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Food Chemistry 97 (2006) 56–64
Food Chemistry
www.elsevier.com/locate/foodchem

Rapid communication

Effects of *Amelanchier* fruit isolates on cyclooxygenase enzymes and lipid peroxidation

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Received 4 January 2005; received in revised form 16 March 2005; accepted 16 March 2005

Abstract

Bioassay-directed isolation and purification of the ethyl acetate and methanol extracts of *Amelanchier canadensis* resulted in 1,3-dilinoleoyl 2-olein (**1**), 1,3-dioleoyl 2-linolein (**2**), 5-hydroxymethyl-2-furfural (**3**), 5-(sorbitoloxymethyl)-furan-2-carboxaldehyde (**4**), 5-(mannitoloxymethyl)-furan-2-carboxaldehyde (**5**), and 5-(α-D-glucopyranosyloxymethyl) furan-2-carboxaldehyde (**6**). Four compounds, oleanolic acid (**7**), ursolic acid (**8**), kaempferol-3-*O*-α-L-rhamnopyranosyl (1 ← 2) rhamnopyranoside (**9**), and kaempferol-3-*O*-α-L-rhamnopyranoside (**10**) were isolated from the ethyl acetate extract of fresh fruits of *Amelanchier arborea*. The compounds were isolated and purified by various chromatographic techniques and characterized by NMR and GC/MS methods. The isolated compounds inhibited lipid peroxidation (by 85%) at 100 ppm when compared to 89%, 87%, and 98% for the commercial antioxidants butylated hydroxy anisole (BHA), butylated hydroxytoluene (BHT), and *tert*-butylhydroxyquinone (TBHQ) at 1.67, 2.2, and 1.67 ppm, respectively. Although not selective, some of these compounds inhibited cyclooxygenase (COX)-1 and -2 enzymes. Compounds **3**–**6** were isolated for the first time from *A. canadensis* and compounds **7**–**10** were isolated for the first time from *A. arborea* fruits.

Keywords: *Amelanchier arborea*; *Amelanchier canadensis*; Antioxidant; Lipid peroxidation; Anti-inflammatory

1. Introduction

Amelanchier canadensis (Rosaceae), commonly called Shadblow Serviceberry or Shadbush, is a stoloniferous shrub or small tree reaching 8 m. The fruits, purplish-black, slightly sweet, and ripe in late June, are consumed in North America. *Amelanchier arborea* bears the edible fruits, which are also consumed in North America and its fruits are 1/4 to 3/8 inch in diameter, rounded, purplish-black, slightly sweet, and ripen in late June. The fruits of *A. arborea* were important to the early settlers and the Native Americans and have been used in making juice, jelly and pies (Berkheimer & Hanson, 2001).

There are 25 deciduous shrubs or small trees of the genus *Amelanchier* indigenous to North America, Europe and Asia (Griffiths, 1994). The *Amelanchier* species are prevalent in the nursery trades and used as ornamentals in landscape development and in commercial fruit production. Breeding programmes focus on selections for ornamental quality and fruit character. *Amelanchier alnifolia* is a commercial fruit crop in the Canadian Prairie provinces with approximately 25 commercially available selections (Zatylny & St. Pierre, 2003).

The ethanolic extracts of *A. canadensis* fruits are reported to yield 3-feruloylquinic, 5-feruloylquinic, chlorogenic, neochlorogenic, isochlorogenic and caffeic acids

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doi:10.1016/j.foodchem.2005.03.023

and the total phenolic acid content is 222 mg/100 g fresh fruit (Sergeeva, Bandyukova, Shapiro, Narizhnaya, & Anikhimovskaya, 1980). Similarly, previous research on *A. arborea* fruits was focussed on the identification of organoleptic compounds by using GC/MS and sensory evaluation and led to the identification of benzaldehyde, phenyl-acetaldehyde, 2-hexenal and hexanal (Parliment, 2001). The analysis of *A. canadensis* seed extracts by GC/MS showed that it contained 7.2% and 0.9% oil and sterol, respectively. The major sterol present in the seed was β-sitosterol, in addition to campesterol, stigmasterol, brassicasterol, cholesterol, Δ7-stigmasterol, Δ5-avenasterol and Δ7-avenasterol (Zlatanov & Vazvazova, 1999). The phospholipids present in the oils were phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine and phosphatidic acids (Zlatanov & Vazvazova, 1999).

The phytochemistry and bioactivities of compounds present in *A. arborea* and *A. canadensis* fruits are not known. In this paper, we report the isolation and characterization of lipid peroxidation and COX enzyme inhibitory compounds from *A. canadensis* and *A. arborea* fruits.

2. Materials and methods

2.1. Amelanchier fruits

Fully ripened fruits of *A. arborea* and *A. canadensis* were collected in mid July, 2001, in Eaton Rapids, MI. The locations of trees were recorded in the Data Base maintained by the Department of Horticulture at Michigan State University. The fruits were stored in plastic zip lock bags at −20 °C prior to analyses.

