Inhibition of Advanced Glycation End Product Formation by Medicinal Plant Extracts Correlates with Phenolic Metabolites and Antioxidant Activity

Authors Cory S. Harris[1,2,3], Louis-Philippe Beaulieu[1,3], Marie-Hélène Fraser[3,4,5], Kristina L. McIntyre[1,3], Patrick L. Owen[3,7], Louis C. Martineau[3,6], Alain Cuerrier[3,4], Timothy Johns[3,5,7], Pierre S. Haddad[3,6], Steffany A.L. Bennett[2,3], John T. Arnason[1,3]

Affiliations The affiliations are listed at the end of the article

Key words
- advanced glycation end products
- diabetes
- diabetic complications
- plant phenolics
- oxidative stress
- antioxidants

Abstract

Nonenzymatic formation of advanced glycation end products (AGEs) is accelerated under hyperglycemic conditions characteristic of type 2 diabetes mellitus and contributes to the development of vascular complications. As such, inhibition of AGE formation represents a potential therapeutic target for the prevention and treatment of diabetic complications. In the present study, ethanolic extracts of 17 medicinal plants were assessed for inhibitory effects on *in vitro* AGE formation through fluorometric and immunochemical detection of fluorescent AGEs and N^{ε}-(carboxymethyl)lysine adducts of albumin (CML-BSA), respectively. Most extracts inhibited fluorescent AGE formation with IC_{50} values ranging from 0.4 to 38.6 µg/mL and all extracts reduced CML-BSA formation but to differing degrees. Results obtained through both methods were highly correlated. Antiglycation activities were positively correlated with total phenolic content, free radical scavenging activity and reduction in malonyldiadehyde levels following oxidation of low-density lipoprotein, but negatively correlated with lag time to formation of conjugated dienes. Together, these results provide evidence that antioxidant phenolic metabolites mediate the antiglycation activity of our medicinal plant collection, a relationship that likely extends to other medicinal and food plants.

Introduction

Macro- and microvascular complications of diabetes, such as coronary heart disease, stroke, nephropathy, neuropathy, retinopathy, cataract development and atherosclerosis, are major causes of morbidity and mortality among patients with type 2 diabetes mellitus [1]. Elevated levels of advanced glycation end products (AGEs) in diabetic patients contribute to the development of many of these complications and represent a promising therapeutic target for the management of vascular complications. AGEs are formed through nonenzymatic reactions between proteins, nucleic acids, or lipids and reducing carbohydrates such as glucose and fructose. AGEs accumulate more readily in diabetics as hyperglycemia and oxidative stress promote glycation reactions that impair protein function and clearance, often leading to cell and tissue damage [2,3]. Certain AGEs, such as N^{ε}-(carboxymethyl)lysine (CML), activate the cell surface AGE receptor (RAGE) that, in turn, activates NF-κB and related proinflammatory pathways implicated in the initiation and progression of diabetic vascular damage [2,4].

As the global prevalence of type 2 diabetes continues to rise, it remains consistently higher among many of the world's indigenous populations. In Canada, First Nations men and women experience 3.6 and 5.3 times higher rates of diabetes than nonnative Canadians, respectively [5]. Because access to medical services, daily monitoring of blood-glucose levels and/or compliance with modern management regimens are limited or lower in First Nations and other aboriginal communities [6,7], uncontrolled hyperglycemia increases the incidence and severity of diabetic complications [8,9]. Since oxidative processes are implicated in both the formation and toxicity of AGEs, dietary antioxidants could play an important role in mitigating both processes. Repeatedly identified as effective antioxidants and inhibitors of AGE formation [10], plant phenolics, which are widely distributed among vascular plants, are of particular interest. The wealth of food and medicinal plants used in aboriginal and popular cultures worldwide offers a unique opportunity to

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Correspondence
Dr. John T. Arnason
Department of Biology
Centre for Research in Biopharmaceuticals and Biotechnology
University of Ottawa
30 Marie Curie
Ottawa, Ontario
Canada K1N 6N5
Phone: + 1 613 562 52 62
Fax: + 1 613 562 57 65
John.Arnason@uOttawa.ca

provide management options that are pharmacologically, culturally and economically relevant to diverse at-risk populations.

Since 2003, the Canadian Institutes of Health Research Team in Aboriginal Anti-Diabetic Medicines, a collaboration among basic researchers, health professionals and the Cree of Eeyou Istchee of Northern Quebec, has investigated numerous locally used plant medicines for treating symptoms of diabetes [11–14] in an array of experimental models. Recently, we described the inhibitory effect of mountain cranberry (*Vaccinium vitis-idaea* L.) extract on AGE formation and, based on phytochemical analysis, identified several phenolic metabolites that effectively inhibited formation of AGEs [15]. Here, employing complementary *in vitro* assays, we extend this study to a collection of 17 medicinal plant extracts and explore the potential relationships between observed antiglycation effects, phenolic metabolites and antioxidant activities.

Materials and Methods

Plant materials and extraction

Seventeen medicinal plant species were collected in the regions of Mistissini and Whapmagoostui, Québec, Canada, in accordance with instructions provided by Elders. The most commonly used organ(s) of the most frequently cited antidiabetic plant species were selected for analysis [14]. Dr. Alain Cuerrier ascertained botanical identity of the plant species and voucher specimens were deposited in the Marie-Victorin Herbarium of the Montréal Botanical Garden in Montréal, Canada. Dried plant samples were processed and extracted twice with 80% ethanol (10 mL/g dry material) as described previously [12]. Dried extracts were stored in darkness at 4 °C.

Sample preparation

For AGE formation assays, extracts were dissolved and serially diluted in 80% ethanol for testing on the day of experimentation. Pure compounds were prepared in the same manner; caffeic acid, gallic acid, taxifolin, quercetin, quercetrin, and rutin were purchased from Sigma, chlorogenic acid, catechin and myricetin from Extrasynthase: procyanidins B1 and B2 were graciously provided by Dr. Ferreira (University of Mississippi), and 24-hydroxybetulinic acid and morronoside were isolated in house from *Sorbus decora* and *Sarracenia purpurea*, respectively. Purity of purchased standards and isolated compounds were greater than 95% and 90%, respectively, as determined by HPLC analysis. Extracts and controls, including Trolox (Sigma, 97% purity), for the oxygen radical absorbance capacity (ORAC) assay were prepared in 50% (v/v) MeOH/PBS.

