavenging capacity, meat model system

Blueberry leaves were extracted with ethanol (95%, v/v) or aqueous acetone (70%, v/v). The crude extracts so obtained were evaporated to near dryness and then lyophilized. The antioxidant activity of these phenolic extracts was evaluated using the β-carotene-linoleate, 2,2-diphenyl--1-picrylhydrazyl radical (DPPH•) and 2-thiobarbituric acid reactive substances (TBARS) assays. The content of crude tannins in ethanol and acetone extracts of blueberry leaves was 181 and 213 mg catechin equivalents/g extract, respectively. The antioxidant activity of phenolic extracts using the β-carotene-linoleate model system was comparable to that exhibited by the synthetic antioxidant butylated hydroxyanisole (BHA). The DPPH• scavenging effect of crude phenolic extracts, at 100 μg/assay, was over 90%. Inhibition of meat lipid oxidation ranged from 36.6 to 100% for crude phenolics of the ethanol extract at 200- and 1 000-ppm addition levels, respectively. Similar inhibitory effects were observed for crude extracts of blueberry leaves phenolics from the aqueous acetone preparation.

INTRODUCTION

There is a growing interest in replacing synthetic antioxidants such as butylated hydroxyanisole (BHA), propyl gallate (PG), and *tert*-butylhydroquinone (TBHQ) with natural alternatives. Constituents of fruits, vegetables, spices, leaves, roots and barks have been exploited as potential sources of natural antioxidants [Shahidi & Naczk, 2003; Sherwin, 1990].

The antioxidant activities of plant extracts are associated with the presence of phytochemicals such as anthocyanins, phenolic acids, flavonoids and tannins [Cao *et al.*, 1996]. Prior *et al.* [1998] noticed an increase in the antioxidant activity of blueberry extracts with maturity of the berries. Wang and Lin [2000] evaluated the antioxidant activity of fruits and leaves of blackberry, raspberry and strawberry by their oxygen radical absorbance capacity (ORAC); extracts of phenolics from leaves exhibited significantly ($p<0.05$) higher ORAC values than those from the corresponding berries. Recently, Siddhuraju and Becker [2003] reported that leaves from the drumstick tree (*Moringa oleifera* Lam.) could be used as a potential source of natural antioxidants due to their marked antioxidant activity.

The leaves of blueberry are by-products from mechanical harvesting of wild berries, and these are not commercially utilized. In this contribution we investigate and discuss the possible utilization of blueberry leaves as a source of natural food antioxidants.

MATERIALS AND METHODS

All solvents used were of analytical grade. Butylated hydroxyanisole (BHA), *tert*-butylhydroquinone (TBHQ), β-carotene, linoleic acid, 2,2-diphenyl-1-picrylhydazyl radical (DPPH•), (+) catechin, polyoxyethylenesorbitan monopalmitate (Tween 40), trichloroacetic acid, potassium ferricyanide, ferric chloride, and 2-thiobarbituric acid were obtained from Sigma Chemical Co. (St. Louis, MO).

Blueberry leaves, a by-product of mechanical harvesting, were collected from a wild blueberry farm located in Antigonish county, Nova Scotia, Canada. The leaves were separated from other debris, dried at room temperature and then stored in sealed polyethylene bags at -18°C until examined. Crude polyphenolics were extracted from dried leaves with 95% (v/v) ethanol three times at 50°C for 30 min at a solids-to-solvent ratio of 15:100 (w/v). The ethanolic extracts were combined, evaporated to near dryness under vacuum at 40°C and then lyophilized. Crude polyphenolics were also extracted from the leaves with 70% (v/v) aqueous acetone in a similar fashion as that described above. Chlorophyll was removed from the extracts as described by Amarowicz *et al.* [2003].

The total content of phenolic compounds in the extracts was estimated using the *Folin-Denis* reagent [Swain & Hillis, 1959] and expressed as absorbance units per 1 g of lyophilized extract. The content of condensed tannins (CT) in the extracts was measured using the modified vanillin

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assay and expressed as catechin equivalents per 1 g of lyophilized extract [Naczk *et al.*, 1994].

The antioxidant activity of crude phenolic extracts of wild blueberry leaves was evaluated in a β-carotene--linoleate model system [Miller, 1971; Amarowicz, 1999 a]. A methano-lic solution (0.2 mL) containing 2 mg of crude extract was added to a series of tubes containing 5 mL of an emulsion of linoleic acid and β-carotene stabilized by Tween 40, prepared as described by Amarowicz *et al.* [1999b]. A controlled experiment was carried out using 0.5 mg BHA. Immediately after the addition of the emulsion to tubes, the zero-time absorbance at 470 nm was recorded. Samples were held in a water bath at 50°C and their absorbances read over a 120 min period at 15 min intervals.