2.2. General experimental procedures

^{1}H and ^{13}C NMR spectra were recorded on Varian INOVA (300 MHz) and VRX (500 MHz) instruments. ^{13}C NMR spectra were recorded at 75 and 125 MHz, respectively. ^{1}H NMR chemical shifts are reported in ppm relative to $CDCl_3$ at 7.24, CD_3OD at 3.31, DMSO-d_6 at 2.54, and D_2O at 4.80. Coupling constants, *J*, are in Hertz. ^{13}C NMR chemical shifts are reported in ppm relative to $CDCl_3$ at 77.0, CD_3OD at 49.0, DMSO-d_6 at 39.50. Standard pulse sequences were employed for all 1D (^{1}H, ^{13}C, DEPT) and 2D (HMBC and HMQC) NMR experiments. Mass spectra were recorded at the Michigan State University Mass Spectrometry Facility using a JOEL HX-110 double focusing mass spectrometer (Peabody, MA) operating in the positive ion mode for FABMS experiments.

A Recycling Preparative Liquid Chromatograph model LC-20, equipped with a model AS-20 fraction collector (both Japan Analytical Industry Co., Tokyo) and two JAIGEL-ODS columns (A-343-10, 250 × 20 mm, 10 μm, Dychrom, Santa Clara, CA) in tandem were used for purification of extracts. Peaks were detected using UV and refractive index detectors attached to a model D-2500 Chromato-integrator (Hitachi, Tokyo). The HPLC system (Waters Corp., Milford, MA) used was equipped with a Controller, a 717 Autosampler, a 2410 RI detector and a 996 photodiode array detector and an Xterra Prep RP-18 and/or RP-8 column (250 × 19 mm i.d. 10 μm). Data were recorded and processed using Empower Pro (Waters Corp., Milford, MA) software. Merck silica gel (60 mesh size, 35–70 μm) and C18 with a particle size of 60 μm (Dychrom, Santa Clara, CA) were used for preparative medium-pressure liquid chromatography (MPLC). For preparative TLC separation, 250, 500, 1000 μm silica gel plates (Analtech, Inc., Newark, DE) were used. TLC plates were viewed under UV light at 254 nm and 366 nm or sprayed with 10% sulfuric acid solution. Gas chromatographic-mass spectrometric (GC-MS) analysis was carried out using an HP 6890 system equipped with an electron capture detector operating at 250 °C, an HP-5MS (30 m × 250 μm × 0.25 μm) column and a 7673 model injector operating at 250 °C in the splitless mode; the injection volume was 1.0 μl. Helium was used as carrier gas at 0.8 ml/min. The initial temperature was started at 50 °C, held for 2 min, elevated to 250 °C at 10 °C/min and held until the end of analysis. The quadrupole mass filter was set to scan from 40 to 550 *m/z* units. The samples were dissolved in hexane to yield a solution of 1 mg/ml.

The solvents used were of ACS reagent grade and purchased from Spectrum Chemical Co. (Gardena, CA). Butylated hydroxytoluene (BHT), dimethyl sulfoxide (DMSO), and acetic acid were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). Celebrex and Vioxx were a physician's professional samples supplied by Dr. S. Gupta, Sparrow Hospital, Michigan. 1-Stearoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine was purchased from Avanti Polar Lipids, Inc. (Alabastaer, AL), 3-[*p*-(6-phenyl)-1,3,5-hexatrienyl]-phenylpropionic acid from Molecular Probes (Eugene, OR). COX-1 and COX-2 enzymes were prepared in the Bioactive Natural Products and Phytoceuticals Laboratory (BNPP) from ram seminal vesicles and prostaglandin endoperoxide H synthase-2 (PGHS-2) cloned insect cell lysate, respectively.

2.3. Extraction and purification

Fresh fruits of *A. canadensis* (750 g) were blended with water (2 × 750 ml) in a Waring Blender (5 min) at high speed and centrifuged at 10,000*g* at 4 °C for 20 min. The residue was then extracted with acidic methanol (1% HCl, 3 × 1000 ml) and ethyl acetate (3 × 1000 ml), respectively. The yields of aqueous, methanol and ethyl acetate extracts, after removal of the solvents, were 55, 90, and 6 g, respectively. An aliquot of the ethyl ace-

tate extract (4.8 g) was fractionated into $CHCl_3$ soluble (3 g) and insoluble fractions (1.8 g) by stirring with $CHCl_3$ (30 ml). The chloroform-soluble fraction (1.9 g) was subjected to silica gel MPLC column chromatography (350 × 40 mm) and eluted with 100% hexane, followed by gradients of acetone/hexane, chloroform, chloroform/methanol, and methanol. A total of sixteen 200 ml fractions were collected. Based on TLC profiles, the fractions were combined to yield fractions I (160 mg, hexane, 300 ml, hexane/acetone/20/80, 500 ml), II (1.05 g, hexane/acetone/20/80, 150 ml), III (32 mg, hexane/acetone/40/60, 250 ml), IV (74 mg, hexane/acetone/40/60, 150 ml, $CHCl_3$ 225 ml, MeOH/$CHCl_3$/25/75 500 ml), V (89 mg, MeOH/$CHCl_3$/25/75 300 ml, MeOH 200 ml) and VI (367 mg, MeOH 260 ml).