AGE formation assay

AGE formation was assessed as previously described [15, 16] with the following modifications. Bovine serum albumin (BSA, Sigma) (1 mg/mL) was incubated with 100 mM glucose/100 mM fructose (BDH Chemicals, Ltd.) in 100 mM sodium phosphate monobasic monohydrate buffer (pH 7.4) with 80% ethanol vehicle (negative control), with extract or compound (experimental treatment), or with quercetin (positive control). Replicates of each treatment without BSA were included to control for analyte autofluorescence and replicates with BSA and vehicle (no sugar) was included to control for the fluorescence of BSA and as a negative control for immunochemical assessments (BSA control). Multiple concentrations of each extract (0.39–200 µg/mL) or compound (0.05–50 µg/mL) were tested in quadruplicate in sterile opaque polystyrene 96-well clear bottom plates (Corning, Inc.). Plates were covered, sealed with parafilm, and incubated for 7 days at 37 °C in darkness on a mechanical shaker (Series 25 Incubator Shaker, New Brunswick Scientific Co., Inc.).

Fluorescence-based quantitation of AGE formation

Following incubation, fluorescence was measured using a microplate reader (SpectraMax M5, Molecular Devices) at excitation and emission wavelengths of 355 nm and 460 nm, respectively. Plates were then stored at − 20 °C until immunochemical analyses. Fluorescence readings for control and experimental treatments were blanked against the BSA and appropriate treatment controls. The corrected fluorescence readings (F) for the negative control ($F_{negative}$) and experimental treatments ($F_{experimental}$) were used to determine the percent inhibition of AGE formation as follows: % inhibition = $[(F_{negative} - F_{experimental}) / (F_{negative})] \times 100\%$. IC_{50} values were defined as the amount of extract (µg/mL) or compound (µM) required to reduce AGE formation by 50% relative to the negative control as determined by regression analysis [% inhibition vs. log (concentration), n = 3–4].

Immunochemical detection of CML-BSA adducts

Protein samples collected from control and experimental treatments were first concentrated using Ultracel YM-10 membrane centrifugal filters (Millipore) then quantified using a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Ltd.) according to manufacturer instructions. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Western blot analyses were performed using mouse monoclonal antibodies targeted against CML-BSA adducts (1 : 1000, Clone 318 003, R & D Systems) detected using goat anti-mouse IgG polyclonal antibodies conjugated with horseradish peroxidase (1 : 1000, R & D Systems). CML-BSA adducts were visualized with SuperSignal West Pico (MJS BioLynx, Inc.). Proteins treated with multiple concentrations of a single extract were initially analyzed to observe concentration-dependent effects. In these experiments, a duplicate gel stained with Coomassie Blue following electrophoresis served as a loading control. For quantitative comparison, samples treated with different extracts at the same concentration (6.25 µg/mL) were analyzed simultaneously (11 samples per gel, n = 4–6). Immunoreactive bands were quantitated by image densitometry (ImageJ software v. 1.38X, National Institutes of Health). Membranes were reversibly stained with Ponceau S and electronically scanned to accurately control for protein loading. Percent inhibition was calculated relative to the negative control (vehicle alone).

Assessment of total phenolic content

Total phenolic content (TPH) was measured by the Folin-Ciocalteau method [17] with modifications as previously described [12]. Extracts in 80% ethanol were combined with freshly diluted Folin-Ciocalteau reagent (BDH) allowing 5 min for the reaction to equilibrate before adding 7.5% anhydrous $NaHCO_3$ solution. After an additional 2 h of incubation at room temperature, absorbance was measured at 725 nm with a SpectraMax M5 microplate reader. TPH was calculated relative to a quercetin standard and expressed as quercetin equivalents.

Oxygen radical absorbance capacity (ORAC) assay

ORAC was measured using a microplate-based assay developed by Gillespie et al. [18]. Briefly, 0.08 µM fluorescein was added to 75 mM PBS (blank), Trolox standard (6.25–50 µM) or plant ex-

tract (0–25 mg/mL) in 96-well black microplates. After equilibration at 37 °C for 10 minutes, a 150 mM solution of 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) was added to each well. Fluorescence was subsequently monitored every three minutes for 90 minutes at excitation and emission wavelengths of 485 nm/530 nm. Final ORAC values were calculated as µM Trolox equivalents (TE) at 1 µg/mL (extracts) or 1 µM (controls) and expressed as mean ± SEM (n = 3–4).

Other antioxidant assays

We extracted and summarized data from our previous study on the antioxidant activity of a larger collection of boreal plants that included the same set of Cree extracts used herein [11]. Free radical scavenging activity was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical assay as described previously [11, 19]. Different concentrations of each extract, quercetin or ascorbic acid (positive controls) were combined with 100 µM DPPH and allowed to stand at room temperature for 10 min before measuring absorbance at 517 nm using a Beckman DU 640 spectrophotometer. IC_{50} values were calculated from the linear portion of the response curve for ascorbic acid (n > 3).

Lag time before the appearance of conjugated dienes on low-density lipoprotein (LDL) was determined following Cu^{2+}-mediated oxidation as described by Esterbauer et al. [20]. $CuSO_4$ (15 µM), LDL (100 µg/mL) and plant extract (5 µg/mL), Trolox (20 µM, positive control) or vehicle were mixed in 1 mM PBS and continuously monitored for absorbance at 234 nm for up to 15 h with a µQuant universal microplate spectrotometer (Bio-Tek Instruments, Inc.). Lag time was calculated as the intersection of baseline absorbance with the propagation phase. Malonyldialdehyde end products of lipid peroxidation were quantified using the thiobarbituric acid-reactive substances (TBARS) assay according to established methods [21]. Combined extract (5 µg/mL) or control (Trolox, 20 µM) and LDL (100 µg/mL) in 1 mM PBS was incubated for 1 h at room temperature before initiating oxidation by adding $CuSO_4$ and subsequently sampling after 4 hours for TBARS analysis [11].