The scavenging effect of crude phenolic extracts on 2,2--diphenyl-1-picrylhydazyl radical (DPPH•) was monitored according to the method of Hatano *et al.* [1988]. An aliquot of (0.1 mL) methanolic solution containing 20-100 μg of crude phenolic extract of wild blueberry leaves was mixed with 2 mL of methanol and then added to a methanolic solution of DPPH• (1 mmol/L, 0.25 mL). The mixture was vortexed for 10 s, left to stand at room temperature for 30 min and then its absorbance was recorded at 517 nm.

The reducing power of crude phenolic extracts of wild blueberry leaves was measured as described by Oyaizu [1986]. Briefly, crude extracts (100-500 μg), dissolved in 1 mL of distilled water, were mixed with 2.5 mL of a 0.2 mol/L phosphate buffer (pH 6.6) and 2.5 mL of a 1% (w/v) solution of potassium ferricyanide. The mixture was incubated at 50°C for 20 min. Following this, 2.5 mL of 10% (w/v) trichloroacetic acid were added and the mixture was centrifuged at 1 750 x g for 10 min. A 2.5-mL aliquot of the upper layer was combined with 2.5 mL of distilled water and 0.5 mL of a 0.1% (w/v) $FeCl_3$ solution; the absorbance of the reaction mixture was read at 700 nm.

The antioxidant activity of crude phenolic extracts of wild blueberry leaves was also evaluated using a pork model system; the extent of lipid oxidation was determined according to the 2-thiobarbituric acid reactive substances (TBARS) assay as described by Pegg [2001]. Meat model systems, containing 0 (control), 200, 500 and 1 000 ppm of crude extracts or 50 ppm of TBHQ, were prepared as described by Shahidi and Pegg [1990]. The oxidation of meat lipids was monitored after 1, 3, 5 and 7 days of storage at 4°C. The antioxidant capacity of tested phenolic extracts was expressed as % inhibition of lipid oxidation on day seven using the following general equation:

$$\%\text{Inhibition} = \left(1 - \frac{\textit{Sample absorbance}_t - \textit{TBHQ absorbance}_{t=1}}{\textit{Non additive absorbance}_t - \textit{TBHQ absorbance}_{t=1}}\right) \text{x } 100$$

where, sample absorbance t is the absorbance reading for the TBA-TBARS complex of the test sample at day t, TBHQ absorbance $_{t=1}$ is the absorbance reading for the synthetic antioxidant treated control at day 1, and non additive absorbance $_t$ is the absorbance reading for the control sample containing no additives at day t.

The results presented in Figures 1 to 4 are mean values of at least three experiments (with three replicates per experiment). Statistical analysis of data (analysis of variance (ANOVA) test, t-test) was carried out using SigmaStat v. 2.03 (SSPS Science Inc., Chicago, IL).

RESULTS AND DISCUSSION

The crude phenolic extracts from plant materials are composed of a complex mixture of phenolics differing in the number and arrangement of both hydroxy and methoxy groups on the aromatic rings of phenolic acids, flavonoids and other related compounds, as well as their degree of polymerization. The total content of phenolics was determined using the *Folin-Denis* reagent, which is sensitive to many classes of phenolic compounds. Additionally, the total content of condensed tannins was estimated by the vanillin and proanthocyanidin assays; these protocols are routinely employed for the quantification of condensed tannins. Rice-Evans *et al.* [1996] and Brand-Williams *et al.* [1995] demonstrated that the antioxidant activities of polyphenolic compounds are largely dictated by their molecular structure. This complicates both the determination of the antioxidant activity for complex mixtures of phenolics such as plant extracts and the interpretation of experimental data. Consequently, evaluation of the antioxidant activity of plant phenolic extracts is limited to an estimation of the total antioxidant activity of the system. Numerous methods have been proposed to evaluate/estimate the antioxidant potential of natural sources of antioxidants [Shahidi & Naczk, 2003]. Of these, the β-carotene-linoleate model system, the meat model system as well as the DPPH• and reducing power assays are commonly employed.