The fraction II (1.012g) was further fractionated by MPLC silica gel column (350 × 40 mm) and eluted successively with hexane, hexane/acetone and methanol. Aliquots of 15 ml fractions (180) were collected. Based on TLC, these fractions were combined to yield fractions IIA (805 mg, acetone/hexane/4/96, 300 ml), IIB (20 mg, acetone/hexane/8/92, 225 ml), IIC (18 mg, acetone/hexane/8/92 ml ,150 ml), IID (9.4 mg, acetone/hexane/4/96, 75 ml), IIE (100 mg, acetone/hexane/8/92, 150 ml, acetone/hexane/16/84, 75 ml) and IIF (54 mg, acetone/hexane/16/84, 600 ml).

The lyophilized fruits (148 g) of *A. arborea* were blended with hexane (500 ml) in a Waring Blender (5 min) at high speed and extracted sequentially with hexane (3 × 500 ml), ethyl acetate (3 × 1000 ml) and methanol (3 × 1000 ml). The yields of hexane, ethyl acetate, and methanol extracts after removal of the solvents were 2, 1.2, and 98 g, respectively. The TLC comparison of the hexane extract showed that it contained mainly triglycerides. Therefore, it was not further analyzed. The ethyl acetate extract (1.1 g) was subjected to MPLC using a silica gel column (350 × 40 mm). The column was eluted with chloroform, methanol/chloroform gradients and finally with methanol at 4 ml/min. A total of 180 fractions in 15 ml were collected. Based on TLC profiles, fractions were combined to yield Fraction i (186 mg, $CHCl_3$, 100 ml), ii (115 mg, $CHCl_3$, 50 ml), iii (37 mg, MeOH/$CHCl_3$/1/10, 100 ml), iv (66 mg, MeOH/$CHCl_3$/1/10, 340 ml), v (14.5 mg, MeOH/$CHCl_3$/1/5, 150 ml), vi (34 mg, MeOH/$CHCl_3$/1/5, 200 ml), vii (18 mg, MeOH/$CHCl_3$/1/5, 45 ml), viii (61 mg, MeOH/$CHCl_3$/1/5, 200 ml MeOH/$CHCl_3$/1/2, 300 ml), ix (23 mg, MeOH/$CHCl_3$/1/2, 150 ml), x (88 mg, MeOH/$CHCl_3$/1/1, 120 ml), xi (52 mg, MeOH, 130 ml), and xii (182 mg, MeOH, 350 ml). The fraction i contained mostly triglycerides, as indicated by TLC, and was not analyzed further.

2.4. Isolation of compounds *1, 2 and 3*

Fraction IID was pure by TLC analysis and gave compound **2**. Repeated silica gel preparative TLC of fraction IIA (230 mg), using acetone/hexane (5/95) and acetic acid/diethylether/hexane (1/20/80) as mobile, phases yielded compound **1** (40 mg). The structures of these compounds were determined as 1,3-dilinoleoyl 2-olein (**1**) and 1,3-dioleoyl 2-linolein (**2**) by ^1H and ^{13}C NMR spectral analyses and by comparison with reported spectral data (Chandra & Nair, 1993; Ramsewak, Nair, Murugesan, Mattson, & Zasada, 2001).

2.5. Isolation of compounds *3–6*

The methanol extract (31.4 g) of *A. canadensis* fruits was suspended in water (150 ml) and partitioned with ethyl acetate (4 × 150 ml) to yield ethyl acetate (1.3 g) and water-soluble fractions. The water-soluble fraction was lyophilized (26.8 g). An aliquot (25.4 g) of this lyophilized powder was subjected to C-18 MPLC (450 × 51 mm) and eluted with water/methanol gradient (2–100% methanol). A total of 24 fractions in 200 ml aliquots were collected. The fractions were combined based on TLC profiles and afforded fractions I (21.79 g, 2% MeOH in water, 1200 ml), II (1.032 g 10% MeOH in water, 650 ml 30% MeOH in water, 300 ml), III (473 mg, 30% MeOH in water, 200 ml), IV (581 mg, 30% MeOH in water, 75 ml), V (422 mg, 30% MeOH in water 175 ml), VI (192 mg, 30% MeOH in water, 825 ml) and VII (400 mg, 50% MeOH in water, 800 ml, MeOH, 900 ml). The TLC analyses revealed that fractions I and II contained mainly sugars. Similarly, HPLC analysis showed that fraction VII contained mainly anthocyanins. Therefore, fractions I, II and VII were not purified further. Fraction VI (414 mg) was purified by LC-20 using 30% methanol in water. The fractions collected were VIA (19.4 mg, R_t = 20 min), VIB (54 mg, R_t = 22 min), VIC (172 mg, R_t = 26 min), VID (123 mg, R_t = 36 min), and VIE (47 mg, R_t = 44 min). The fraction VIC was purified by silica gel preparative TLC, using MeOH/$CHCl_3$ (10/90) as the mobile phase, to yield compound **3** (22 mg). Compound **3** (60 mg) was also obtained from fraction V. The purification of fraction IV (260 mg), by repeated silica gel preparative TLC (4 × 20 × 20 cm, 1000 μm) using MeOH/$CHCl_3$ (20/80) and (10/90) as mobile phases, yielded an inseparable mixture of compounds **4** and **5** (32 mg), and compound **6** (26 mg). ^1H and ^{13}C NMR spectra of compounds (**4**) and (**6**) were identical to 5-hydroxymethyl-2 furfural, and 5-(α-D-glucopyranosyloxymethyl)furan-2-carboxaldehyde (**6**) (Angyal, 1972; Hearn, 1976; Lichtenthaler, Martin, Weber, & Schiweck, 1993; Urashima, Suyama, & Adachi, 1988).