Statistical analyses

IC_{50} calculations and Spearman nonparametric correlation analyses employed to test associations between antiglycation activity, ORAC results and previously reported experimental data were performed using GraphPad InStat (version 3).

Results

The current study evaluated the antiglycation potential of 17 medicinal plant extracts used by the Cree of Eeyou Istchee to treat symptoms of diabetes (**Table 1**). Each extract has been analyzed phytochemically, as described in separate published [15, 22–24] and forthcoming reports, with more than 50 different phenolic and nonphenolic compounds identified within the collection to date. Total phenolic content (TPH) ranged from 14–318 mg of quercetin equivalents/g extract (data summarized in **Table 1**).

All extracts reduced AGE formation as determined by fluorometry, immunochemistry, or both. Fourteen extracts inhibited the production of fluorescent AGEs with data allowing for IC_{50} determination for 13 extracts (**Table 2**). Representative responses are illustrated in **Fig. 1**. Quercetin, a known AGE inhibitor, served as a positive control (**Fig. 1A**). *V. vitis-idaea* extract (**Fig. 1B**), along with five others *(A. balsamea, J. communis, P. banksiana, R. tomentosum, S. planifolia)* exhibited concentration-dependent inhibition of fluorescent AGE formation. Seven extracts *(G. hispidula, K. angustifolia, P. glauca, P. mariana, P. balsamifera, R. groenlandicum, S. purpurea)* demonstrated biphasic activity with concentration-dependent inhibition at low but not high concentrations (**Fig. 1C**). Although a similar response was seen with *L. laricina*, no IC_{50} was established as maximum inhibition did not reach 50% (data not shown). Like the *L. clavatum* extract (**Fig. 1D**), *S. decora* had no effect on antiglycation activity in this assay.

Immunochemical detection of CML-BSA adducts was conducted on protein purified from control and experimental treatments and separated by SDS-PAGE. Two major CML-BSA adducts were detected by the monoclonal antibody, a 66 kD product representing CML-BSA and a higher molecular weight band corresponding to highly aggregated CML-reactive products (**Fig. 2**). Control reactions included incubation of BSA alone, which yielded no CML products, and with glucose and fructose in the presence of quercetin (16 µM), which resulted in significantly reduced CML-BSA adduction (**Fig. 2A**). All extracts inhibited CML-BSA adduct formation in a concentration-dependent manner (see **Fig. 2** for representative responses). As shown for *L. clavatum* (**Fig. 2D**), extracts that showed little effect on fluorescent AGE formation reduced CML-BSA levels albeit less effectively than most other extracts.

To quantitatively compare the effects of different extracts on CML production, protein from samples treated with 6.25 µg/mL of each extract were evaluated concomitantly (**Fig. 3A**). Inhibition of CML-BSA adduct formation ranged from 20% for *L. clavatum* to 87% for *S. planifolia* (**Table 2**) and was highly correlated with results from the fluorescence assay (**Fig. 3B**).

Because free radicals and oxidative reactions can accelerate glycation, the radical scavenging and antioxidant properties of each extract were next considered. A broad range of ORAC values was observed with a 40-fold difference in TE between the most active extract, *K. angustifolia,* and the least, *J. communis* (**Table 3**). The different leaf and bark extracts displayed variable activity but were generally more active than fruit (*J. communis* and *V. vitis-idaea*) or cone (*P. mariana* and *P. banksiana*) extracts. In addition to ORAC data, previously determined antioxidant activities summarized in **Table 3** [11] were included in correlation analyses.

Spearman nonparametric correlation analysis was used to investigate potential relationships between observed antiglycation activities and the phenolic content or antioxidant properties of individual extracts. IC_{50} values obtained by fluorometry and % inhibition obtained by immunochemistry were considered relative to TPH, ORAC values, DPPH scavenging activity, and the ability to 1) delay the appearance of conjugated dienes, and 2) prevent the production of TBARS following Cu^{2+} oxidation of human low-density lipoprotein (LDL). TPH correlated positively with both measures of AGE inhibition as phenolic-rich extracts generally exhibited stronger inhibitory effects (**Fig. 4A**, **Table 4**). Positive correlations were also observed between antiglycation activities and ORAC, DPPH and TBARS data (**Fig. 4**, **Table 4**). In contrast, the antiglycation activity was negatively correlated with the delay of conjugated diene formation following LDL oxidation (**Fig. 4**, **Table 4**). The same pattern was observed between TPH and antioxidant activities; phenolic content correlated positively with all measures save conjugated diene formation.

Given the apparent role of phenolic metabolites in the antiglycation activity of Cree medicinal plants, 11 phenolic compounds representative of those found in the extracts were individually

Table 1 Plant species used by the Cree for the treatment of diabetic symptoms*.