Due to a lack of appropriate standards, the total phenolics content in the extracts of wild blueberry leaves was expressed as absorbance units/g (A/g) of extracts. The total phenolics content in ethanolic extracts was slightly higher (2 893±31 A/g) than that in the acetone extract (2 779±41 A/g). On the other hand, a higher level of condensed tannins was detected in the acetone extract (213.4±3.2) compared with the ethanolic preparation (180.6±3.1).

The effect of crude phenolic extracts of wild blueberry leaves on the coupled oxidation of β-carotene and linoleic acid was compared to that of BHA (Figure 1). The antioxidant activity of crude phenolic extracts was somewhat lower than that displayed by BHA. However, acetone extracts of blueberry leaves phenolics were more effective ($p<0.05$) compared to the corresponding ethanolic extracts; a higher level of proanthocyanidins, which are more soluble in acetone, may have brought about the stronger antioxidant activity observed for acetone extracts. More research is needed to assess the contribution of tannin and non-tannin fractions of phenolics to the observed antioxidant activity of the prepared extracts from blueberry leaves.

The DPPH• assay is commonly employed to evaluate the ability of antioxidants to scavenge free radicals. The change in absorbance at 517 nm is used as a measure of the scavenging effect of a particular extract for DPPH radicals. The absorbance at 517 nm decreases as the reaction between antioxidant molecules and DPPH• progresses. The scavenging effect of antioxidants is influenced by their concentration and the type of radical in question as well as the molecular structure and kinetic behavior of the phenolics involved. Amarowicz *et al.* [2003] pointed out, however, that caution must be exercised when interpreting such results. The reactions that DPPH• can undergo are not simple and straightforward, and much is

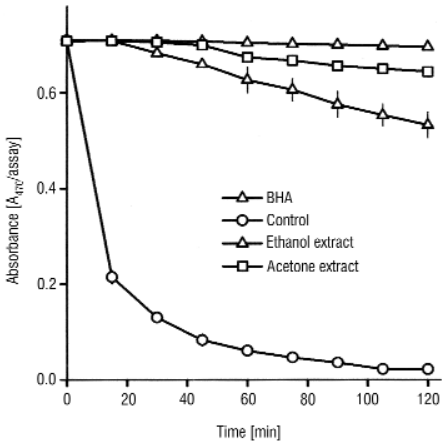

FIGURE 1. Antioxidant activity of crude phenolic extracts of wild blueberry leaves in a β-carotene-linoleate model system, as measured by changes in the absorbance at 470 nm.

still to be learned about its chemistry. Moreover, one cannot arbitrarily assume that the decrease in absorbance at the 517 nm absorption maximum is solely attributed to the antioxidant donating a hydrogen atom or an electron to DPPH•. Figure 2 shows the scavenging effect of crude phenolic extracts from wild blueberry leaves. No statistically significant difference ($p>0.05$) was noted between the crude phenolic extracts prepared using aqueous acetone and ethanol. The scavenging effect of extracts, at 100 μg/assay, on DPPH• was over 90%. The strong free--radical scavenging capacities displayed by crude phenolic extracts of wild blueberry leaves suggest that these extracts may also possess a strong antimutagenic activity. Hochstein and Atallah [1988] associated the ability of antioxidants to scavenge free radicals with their antimutagenic activity.

Figure 3 depicts the reducing power of crude phenolic extracts of wild blueberry leaves as a function of concentration. Crude phenolic extracts of blueberry leaves displayed a considerable reducing power, primarily due to their effect as electron donors and thereby halting radical chain reactions by converting free radicals to more stable products. No statistically significant ($p>0.05$) difference in reducing power among the crude phenolic extracts from the extraction solvents employed was noted. The reducing power of blueberry leaves extracts was similar to those reported by Amarowicz *et al.* [1999b, 2000] for crude phenolic extracts of canola hulls and ethanolic extracts of evening primrose seeds.

Figure 4 shows the inhibitory effect of crude phenolic extracts of blueberry leaves on lipid oxidation of a meat model system as displayed by their content of TBARS. The results indicate that extracts added at 500- and 1000-ppm levels exhibited an antioxidant activity comparable to that of the TBHQ sample. These addition levels correspond to approximately 100 and 200 ppm of proanthocyanidins in the extracts. No statistically significant difference ($p>0.05$) was found between antioxidant activities displayed by the

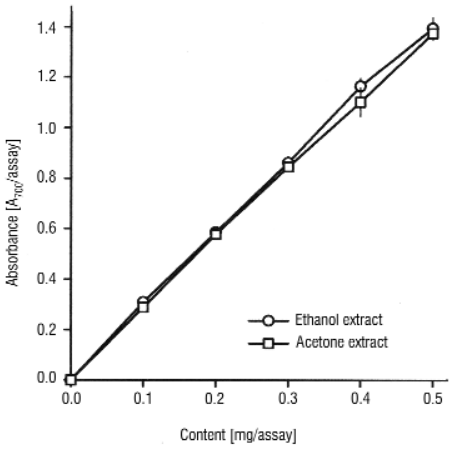

FIGURE 3. Reducing power of crude phenolic extracts of wild blueberry leaves, as measured by changes in absorbance at 700 nm.