Compounds **4**. Colourless syrupy liquid; FABMS (relative intensity): $[M^+ + Na]^+$ 313 (20), $[M^+ + H]^+$ 291 (40), $[M^+ - HMF + H]^+$ 183 (10), $[M^+ - sorbitol + H]^+$ 110 (10), $[M^+ - alditol]^+$ 109 (30). ^1H NMR (500 MHz, CD_3OD): δ 9.54 (1H, s, 6-H), 7.38 (1H, d, J = 3.5 Hz, 3-H), 6.68 (1H, d, J = 3.5 Hz, 4-H), 4.62 (2H, s, H-7), 3.4–4.0 (8H, m, 1′, 2′, 3′, 4′, 5′, 6′-H).

^{13}CNMR (125 MHz, CD_3OD): δ 179.55 (C-6), 160.26 (C-5), 154.17 (C-2), 124.33 (C-3), 112.68 (C-4), 75.03 (C-2′), 73.68 (C-1′), 73.49 (C-4′), 73.08 (C-5′), 71.68 (C-3′), 66.15 (C-7), 64.19 (C-6′).

Compounds **5**. Colourless syrupy liquid; FABMS (relative intensity): $[M^+ + Na]^+$ 313 (20), $[M^+ + H]^+$ 291 (40), $[M^+ - HMF + H]^+$ 183 (10), $[M^+ -$ mannitol + H$]^+$ 110 (10), $[M^+ -$ alditol$]^+$ 109 (30). ^{1}H NMR (500 MHz, CD_3OD): δ 9.54 (2H, s, 6-H), 7.38 (1H, d, J = 3.5 Hz, 3-H), 6.68 (1H, dd, J = 3.5, 1.0 Hz, 4-H), 4.61 (2H, s, H-7), 3.50–4.01 (8H, m, 1′, 2′, 3′, 4′, 5′, 6′-H). ^{13}CNMR (125 MHz, CD_3OD): δ 179.56 (C-6), 160.12 (C-5), 154.17 (C-2), 124.31 (C-3), 112.62 (C-4), 73.27 (C-2′), 73.27 (C-1′), 73.05 (C-5′) 70.99 (C-3′), 70.74 (C-4′), 66.15 (C-7), 64.82 (C-6′).

2.6. Acetylation of compounds *4* and *5*

An aliquot (5 mg) of the mixture of compounds **4** and **5** was dissolved in pyridine (1 ml) and acetic anhydride (2 ml) was added. The solution was kept at room temperature for 72 h. Deionized water was added to the reaction mixture and the product was extracted with $CHCl_3$ (3 × 10 ml). The solvent was evaporated under vacuum and purified by preparative silica TLC, using hexane/acetone (2:1) as the mobile phase. The UV-active band was removed, eluted with chloroform and removal of the solvent afforded an inseparable mixture of acetylated products **4A** and **5A** (8 mg) as a colourless liquid.

Compound **4A**. Colourless liquid. ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.62 (1H, s, H-6), 7.26 (1H, d, J = 3.5 Hz, H-2), 6.58 (1H, d, J = 3.5 Hz, H-3), 5.50 (1H, dd, J = 6.5, 4.0 Hz, H-3′), 5.43 (1H, dd, J = 11.0, 5.0 Hz, H-4′), 5.28 (1H, m, H-2′), 5.06 (1H, m, H-5′), 4.54 (2H, s, H-7), 4.34 (1H, dd, J = 11.5, 4.5 Hz, H-6a′), 4.11 (1H, dd, J = 11.5, 6 Hz, H-6b′), 3.70 (1H, dd, J = 11, 4.5 Hz, H-1a′), 3.56 (1H, dd, J = 10.5, 5Hz, H-1b′), 2.09 (3H, s), 2.06 (3H, s), 2.04 (3H, s), 2.02 (3H, s), 2.01 (3H, s).

Compound **5A**. Colourless liquid. ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.62 (1H, s, H-6), 7.19 (1H, d, J = 3.5 Hz, H-2), 6.54 (1H, d, J = 3.5 Hz, H-3), 5.43 (1H, dd, J = 11.0, 5.0 Hz, H-4′), 5.35 (1H, m, H-3′), 5.06 (1H, m, H-2′), 5.04 (1H, m, H-5′), 4.54 (2H, s, H-7), 4.24 (1H, dd, J = 11.5, 4.5 Hz, H-6a′), 4.01 (1H, dd, J = 11.5, 6 Hz, H-6b′), 3.66 (1H, dd, J = 10.5, 5.5 Hz, H-1a′), 3.56 (1H, dd, J = 10.5, 5Hz, H-1b′), 2.08 (3H, s), 2.05 (3H, s), 2.03 (3H, s), 2.02 (3H, s), 2.00 (3H, s).