Species (Family)	Family	Common name	Cree name	Voucher number	Plant part	Identified compounds	TPH (rank)[a]
Abies balsamea (L.) Mill.	Pinaceae	Balsam fir	Inaast	Mis 03-1	Bark	Gallocatechin derivatives	12
Alnus incana subsp. rugosa (Du Roi) R. T. Clausen	Betulaceae	Speckled alder	Atuuspiih	Mis 03-11	Bark	Oregonins, rubranoside A & B, hirsutanone	2
Gaultheria hispidula (L.) Muhl.	Ericaceae	Creeping snowberry	Piyeumanaan	Mis 03-7	Leaf	Chlorogenic acid, (−)-catechin, (+)-epicatechin, taxifolin glycoside, myricitrin, quercetin glycosides	9
Juniperus communis L.	Cupressaceae	Ground juniper	Kaakaachuminatukw	Whap 04-6	Cone	(−)-Catechin, 2 kaempferol glycosides, quercetin glycosides	11
Kalmia angustifolia L.	Ericaceae	Sheep laurel	Uischichipukw	Mis 03-12	Leaf	(+)-Catechin, (−)-epicatechin, myricetin, quercetin glycosides	3
Larix laricina Du Roi (K. Koch)	Pinaceae	Tamarack	Waatinaakan	Mis 03-47	Bark	(+)-Catechin, (−)-epicatechin, 2 diterpenes, resin acids, piceatannol hydroxystilbenes	5
Lycopodium clavatum L.	Lycopodiaeae	Common clubmoss	Pastinaakwaakin	Mis 03-43	Whole plant	Ferulic acid derivatives, apigenin derivatives	17
Picea glauca (Moench.) Voss	Pinaceae	White spruce	Miinhikw	Whap 04-12	Needle	(+)-Catechin, taxifolin, kaempferol, quercetin and isorhamnetin glycosides, hydroxystilbenes, lycopodine	10
Picea mariana (P. Mill) BSP	Pinaceae	Black spruce	Iinaatikw	Mis 03-15	Cone	Pungenin, hydroxy- and hydroxymethoxystilbenes	7
Pinus banksiana Lamb.	Pinaceae	Jack pine	Uschisk	Mis 03-14	Cone	(+)-Catechin, procyanidin B, taxifolin	1
Populus balsamifera L.	Salicaceae	Balsam poplar	Mash-mitush	Mis 03-49	Bark	Salicin, salicortin, salireposide, populoside, rubranoside	13
Rhododendron groenlandicum (Oeder) Kron & Judd	Ericaceae	Labrador tea	Kaachepukw	Mis 03-2	Leaf	Chlorogenic acid, (+)-catechin, (−)-epicatechin, procyanidins B1, B2, B3, quercetin glycosides, *p*-coumaric acid	6
Rhododendron tomentosum ssp. *subarcticum* (Harjama) G. Wallace	Ericaceae	Northern labrador tea	Wiisichipukw	Whap 04-33	Leaf	Chlorogenic acid, *p*-coumaric acid, caffeic acid derivatives, (+)-catechin, (−)-epicatechin, taxifolin glycoside, quercetin glycosides, procyanidins B1, B2, B3, myricetin	8
Salix planifolia Pursh	Salicaceae	Tealeaf willow	Piyeuwaatikw	Whap 03-37	Bark	Salicin, isosalireposide derivatives, tremulacin	4
Sarracenia purpurea L.	Sarraceniaceae	Pitcher plant	Aygadash	Mis 03-5	Leaf	Kaempferol, quercetin and cyanidin glycosides, goodyeroside, morronoside	14
Sorbus decora (Sarg.) C. K. Schneid.	Rosaceae	Showy mountain ash	Muskumanaatikw	Mis 03-10	Bark	(+)-Catechin, (−)-epicatechin, uvaol, 24-hydroxyuvaol, beta-amyrin, betulin, 24-hydroxybetulin, betulinic acid	15.5
Vaccinium vitis-idaea L.	Ericaceae	Mountain cranberry	Wiishichimanaanh	Whap 04-20	Fruit	Benzoic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, *p*-coumaroyl-D-glucose, (+)-catechin, quercetin and cyanidin glycosides	15.5

* Based on ethnobotanical interviews detailed in Leduc et al. [13] and Fraser et al. [11]; [a] Total phenolic content ranked according to quercetin equivalents [12, 14]

assessed for inhibitory effects on fluorescent AGE formation. All proved effective with IC_{50} values in the low µM range, including simple phenolic acid derivatives, flavonoids and procyanidins (**Table 5**). Two nonphenolic metabolites, a terpene (24-hydroxybetulin) isolated from *S. decora* and an iridoid (morronoside) from *S. purpurea* displayed no activity (data not shown).

Discussion

Ethanolic extracts of 17 medicinal plants were evaluated for their inhibitory effects on *in vitro* formation of AGEs, which have been implicated in the pathophysiology of macro- and microvascular complications of diabetes as well as other age-related cardiovascular and neurodegenerative conditions [25, 26]. Given the abundance of phenolic metabolites identified within the various extracts (**Table 1**) and the known antiglycation activities of certain plant phenolics [10, 27], it was not surprising that the majority of extracts were effective AGE inhibitors. The observed potency of many extracts, however, was superior to other reports [28]. Our results demonstrate that phenolic-rich plant extracts are capable of directly preventing AGE formation in aqueous environments such as the blood, where glycation of long-lived proteins and other macromolecules contribute to vascular damage and the development of diabetic complications. In many cases, substantial inhibition was observed at concentrations of physiological relevance; IC_{50} values for fluorescent AGEs were below 2.5 µg/mL for six extracts while 12 of the 17 extracts inhibited