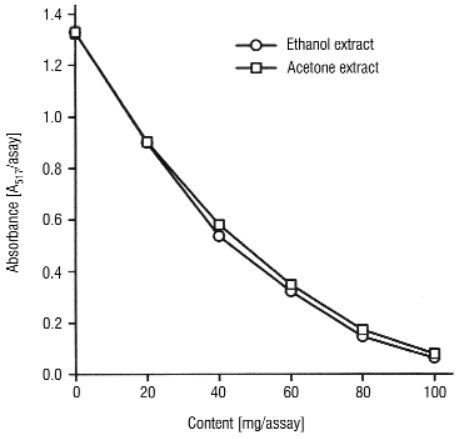

FIGURE 2. Scavenging effect of crude phenolic extracts from wild blueberry leaves on the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•), as measured by changes in absorbance at 517 nm.

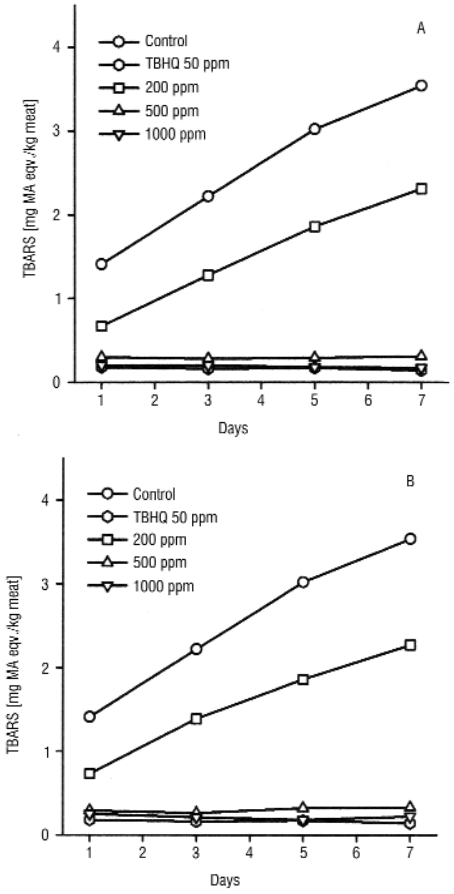

FIGURE 4. Antioxidant activity of crude phenolic extracts of wild blueberry leaves in pork model systems, as measured by changes in the content of TBARS (results expressed as mg malonaldehye equivalents/kg meat). A: ethanol extract; B: acetone extract.

ethanolic and acetone extracts. The antioxidant activity resulting from a 500-ppm addition of extract to pork, expressed as % inhibition of meat lipid oxidation, ranged from 95.5% for the acetone to 100% for the ethanol preparations over the seven day refrigerated storage period. Tang *et al.* [2001] reported that addition of tea catechins at a concentration of 300 mg/kg minced muscle reduced lipid oxidation by above 55%. Tea catechins were also reported to function as potential antioxidant in pork [Shahidi & Alexander, 1998]. Application of the bearberry (*Arctostaphylos uva-ursi*) extract at a 500 ppm addition level protected the meat against lipid to a similar extent as that of TBHQ [Amarowicz *et al.*, 2001].

The antioxidant action of the blueberry leaves extracts may be ascribed to their free-radical scavenging capacity as well as their chelation of metal ions in the presence of ortho-, dihydroxy- and ketol subsistents.

CONCLUSIONS

Crude extracts of phenolics of wild blueberry leaves exhibited strong antioxidant properties. Ethanol was found to be an effective solvent at extracting potential antioxidant compounds from wild blueberry leaves. Further research is needed to evaluate the antioxidant activity of various phenolic fractions of these extracts and to isolate and identify the most active components.

ACKNOWLEDGEMENT

Marian Naczk thanks the Natural Sciences and Engineering Research Council (NSERC) of Canada for financial support in the form of a discovery grant.

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