2.7. Isolation of compounds *7–10*

Silica gel preparative TLC (1000 μm) of fraction ii using acetone/hexane (15/60) as the mobile phase, and developed twice, yielded an inseparable mixture of compounds **7** and **8** (10.2 mg). Fractions ix and viii were dissolved, separately, in methanol/water (1:1, 3 ml), filtered (0.22 μm) and purified by using an Xterra RP-18/8 column (250 × 19 mm, 5 μm, Waters Corp.) at 35 °C. Fraction ix (23 mg) was subjected to repeated preparative HPLC using 20% CH_3CN in water at 3 ml/min to yield compound **9** (R_t = 44 min, 1.7 mg). Similarly, compound **10** was purified from fraction viii (61 mg) by repeated preparative HPLC using 30% CH_3CN in water at 3 ml/min (R_t = 48 min, 1.1 mg). The structures of compounds **7–10** were determined by ^{1}H NMR spectral analysis, in conjunction with the literature data (Faizi, Ali, Saleem, Bibi, & Bibi, 2001; Fossen, Larsen, Kiremire, & Anderson, 1999; Hung & Yen, 2001; Rao, Damu, Jayaprakasam, & Gunasekar, 1999; Urashima et al., 1988).

2.8. Saponification of compounds *1* and *2* and methylation of the resulting fatty acids

Compounds **1** and **2** (2 mg each) were reacted with 5% NaOH in methanol (1 ml) separately. HCl (6 N in MeOH) was then added to acidify the solution and they were dried under nitrogen. Diazomethane was prepared according to a previously published method (Kelm & Nair, 1997). Briefly, *N*-nitroso-*N*-methyl urea was reacted with concentrated KOH solution in ether. The resulting yellow-coloured ethereal solution containing CH_2N_2 was separated and used to methylate the free fatty acids obtained from the hydrolysis of **1** and **2**. The methylated products, dissolved in hexane, were used for GC-MS analysis.

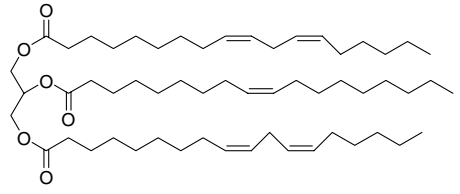
2.9. Cyclooxygenase enzyme inhibitory assay

Cyclooxygenase enzyme inhibitory activities of the compounds isolated from *Amelanchier* fruits were evaluated using COX-1 and COX-2 enzymes according to the previously published procedures (Wang et al., 1999). The rate of oxygen consumption during the initial phase of the enzyme-mediated reaction, with arachidonic acid as substrate was measured using a Model 5300 biological oxygen monitor (Yellow Spring Instruments, Inc., Yellow Springs, OH). The reaction mixture consisted of 0.1 M Tris, 1.0 mM phenol, 17 μg hemoglobin, the enzyme, and 6 μl test sample dissolved in DMSO at 1.5% (DMSO alone as solvent control). The reaction was performed in a 600 μl micro chamber (Instech Laboratory, Plymouth Meeting, PA) at 37 °C. After 2 min of incubation of the enzyme and test samples, 10 μl of arachidonic acid (0.25 mg/0.5 ml of Tris buffer) were added to initiate the reaction. Data were recorded using Quicklog for Windows (Strawberry Tree Inc., Sunnyvale, CA). Aspirin, Celebrex and Vioxx at 180, 1.67 and 1.67 ppm, respectively, were used as positive controls.

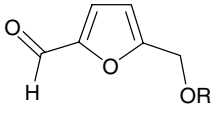
2.10. Lipid peroxidation inhibitory assay

Lipid peroxidation inhibitory assay was performed according to the previously published method (Wang

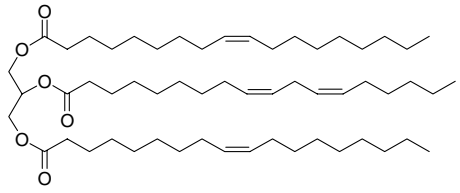
et al., 1999). Inhibition of lipid peroxidation was measured by fluorescence spectroscopy using large unilamellar vesicles (LUVs) of 1-stearoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine containing a fluorescent probe (3-[*p*-(6-phenyl)-1,3,5-hexatrienyl]-phenylpropionic acid) and reported after 21 min as relative fluorescence compared to control. BHT, TBHQ and BHA were used as positive controls. Briefly, the lipid and probe were dissolved in DMF and evaporated in vacuo. The solvent-free mixture was resuspended in 0.15 M NaCl, 0.1 mM EDTA and 0.01 M MOPS (kept over Chelex resin), subjected to 10 freeze-thaw cycles using a dry ice–ethanol bath, and extruded 29 times through a 100 nm pore size membrane to form the LUVs. The assay buffer used consisted of a mixture of 100 μl HEPES buffer, 200 μl 1 M NaCl, and 1.64 ml nitrogen-sparged Millipore water. An aliquot of 20 μl of test sample in DMSO or DMSO (control) and 20 μl of liposome suspension were mixed with the assay buffer to achieve the desired concentration of the test compounds. Peroxidation was initiated by adding 20 μl of $FeCl_2 \cdot 4H_2O$ (0.5 mM) and the samples were gently vortexed. The fluorescence was monitored using a Turner fluorometer with a narrow band pass 360 nm filter and a sharp cut 415 nm filter at 0, 1, 3, 6, and every 3 min thereafter for 21 min. Analyses of samples and controls were performed in duplicate. Relative fluorescence (F_t/F_0) was calculated by dividing the fluorescence value at a given time (F_t) by that time at $t = 0$ min (F_0). The lipid peroxidation inhibitory activity reported is for the final reading at 21 min.