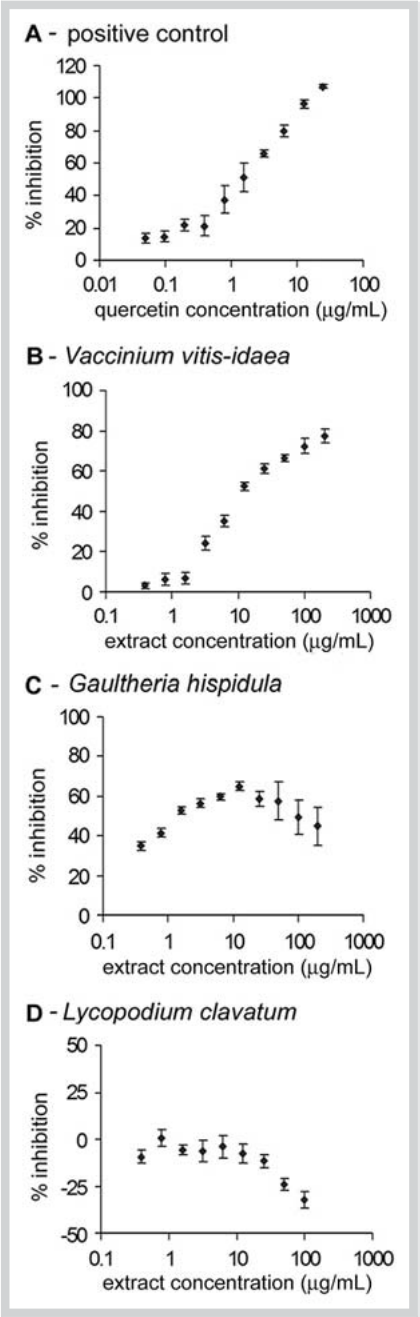

Fig. 1 Concentration-dependent effects of representative Cree medicinal plant extracts on *in vitro* formation of fluorescent AGEs. Results are expressed as the percent inhibition relative to negative control versus the log concentrations of (**A**) the positive control quercetin or extracts of (**B**) *Vaccinium vitis-idaea*, (**C**) *Gaultheria hispidula,* and (**D**) *Lycopodium clavatum* (means ± SEM, n = 3–4). Where applicable, a logarithmic regression was fitted to the data with IC_{50} values calculated from the corresponding regression equation. IC_{50} was defined as the amount of extract (µg/mL) or control (µM) required to reduce AGE formation by 50% relative to the negative control (maximum glycation). Note that the x-axis does not always intersect the y-axis at zero.

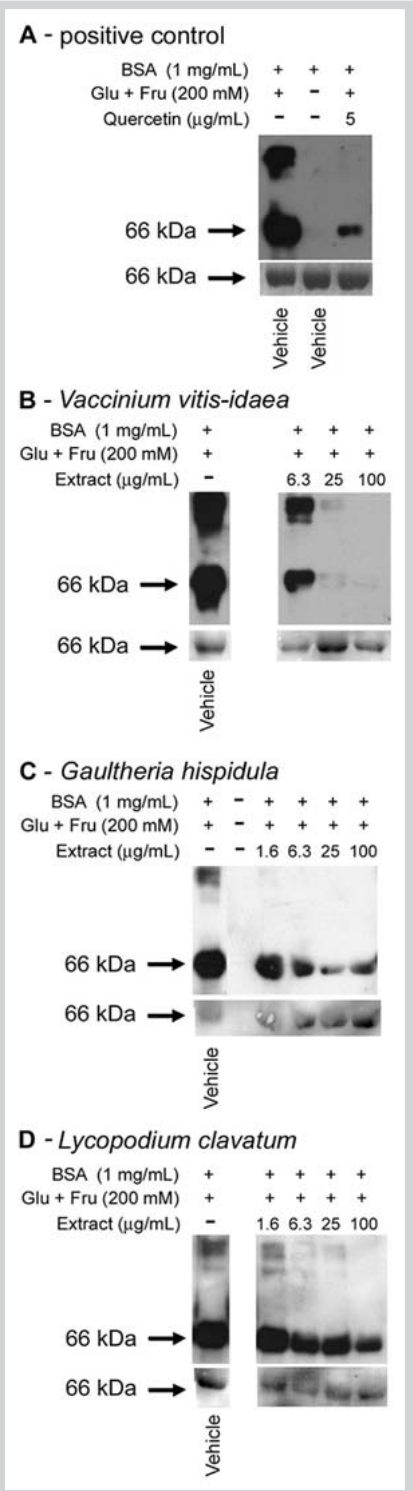

Fig. 2 Concentration-dependent effects of representative Cree medicinal plant extracts on *in vitro* formation of N^{ε}-(carboxymethyl)lysine-protein adducts. Western blot analyses using CML-specific antibodies were conducted on proteins incubated for 7 days with BSA, glucose/fructose, vehicle and (**A**) the positive control quercetin (16 µM), or various concentrations of (**B**) *Vaccinium vitis-idaea*, (**C**) *Gaultheria hispidula* and (**D**) *Lycopodium clavatum* extract. BSA incubated without sugar was used as a negative control for the CML-BSA antibody. Two simultaneous SDS-PAGE separations were performed, in which 1.9 µg of protein was loaded to both. The first gel was used for Western blotting with a primary monoclonal anti-CML-BSA antibody (upper panels) and the second was used to verify equal protein loading with Coomassie Blue staining (lower panels).

CML production by > 50% at 6.25 µg/mL (**Table 2**). Due to limited absorption and/or rapid metabolic modification, the bioavailability of individual phenolics consumed in the diet is generally in the nM to low µM range [29–31]. Though the bioavailability of phenolics from the specific extracts have yet to be determined, their abundance in certain samples suggests that the cumulative concentration of phenolics (and metabolic products) with antiglycation activity likely provides some degree of protection against endogenous AGE formation.

Importantly, inhibitory effects were not limited to fluorescent AGEs, which account for only a small percentage of physiologically occurring cross-linking structures, but extend to CML and possibly other nonfluorescent AGEs implicated in the pathophysiology of diabetic complications [32]. Though results from the two assays were strongly correlated, some differences were observed. These differences may reflect selective inhibition of different classes of AGEs, depending on their mechanism of formation (e.g. *S. purpurea* and *S. decora* appear to exhibit some specificity toward inhibiting CML formation). Alternatively, the production of fluorescent non-AGE structures formed between plant metabolites and sugar, BSA or their breakdown products may have complicated fluorescence readings and contributed to the observed discrepancies between assays.

As predicted, the antiglycation activity of extracts correlated positively with total phenolic content and three of four antioxidant scores. Negative correlations were unexpectedly identified with

Table 2 Summary of the antiglycation activities of Cree medicinal plant extracts and their relative rankings.

