Antioxidant and anti-inflammatory activities of methanol extracts from *Solidago canadensis*

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Abstract

Solidago canadensis L. is an herbaceous plant of the family of Asteraceae. In North America, this plant has been used in native medicine for the treatment of pain, burns, ulcers, fever, gastrointestinal and liver diseases. Flowers, leaves and stems methanol extracts have shown antioxidant activities with ORAC values of 2.7 ± 0.9 μmol TE/mg for flowers, 10.0 ± 0.2 μmol TE/mg for leaves and 3.6 ± 0.8 μmol TE/mg for stems. The flowers extract have also shown an anti-inflammatory activity (IC_{50}: 8 ± 2 μg/mL) on LPS-activated RAW 264.7 macrophages nitric oxide inhibition. However, leaves and stems methanol extracts were found to be inactive. Through fractionation of the flowers extract, 5 known caffeoylquinic acid derivatives were isolated. To our knowledge, compound **5** (3,4-di-*O*-caffeoylquinic acid) is reported for the first time in *Solidago canadensis*. All isolated compounds were found to be inactive on LPS-activated RAW 264.7 macrophages nitric oxide inhibition.

1. Introduction

Solidago canadensis L. is an Asteraceae widely distributed across North America, occurring in almost every state in United States and throughout Canada. *Solidago canadensis* was used in American native medecine to treat pain (Rousseau, 1945), burns, ulcers (Arnason et al., 1981), fever (Moerman, 2000), gastrointestinal (Turner et al., 1980; Moerman, 2000) and liver (Moerman, 2000) diseases. Anti-inflammatory and antioxidant actions have been reported for extracts of the genus *Solidago* (Arens-Corell and Okpanyi, 1990; Leuschner, 1995). Previous studies on *Solidago canadensis* herbs extracts reported DPPH and luminol free radical scavenging antioxidant activities (Apáti et al., 2003; Papp et al., 2004). Furthermore, roots where demonstrated active when tested for DPPH free radical scavening, NBT/XO superoxide scavenging and DCF/AAPH peroxyl radical scavenging (McCune and Johns, 2002). However, to our knowledge, no antioxidant activity has been reported for *Solidago canadensis* flowers, leaves and stems. The genus *Solidago* contain numerous interesting secondary metabolites such as: flavonoids, phenolic acids and glucosides, polysaccharides, diterpenes, triterpenoid saponosides, tannins, essential oils and others (Thiem et al., 2001). Previous phytochemical studies of *Solidago canadensis* reported the presence of flavonoids (Krepinsky and Herout, 1962; Apáti et al., 2003), phenolic acids (Kalemba, 1992), sesquiterpenes (Bohlmann et al., 1979), diterpenes (Anthonsen et al., 1969; Bohlmann et al., 1979) and saponins (Reznicek et al., 1990).

In this study, we evaluate antioxidant and anti-inflammatory activities of flowers, leaves and stems methanol extracts of *Solidago canadensis*.

2. Materials and methods

2.1 General experimental procedures

NMR spectra were recorded in methanol-d_4 on a Bruker Avance 400 spectrometer (5 mm QNP with Z-gradient probe) operating at 400.13 MHz (^{1}H) or 100.61 MHz (^{13}C). Chemical shifts were referenced relative to the corresponding residual solvent signals (δ 3.31/49.0 ppm, respectively). The accurate mass determination was carried out with an Applied Biosystems QSTAR XL Hybrid LC/MS/MS system. All HPLC separations were performed on a preparative Agilent 1100 series with a ZORBAX ODS column C18 (2.1 x 25 cm; 7 µm) at a flow rate of 16 mL/min. Compounds were detected by UV absorption at 254 nm. For all HPLC procedures, solvent A was H_2O + 0.1% HCOOH and solvent B was ACN + 0.1% HCOOH. Silica gel 60 (230-400 Mesh, Silicycle), Diaion HP-20 (Supelco) and silica gel 60 C18 (230-400 Mesh, EMD) were used for column chromatography. The solvents were purchased from EMD. Thin-layer chromatography (TLC) was performed on silica gel 60 F_{254} aluminium sheets (Silicycle) using EtOAc-MeOH-H_2O (100:16.5:13.5) as solvent system. Detection of the phenolic compounds was carried out by spraying TLC plates with NP/PEG reagent (1% diphenylboric acid 2-aminoethylester in methanol and 5% polyethylene glycol in ethanol) and observing under UV light before and after revelation (254 and 365 nm).

2.2 Plant material

The flowers, leaves and stems of *Solidago canadensis* L. were collected in september 2004 at St-Félicien (48°40'N, 72°27'W), Québec, Canada. The specimens were identified by Mr. Patrick Nadeau (Département des Sciences Fondamentales, Université du Québec à Chicoutimi, Québec, Canada). A voucher specimen was deposited in the Louis-Marie Herbarium of Université Laval, Québec, Canada (QFA-492115).

2.3 Extraction and isolation

The air-dried flowers, leaves and stems of *Solidago canadensis* (25 g) were extracted with methanol (300 mL) in hot condition under reflux during 2 hours. The methanol extracts were evaporated on a rotary evaporator, under reduced pressure.