1



3 R = H
4 R = Sorbitol
5 R = Mannitol
6 R= Glucoside



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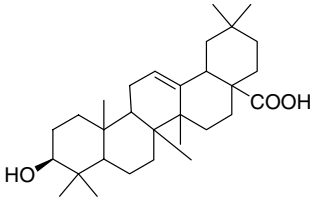

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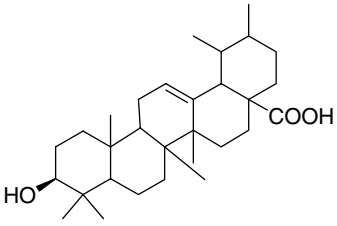

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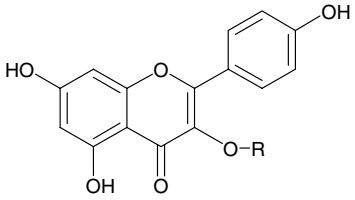

9 R = rhamnopyranosyl (1←2)rhmnopyranoside

10 R = rhamnopyranoside

Fig. 1. Structures of compounds **1**–**10** isolated from *Amelanchier* species.

3. Results

3.1. Compounds from Amelanchier spp.

Six compounds were isolated from the fresh fruits of *A. canadensis*. Compounds **1** and **2** were isolated from chloroform soluble fractions of ethyl acetate extract by successive silica gel MPLC, and preparative TLC purifications. The water-soluble portion of the methanol extract afforded compounds **3**–**6**. Similarly, compounds **7**–**10** were isolated from the ethyl acetate extract of *A. arborea* fruits. The chemical structures of these compounds are shown in Fig. 1, as determined by extensive ^{1}H and ^{13}C NMR studies.

3.2. Lipid peroxidation inhibitory assay

The lipid peroxidation inhibitory activities of crude extracts and pure compounds were determined according to previously published procedures (Wang et al., 1999) (Figs. 2(a) and (b)). Compounds **1** and **2** at 100 ppm, and **3** at 10 ppm, showed only 3.3%, 8.7%, and 8.6% of lipid peroxidation inhibitory activity, respectively. However, compounds **4** and **5** at 100 ppm showed 79% lipid peroxidation inhibitory activity while the acetylated derivative gave 8.3% inhibition at the same test concentration. Compounds **6** at 100 ppm showed 29% inhibitory activity. However, compounds **7/8**, **9** and **10** showed 76%, 85%, and 84% of lipid peroxidation inhibitory activity at 100 ppm, respectively. The commercial antioxidants BHA, BHT and TBHQ at 1.67, 2.2, and 1.67 ppm gave 89%, 87% and 98% of lipid peroxidation inhibitory activity, respectively.

3.3. Cycooxygenase inhibitory assay

Results from COX-1 and COX-2 enzyme inhibitory assays are presented in Fig. 3. Compound **1** showed 39% of COX-1 inhibition but did not inhibit COX-2 enzyme. COX-1 and COX-2 enzyme inhibitory activities for compound **3** at 10 ppm were 5% and 13%, respec-