	Fluorescent AGE formation		CML formation[b]	
	IC_{50} (μg/mL)[a]	Rank	% inhibition	Rank
Quercetin	1.8 ± 0.7	control	85.8 ± 3.7	control
A. balsamea	34.2 ± 12.5	12	38.3 ± 8.0	15
A. incana	N/A	–	72.3 ± 6.4	4.5
G. hispidula	1.5 ± 0.2	4	67.8 ± 5.7	6.5
J. communis	5.4 ± 1.6	7	61.7 ± 4.7	11
K. angustifolia	1.5 ± 0.5	4	72.3 ± 4.6	4.5
L. laricina	N/A	–	48.3 ± 5.4	13
L. clavatum	Inactive	14.5	20.2 ± 7.5	17
P. glauca	6.2 ± 0.6	8	65.9 ± 5.0	8
P. mariana	2.1 ± 0.2	6	64.5 ± 7.9	9
P. banksiana	1.5 ± 0.2	4	76.2 ± 7.8	3
P. balsamifera	21.9 ± 9.7	11	48.0 ± 11.4	14
R. groenlandicum	7.0 ± 1.7	9	55.7 ± 5.3	12
R. tomentosum	1.2 ± 0.4	2	82.0 ± 2.3	2
S. planifolia	0.4 ± 0.2	1	86.9 ± 1.5	1
S. purpurea	38.6 ± 1.9	13	67.8 ± 1.7	6.5
S. decora	Inactive	14.5	63.5 ± 1.8	10
V. vitis-idaea	10.8 ± 2.1	10	32.8 ± 5.2	16

CML = Nε-(carboxymethyl)lysine; N/A = not applicable; [a] IC_{50} concentrations ± SEM were calculated as the amount of extract in μg/mL required to reduce AGE formation by 50% relative to the negative control (maximum glycation) as determined by regression analysis (n = 3–4); [b] % inhibition ± SEM of CML-BSA adduct formation was determined by densitometry of Western blot analyses of purified protein following treatment with sugar, vehicle or 6.25 μg extract/mL (n = 4–6)

Table 3 Summary of the antioxidant activities of Cree medicinal plant extracts and their relative rankings.

Controls	ORAC		DPPH[b]	CD[c]	TBARS[d]
	Trolox equivalents[a]		IC_{50}	Lag time	4 hour
Quercetin	100.7 ± 4.5		13.1 ± 0.6		
Trolox	1			+ 27%	− 76%
Species		Rank	Rank	Rank	Rank
A. balsamea	38.3 ± 8.3	11	11	5	12
A. incana	73.3 ± 13.8	6	3	16	2
G. hispidula	72.9 ± 1.6	7	9	17	10
J. communis	3.4 ± 0.3	17	12	10	13
K. angustifolia	140.5 ± 13.5	1	5	14	6.5
L. laricina	101.8 ± 12.6	2	13	1	14
L. clavatum	4.8 ± 0.4	16	17	2	16
P. glauca	60.6 ± 12.3	10	8	4	8
P. mariana	22.4 ± 2.5	13	2	15	3
P. banksiana	68.6 ± 6.3	8	1	13	1
P. balsamifera	64.7 ± 6.5	9	15	7	15
R. groenlandicum	91.5 ± 7.5	4	6	11	5
R. tomentosum	92.0 ± 11.1	3	7	8	6.5
S. planifolia	87.8 ± 3.2	5	4	9	4
S. purpurea	21.4 ± 2.4	14	10	12	9
S. decora	20.2 ± 1.0	15	14	3	11
V. vitis-idaea	30.7 ± 6.1	12	16	6	17

CD = conjugated dienes; DPPH = 1,1-diphenyl-2-picrylhydrazyl radical; ORAC = oxygen radical absorbance capacity: TBARS = thiobarbituric acid-reactive substances; n/d = not determined; [a] Reported in μM Trolox equivalents at a concentration of 1 μg/mL (extract) or 1 μM (control); [b] IC_{50} value for quercetin expressed in mM. Ranking summarized from data presented in [11]; [c] Lag time for the appearance of conjugated dienes following Cu^{2+}-initiated oxidation of human low-density lipoprotein. Ranking summarized from data presented in Fraser et al. [11]; [d] Accumulation of TBARS 4 hours following Cu^{2+}-initiated oxidation of human low-density lipoprotein. Ranking summarized from data presented in Fraser et al. [11]

regard to conjugated diene formation as extracts of high phenolic content and strong activity in the DPPH and TBARS assays generally accelerated the appearance of conjugated dienes in oxidized LDL. Conjugated dienes represent the first stage of lipid peroxidation while TBARS, specifically malonyldialdehyde, represent final products of the same oxidative process. Despite shorter lag times before the appearance of conjugated dienes, extracts that strongly inhibited glycation also effectively reduced TBARS indicating that peroxidation did not progress through the propagation phase and suggesting that phenolic metabolites likely act as chain-breaking antioxidants downstream of conjugated diene formation. The use of Cu^{2+} to initiate LDL oxidation alongside extracts rich in chelating agents, such as plant phenolics, likely contributed to the negative correlations [33]. Initially, phenolic che-