For the isolation steps, the air-dried flowers of *Solidago canadensis* (285 g) were extracted with methanol in hot condition (3 x 2.5 L) and then, followed with methanol 80% (3 x 2.5 L) during 2 hours. The combined extracts were evaporated under reduced pressure, yielding a residue (103.81 g), which was suspended in water (400 mL) and successively extracted with dichloromethane (3 x 200 mL) and ethyl acetate (6 x 400 mL), yielding dichloromethane, ethyl acetate and aqueous fractions. The aqueous fraction (66.13 g) of *Solidago canadensis* flowers was fractionated into three fractions (I-III) by chromatography over a silica gel column (7 x 60 cm) using an isocratic solvent system of EtOAc-MeOH-

H_2O (100:16.5:13.5). The fraction II (6.70 g) containing the major constituents was divided into six fractions (A - F) using Diaion (3.2 x 40.5 cm) by elution with MeOH-H_2O (20-80). Fraction B (1.03 g) was separated into nine fractions (B1 – B9) by passage over C18 silica gel column (1.6 x 46 cm), eluting with MeOH 10%. From B1 (214.2 mg), compounds **1** (3.8 mg) and **2** (9.3 mg) were isolated by preparative HPLC (6% solvent B). Fractions B5 (135.1 mg) and B6 (64.9 mg) were combined and then, separated by preparative HPLC (16% solvent B) to afford compounds **3** (9.1 mg), **4** (7.0 mg) and **5** (11.4 mg). Identification of these compounds was performed through NMR (^1H, ^{13}C, DEPT, COSY, HSQC and HMBC) and HR-ESI-MS analyses. The data obtained were compared with those reported in the literature (Tatefuji et al., 1996; Tolonen et al., 2002).

2.4 Cell culture

The murine macrophages RAW 264.7 (#TIB-71) cell line was obtained from the American Type Culture Collection (ATCC, Manassas, USA). The RAW 264.7 cell line was grown in Dulbecco's modified Eagle's medium (Mediatech Cellgro®). Medium was supplemented with 10% fetal calf serum (Hyclone, Logan, USA), 1 X solution of vitamins (Mediatech Cellgro®), 1 X sodium pyruvate (Mediatech Cellgro®), 1 X non-essential amino acids (Mediatech Cellgro®), 100 IU of penicillin and 100 mg/mL of streptomycin (Mediatech Cellgro®). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

2.5 Cytotoxic assay

Exponentially growing cells were plated at a density of 5 x 10^3 cells per well in 96-well microplates (BD Falcon) in 100 µL of culture medium and were allowed to adhere for 16 hours before treatment. Then, the cells were incubated for 48 hours in the presence or absence of 100 µL of increasing concentrations of extracts dissolved in culture medium and an appropriate solvent. The final concentration of solvent in the culture medium was maintained at 0.25% (v/v) to avoid toxicity. Cytotoxicity was assessed using the resazurin reduction test (O'Brien et al., 2000). Fluorescence was measured on an automated 96-well Fluoroskan Ascent Fl™ plate reader (Labsystems) using an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Extracts are considered cytotoxic when inhibition of cell growth is observed.

2.6 Measurement of the antioxidant activity by $ORAC_{FL}$ assay

The oxygen radical antioxidant capacity (ORAC) assay was carried out on a Fluoroskan Ascent F1™ plate reader (Labsystems). Quercetin was used as a positive control. The experiment was conducted at 37.5 °C and pH 7.4, with a blank sample in parallel. The fluorimeter was programmed to record the fluorescence of fluorescein every 30 seconds after addition of 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH). The final results were calculated by comparing the net areas under the fluorescein decay curves between the blank and the samples. Trolox was used as a control standard. ORAC values were expressed in micromoles of Trolox equivalents (TE) per milligram (µmol TE/mg).

2.7. Measurement of anti-inflammatory activity by nitric oxide (NO) inhibition on LPS-activated RAW 264.7 macrophages

Exponentially growing cells were plated in 24-well microplates (BD Falcon) at a density of 2 x 10^5 cells per well in 400 µL of culture medium and were allowed to adhere overnight. Cells were then treated or not with positive control N(G)-nitro-L-arginine methyl ester (L-NAME) (250 µmol/L and 1 mmol/L), or increasing concentrations of fractions or pure compounds dissolved in the appropriate solvents, and incubated at 37°C, 5% CO_2 for 24h. The final concentration of solvent in the culture medium was maintained at 0.5% (v/v) to avoid solvent toxicity. Cells were then stimulated with 10 µg/mL lipopolysaccharide (LPS). After 24h, cell-free supernatants were collected and stored at -80°C until NO determination using the Greiss reaction (Green et al., 1990) with minor sulphanilamide and 50 µL of 0.1% N-1-naphtylethylenediamine dihydrochloride in 2.5% H_3PO_4 at room temperature for 20 min. Absorbance at 540 nm was then measured using an automated 96-well Varioskan Ascent plate reader (Thermo Electron) and the presence of nitrite was quantified by comparison with an $NaNO_2$ standard curve. All the samples were tested at the highest concentration without macrophages cytotoxicity. Anti-inflammatory activity was expressed as the concentration of drug inhibiting nitric oxide overproduction by 50% (IC_{50}).