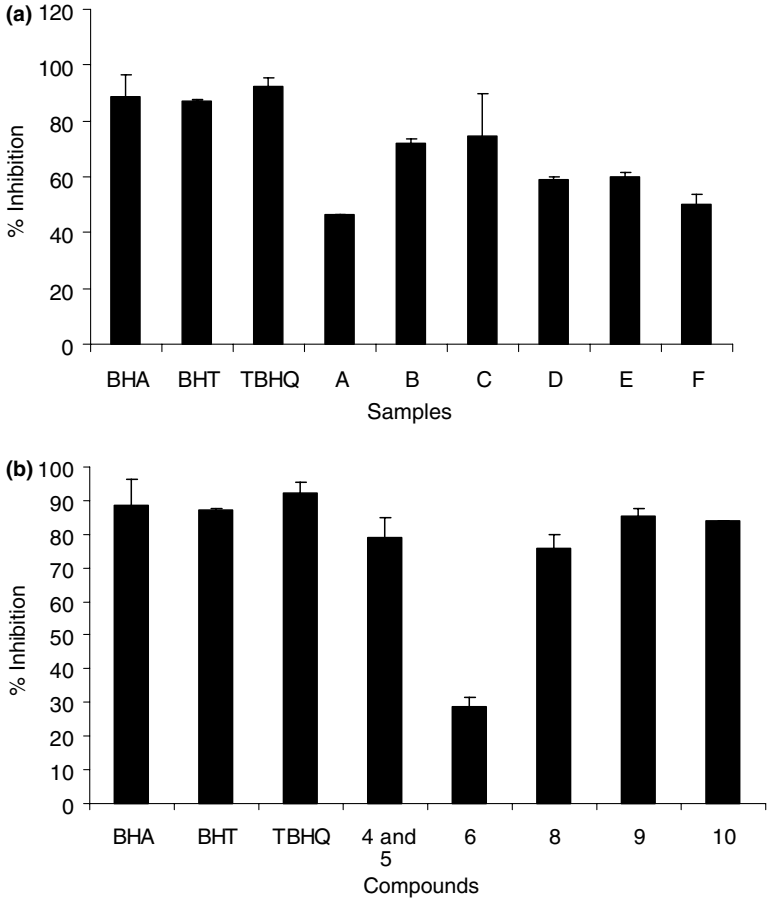

Fig. 2. (a) Lipid peroxidation inhibitory activities from *A. canadensis* fruits (Juice, A), (Methanol extract B), (Ethyl acetate extract, C); *A. arborea* fruits (Hexane extract, D), (Ethyl acetate extract, E), and (Methanol extract F) at 21 min assayed using a liposomal model system. Crude extracts were tested at 250 ppm. Commercial antioxidants BHA, BHT, and TBHQ were assayed at 1.67, 2.20, 1.80 ppm. (b) Lipid peroxidation inhibitory activities of compounds **4**–**10** at 21 min from *Amelanchier* fruits assayed in a liposomal model system. Samples were tested at 100 ppm. Commercial antioxidants BHA, BHT, and TBHQ were assayed at 1.67, 2.20, and 1.80 ppm.

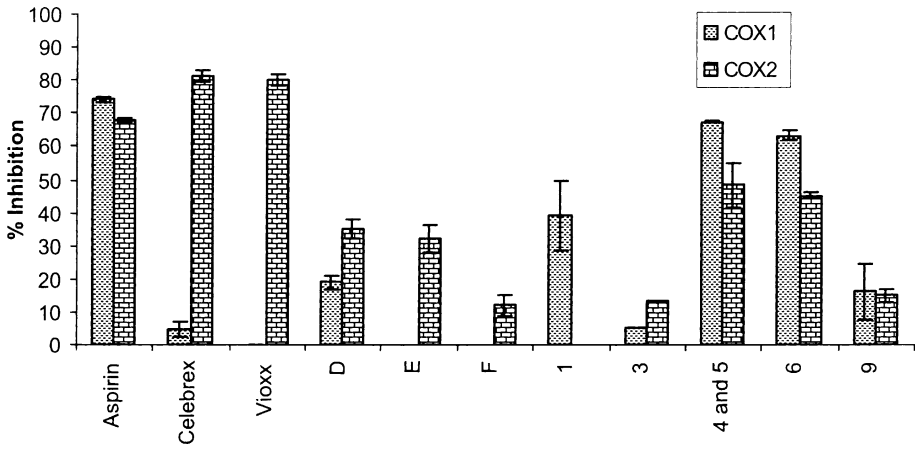

Fig. 3. Cyclooxygenase inhibitory activities of compounds **1**–**9** and extracts from *Amelanchier* fruits (Hexane extract, D), (Ethyl acetate extract, E), and (Methanol extract F). Samples were tested at 100 ppm. Compound **3** was tested at 10 ppm. Aspirin, Celebrex, and Vioxx were tested at 180, 1.67, and 1.67 ppm, respectively.

tively. The mixture of compounds **4** and **5** demonstrated a greater enzyme inhibitory activity, of 68%, inhibition in the COX-1 assay. Similarly, a mixture of compound **6** showed 63% of COX-1 inhibition. The standards Aspirin, Celebrex, and Vioxx at 180, 1.67 and 1.67 ppm, showed 75%, 5% and 0% COX-1 inhibition, respectively. As in the case of COX-1 assay, the mixtures of compounds **4/5**, and **6** at 100 ppm demonstrated COX-2 enzyme inhibitory activity by 49% and 45%, respectively. The standards Aspirin, Celebrex, and Vioxx, at 180, 1.67 and 1.67 ppm, showed 69%, 82% and 85% of COX-2 inhibition, respectively. The acetylated derivatives of compounds **4/5** did not inhibit COX enzymes. Compounds **7/8** and **9** demonstrated 4% and 16% of COX-1 and 6% and 15% of COX-2 enzyme inhibition, respectively, at 100 ppm. Compounds **2** and **10** did not show any COX-1 or -2 enzyme inhibitory activities.

4. Discussion

The structures of known compounds **1**–**3**, and **7**–**10** were determined by ^{1}H and ^{13}C NMR spectral studies. The GC profile of methylated fatty acids resulting from the hydrolysis of compounds **1** and **2** confirmed the presence of oleic and linoleic acid methyl esters with ratios of 2:1 and 1:2, respectively. The retention times of methyl esters of oleic and linoleic acids were identical to those of authentic samples of oleic and linoleic acids. The GC/MS data supported the structures of compounds **1** and **2** as 1,3-dilinoleoyl 2-olein and 1,3-dioleoyl-2-linolein, respectively. Compound **3** was identified as 5-hydroxymethyl-2-furfural from its NMR spectral data. This is the first report of 5-hydroxymethyl-2-furfural from *A. canadensis* although it was reported as a natural product (Chuda et al., 1999; Waruna, Salwa, & Poole, 1994).

The structures of compounds **4/5** were determined from their ^{1}H and ^{13}C NMR, DEPT, HMQC, HMBC and MS spectral analyses, including their acetates. The analysis of ^{1}H and ^{13}C NMR spectra of this product indicated a doubling of proton and carbon signals and suggested that it was a mixture. All attempts to separate **4/5** into pure compounds were not successful. The acetylation of it also gave an inseparable mixture of acetates. Therefore, the structure elucidation was performed on the 1:1 mixture of **4/5**.