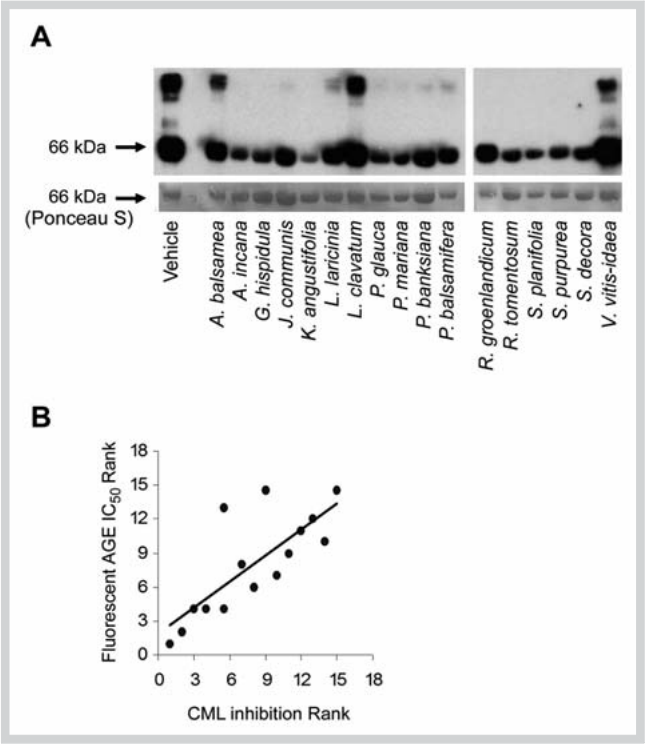

Fig. 3 Quantitative comparison of CML-BSA adduct formation after exposure to a standard concentration of each plant extract. **A** The effect of different extracts on the production of CML was compared immunochemically by simultaneously running protein from samples treated with each extract (6.25 µg/mL) and calculating % inhibition by densitometry of immunoreactive bands relative to the vehicle-treated control (maximum CML formation). Reversible Ponceau S staining applied prior to incubation with primary antibodies served as loading controls. Percent inhibition along with SEM are presented in ▶ **Table 2** (n = 4–6). **B** The antiglycation data for the Cree extracts obtained by fluorometric and immunochemical assays were highly correlated as determined by Spearman's nonparametric correlation analysis (refer to ▶ **Table 3** for statistical details).

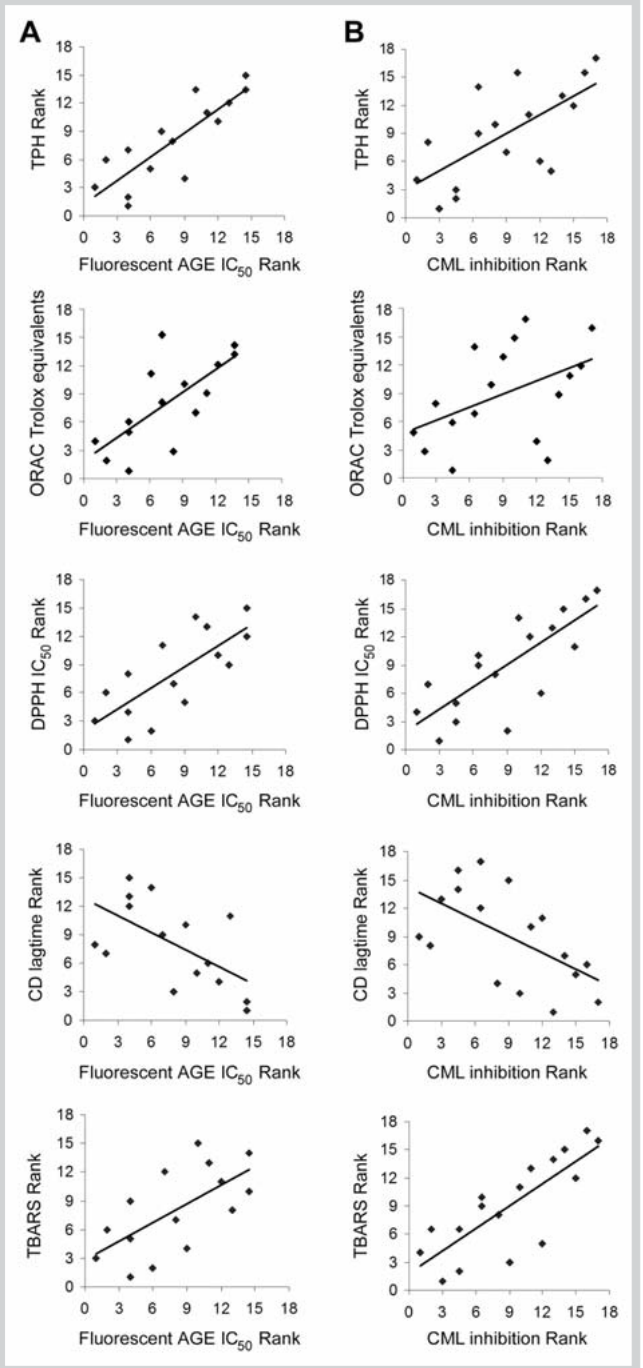

Fig. 4 Inhibition of AGE formation by Cree plant extracts was significantly correlated with total phenolic content and antioxidant activities. Spearman's nonparametric correlation analyses between extract antiglycation activity as determined by IC_{50} values for fluorescent AGEs (left column) and % inhibition for CML adducts (right column) and their ranking in terms of (**A**) total phenolic content (TPH), (**B**) oxygen radical absorbance capacity (ORAC), (**C**) 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity; and inhibition of Cu^{2+}-mediated formation of conjugated dienes (**D**) or thiobarbituric acid-reactive substances (TBARS) (**E**). Statistical details are provided in ▶ **Table 4**.

lation of Cu^{2+} would increase accessibility to LDL leading to accelerated $H^{\cdot}$ extraction from polyunsaturated fatty acids to form conjugated dienes. Phenolic scavenging of the ensuing peroxides, however, would prevent propagation reactions and further malonyldialdehyde formation.

ORAC values correlated with antiglycation and total phenolic results but not with data from other antioxidant assays suggesting that AGE inhibition by phenolic metabolites may involve multiple antioxidant mechanisms. Upon assessing a series of phenolic compounds identified from the extracts for antiglycation effects, the structure-activity requirements appeared similar but distinct from those responsible for antioxidant effects. As previously demonstrated for both antioxidant and antiglycation properties, activity increased with hydroxylation of the phenolic ring or dimerization of catechins and decreased with glycosylation [34, 35]. However, despite possessing inferior antioxidant properties to myricetin, quercetin, catechin and procyanidins [34, 36], taxifolin was the superior AGE inhibitor. Matsuda et al. [37] reported similar results, demonstrating that luteolin possessed greater antiglycation activity than more potent antioxidants such as quercetin and epigallocatechin gallate. Therefore, though phenolic content served as a reasonable indicator of an extract's overall antiglycation and antioxidant potential, activity in individual assays depends on the quantities and properties of specific phenolics in the extract.