3. Results and Discussion

3.1 Extraction yields

The air-dried flowers, leaves and stems of *Solidago canadensis* were extracted with methanol in hot condition under reflux. As shown in Table 1, the extraction yields are 0.14 g/g for flowers, 0.18 g/g for leaves and 0.09 g/g for stems.

3.2 Antioxidant activity of methanol extracts

The antioxidant activity of flowers leaves and stems methanol extracts was assessed using the ORAC assay (Ou et al., 2001). As shown in Table 1, in comparison to quercetin, all extracts exhibited moderate antioxidant activities with ORAC values of 2.7 ± 0.9 μmol TE/mg for flowers, 10.0 ± 0.2 μmol TE/mg for leaves and 3.6 ± 0.8 μmol TE/mg for stems. Hence, leaves methanol extract was found to possess the best antioxidant activity. Previous phytochemical studies of *Solidago canadensis* have shown antioxidant activity for herbs extracts (Apáti et al., 2003; Papp et al., 2004). These results support the ethnopharmacological use of *Solidago canadensis* in pathologies implying reactive oxygen species.

3.3. Macrophages cytotoxicity and anti-inflammatory activity of extracts

The claimed effect of *Solidago canadensis* on inflammatory diseases and related disorders by the American native medicine was challenged by testing flowers, leaves and stems methanol extracts of *Solidago canadensis* for anti-inflammatory activity using LPS-activated RAW 264.7 macrophages nitric oxide (NO) inhibition. Stimulation of RAW 264.7 macrophages by LPS induces iNOS and overproduction of NO which can be detected and quantified photometrically by a simple colorimetric reaction (Green et al., 1990) as described in materials and methods. L-NAME, a NO synthase inhibitor, prevents the formation of NO in LPS-stimulated RAW 264.7 macrophages (Paul et al., 1997) and thus was used as positive control. First, the macrophages cytotoxicity of extracts was investigated. As shown in Table 2, the highest concentration without macrophages cytotoxicity is 10.8 µg/mL for flowers, 13.0 µg/mL for leaves and 111.0 µg/mL for stems methanol extracts. Flowers methanol extract of *Solidago canadensis* inhibited overproduction of NO with an IC_{50} of 8 ± 2 µg/mL. However, leaves and stems methanol extracts were found to be inactive. Thus, the flowers are the most interesting part of *Solidago canadensis* for anti-inflammatory activity. These results support the ethnopharmacological use of *Solidago canadensis* flowers in the treatment of inflammation diseases implying NO and peroxinitrite.

3.4 Isolation of compounds from the flowers extract and evaluation of anti-inflammatory activity

Fractionation was carried out to characterize the active principles responsible of the anti-inflammatory activity of the flowers extract of *Solidago canadensis*. The extract was thus suspended into H_2O and then partitioned successively with DCM and EtOAc. Because of his wealth and his simplicity, as shown by TLC, the aqueous fraction has been chose. Further fractionations have led to the isolation of five known caffeoylquinic acid derivatives (**1-5**) which are the major constituents of the aqueous fraction: neochlorogenic acid (**1**, 3-*O*-caffeoylquinic acid), chlorogenic acid (**2**, 5-*O*-caffeoylquinic acid), 4,5-di-*O*-caffeoylquinic acid (**3**), 3,5-di-*O*-caffeoylquinic acid (**4**) and 3,4-di-*O*-caffeoylquinic acid (**5**). To our knowledge, compound **5** is reported for the first time in *Solidago canadensis*. Caffeoylquinic acid derivatives have been previously reported to have several interesting therapeutic properties, which include reducing inflammation (Facino et al., 1993; Rastrelli et al., 1998); activities on some human leukocyte functions related to inflammatory mechanism such as monocyte migration and reducing of superoxide anion production (Peluso et al., 1995); antioxidative activities (Kwon et al., 2000; Kweon et al., 2001; Stewart et al., 2005); inhibitory activities on lipid peroxidation in mitochondria and microsomes of the liver (Kimura et al., 1984); and finally, anti-viral activity (Zhu et al., 1999). The anti-inflammatory activity of isolated compound was evaluated using LPS-activated RAW 264.7 macrophages nitric oxide (NO) inhibition. All isolated compounds were found to be inactive (data not presented).

In summary, this study show that flowers, leaves and stems methanol extracts of *Solidago canadensis* posses antioxidant activities and flowers methanol extract posses anti-inflammatory activity, which support ethnopharmacological use of *Solidago canadensis* in American native medicine. Isolation procedures of the aqueous fraction of flowers extract have led to the isolation of five known caffeoylquinic acid derivatives (**1-5**) which are the major constituents of the aqueous fraction. To our knowledge, compound **5** (3,4-di-*O*-caffeoylquinic acid) is reported for the first time in *Solidago canadensis*. However, these isolated compounds are not responsible of the anti-inflammatory activity. Further isolation procedures should be done to find the active principle of flowers extract.

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