Current therapeutic efforts targeting AGE production have met with limited success, notably due to side effects associated with

Table 4 Results of Spearman correlation analyses of antiglycation activities, antioxidant activities, and total phenolic content.

Parameters	Relationship	Spearman r^2	p	N
Fluorescent AGEs (IC_{50}) vs. CML (% inhibition)	positive correlation	0.601	0.0006	15
Fluorescent AGEs vs. TPH (quercetin equivalents)	positive correlation	0.702	< 0.0001	15
Fluorescent AGEs (IC_{50}) vs. ORAC (Trolox equivalents)	positive correlation	0.485	0.0039	15
Fluorescent AGEs (IC_{50}) vs. DPPH scavenging (IC_{50})	positive correlation	0.560	0.0013	15
Fluorescent AGEs (IC_{50}) vs. Lag time to CD formation[a]	negative correlation	0.357	0.0187	15
Fluorescent AGEs (IC_{50}) vs. TBARS formation[a]	positive correlation	0.422	0.0087	15
CML (% inhibition) vs. TPH (quercetin eq.)	positive correlation	0.449	0.0032	17
CML (% inhibition) vs. ORAC value (Trolox eq.)	positive correlation[b]	0.267	0.0601	17
CML (% inhibition) vs. DPPH scavenging (IC_{50})	positive correlation	0.611	0.0002	17
CML (% inhibition) vs. Lag time to CD formation	negative correlation	0.331	0.0156	17
CML (% inhibition) vs. TBARS formation	positive correlation	0.636	0.0001	17
TPH (quercetin eq.) vs. ORAC value (Trolox eq.)	positive correlation	0.543	0.0007	17
TPH (quercetin eq.) vs. DPPH scavenging (IC_{50})	positive correlation	0.720	< 0.0001	17
TPH (quercetin eq.) vs. Lag time to CD formation	negative correlation	0.291	0.0255	17
TPH (quercetin eq.) vs. TBARS formation	positive correlation	0.618	0.0002	17
ORAC (Trolox eq.) vs. DPPH scavenging (IC_{50})	positive correlation[b]	0.212	0.0627	17
ORAC (Trolox eq.) vs. Lag time to CD formation	no correlation	0.048	0.4003	17
ORAC (Trolox eq.) vs. TBARS formation	no correlation	0.164	0.1071	17
DPPH scavenging (IC_{50}) vs. Lag time to CD formation	negative correlation	0.498	0.0015	17
DPPH scavenging (IC_{50}) vs. TBARS formation	positive correlation	0.952	< 0.0001	17
Lag time to CD formation vs. TBARS formation	negative correlation	0.446	0.038	17

DPPH = 1,1-diphenyl-2-picrylhydrazyl radical; TPH = Total phenolic content; [a] Conjugated diene (CD) and thiobarbituric acid-reactive substances (TBARS) formation was initiated by Cu^{2+}-mediated oxidization of low-density lipoproteins; [b] statistically insignificant trend

Table 5 Antiglycation activity of selected metabolites identified in Cree medicinal plant extracts.

Metabolite	Fluorescent AGE (IC_{50})[a]	
	µg/mL	µM
Phenolic acid derivatives		
▸ gallic acid	0.56 ± 0.08	3.29 ± 0.61
▸ caffeic acid	1.28 ± 0.37	7.12 ± 2.50
▸ chlorogenic acid	1.31 ± 0.16	3.69 ± 0.57
Flavonoids		
▸ taxifolin	0.51 ± 0.06	1.68 ± 0.26
▸ quercetin	1.69 ± 0.33	5.58 ± 1.08
▸ quercetirin	3.51 ± 1.37	7.82 ± 3.75
▸ rutin	4.14 ± 0.37	6.77 ± 0.74
▸ myricetin	0.86 ± 0.03	2.70 ± 0.12
▸ catechin	3.15 ± 0.32	10.84 ± 1.11
Procyanidins		
▸ procyanidin B1	2.18 ± 0.23	3.77 ± 0.48
▸ procyanidin B2	1.41 ± 0.17	2.43 ± 0.37
Nonphenolic compounds		
▸ 24-hydroxybetulin	Inactive	–
▸ morronoside	Inactive	–

[a] IC_{50} concentrations ± SEM were calculated as the metabolite concentration required to reduce AGE formation by 50% as determined by regression analysis (n = 3)

synthetic inhibitors [38]. Nutritional or herbal interventions based on plant foods and medicines with high phenolic content represent a therapeutic approach with reduced risk of adverse effects and increased appeal to patients inclined toward nonpharmaceutical or alternative healing practices. In the context of aboriginal health, medicinal plants not only provide a source of bioactive compounds but represent an integral part of traditional diet and healing, two elements repeatedly identified by aboriginal peoples as central to personal and community health [39]. Our findings, which support further study of Cree medicinal plants as a means of reducing AGE-associated diabetic complications, suggest that this therapeutic potential is not limited to the plants included in the study. Since phenolic metabolites similar to those found in boreal plants are widely distributed in the plant kingdom, many traditional food and medicine plants around the world possess similar therapeutic potential, local relevance and economic accessibility.

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Affiliations

[1] Centre for Research in Biopharmaceuticals and Biotechnology, Department of Biology, University of Ottawa, Ottawa, ON, Canada
[2] Neural Regeneration Laboratory, Ottawa Institute of Systems Biology, Department of Biochemistry, Microbiology, and Immunology, University of Ottawa, Ottawa, ON, Canada
[3] Canadian Institute of Health Research Team in Aboriginal Anti-diabetic Medicines, Montréal, QC, Canada
[4] Institut de recherche en biologie végétale et le Jardin botanique de Montréal, Montréal, QC, Canada

[5] Department of Plant Science, McGill University, Ste. Anne-de-Bellevue, Canada
[6] Natural Health Products and Metabolic Diseases Laboratory, Department of Pharmacology, Université de Montréal and Montreal Diabetes Research Center, Montréal, QC, Canada
[7] School of Dietetics and Human Nutrition, McGill University, Ste. Anne-de-Bellevue, QC, Canada

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