The positive HRFABMS indicated a single molecular ion at *m/z* 291.1081 ($[M + H]^{+}$, calc. 291.1080) and suggested a molecular formula of $C_{12}H_{18}O_8$. The presence of major fragment ions at *m/z* 183 and 109 suggested the mass of the two major components present in the molecule. The ^{1}H NMR signal at 9.54 ppm, integrated for 1 proton, did not exchange with D_2O. This confirmed an aldehyde moiety in the molecule. Two doublets with a coupling constant of 3.5 Hz, at 7.47 and 6.59 ppm, showed that the compound contained a furan moiety which was further confirmed by its ^{13}C NMR spectrum. It is important to note that the ^{1}H NMR of **4/5** did not show an anomeric proton signal. There were six oxygenated carbon signals between 64 and 74 ppm in addition to the furanoid carbons. Carbon signals at 75.03, 73.68, 73.49, 70.08, 71.68 and 64.19 ppm corresponded to 2′, 1′, 5′, 3′, 4′, and 6′ carbons of the sorbitol moiety, respectively. Similarly, carbon signals at 73.27, 73.27, 73.05, 70.99, 70.74 and 64.82 ppm were assigned to 2′, 1′, 5′, 3′, 4′, 6′ carbons of the mannitol moiety, respectively. This was further confirmed by its DEPT spectral analysis. HMBC correlation between C-4 to H-7 and C-1′ to H-7 confirmed the connectivity of the furfural moiety to an open chain poly hydroxyl moiety. The acetylation of **4/5** gave a total of 10 acetates resulting from sorbitol and mannitol moieties. The chemical shifts of protons at carbons 2′, 3′, 4′, 5′ and 6′ of the

polyhydroxyl moieties were shifted to down field in the acetylated product while protons attached to C-1′ remained unchanged (Angyal, 1972). Therefore, compounds **4/5** were characterized as 5-sorbitol-furan-2-carbaldehyde and 5-mannitol-furan-2-carbaldehyde, respectively.

^{1}H and ^{13}C NMR spectral analyses of compound **6** indicated that a 5-hydroxymethyl-2-furfural moiety was present in the molecule and further suggested that it was connected to an α-glucose moiety. The structure of compound **6**, therefore, was identified as 5-hydroxymethyl-2-furfural-α-glucoside. This was also supported by FABMS as indicated by a $[M + H]^+$ at *m/z* 289. This is the first report of compound **6** as a natural product and its isolation from *Amelanchier* spp.

The ethyl acetate extract of *A. arborea* was fractionated by silica gel MPLC and further purification of the fractions by preparative TLC and HPLC yielded compounds **7**–**10**. The fraction iii yielded an inseparable mixture of compounds **7** and **8**. The ^{1}H NMR spectra of compounds **7** and **10** were found to be in agreement with previously published data for ursolic and oleanolic acids (Hung & Yen, 2001). The HPLC purification of fractions ix and viii gave compounds **9**, and **10** as light yellow powders at R_t = 44 and 48 min, respectively.

Lipid peroxidation inhibitory activities of crude extracts and pure compounds are shown in Figs. 2(a) and (b), respectively. Except for the flavonoids, ursolic and oleanolic acids, all other isolates from *A. canadensis* and *A. arborea* exhibited only marginal lipid peroxidation activity. The antioxidant activities exhibited by ursolic and oleanolic acids were higher in our model system than the reported activity using the thiocyanate method (Hung & Yen, 2001). The flavonoids are well known for their antioxidant activities (Arora, Nair, & Strasburg, 1997; Braca, Sortino, Politi, Morelli, & Mendez, 2002).

Cyclooxygenase enzymes (COX-1 and -2) catalyze the conversion of arachidonic acid to prostaglandins, which are responsible for the onset of inflammation in the body. It has been confirmed that inhibition of COX-1 reduces the production of prostaglandins in the stomach, that cause gastric ulceration and other side effects in the body. The inhibition of COX-2 enzyme reduces the inflammation with minimum side effects since COX-2 enzyme is produced mostly in inflamed tissues. Therefore, selective COX-2 inhibitors are better anti-inflammatory products (Meade, Smith, & DeWitt, 1993; Smith, DeWitt, & Garavito, 2000).

Results from COX-1 and -2 enzyme inhibitory assays are presented in Fig. 3. Compound **1** showed 39% COX-1 inhibition but was not active in COX-2 enzyme assay. This might be due to the hydrolysis of triglyceride into linoleic acid and oleic acid in the assay chamber. The pH of the buffer used in this assay was 7.4 and triglycerides could potentially be hydrolyzed into its corresponding fatty acids. Polyunsaturated fatty acids, such as linoleic and oleic acids, were reported as good COX-enzyme inhibitors (Henry, Momin, Nair, & Dewitt, 2002). COX-1 and COX-2 enzyme inhibitory activities of compound **3** at 10 ppm were 5% and 13%, respectively. The mixture of compounds **4/5** demonstrated a greater enzyme inhibitory activity of 68% inhibition in COX-1 assay. Similarly, compound **7** showed 63% of COX-1 inhibition. The standards, Aspirin, Celebrex, and Vioxx, at 180, 1.67 and 1.67 ppm, showed 75, 5 and 0% COX-1 inhibition, respectively. As in the case of COX-1 assay, the mixture of compounds **4/5**, and **6** at 100 ppm demonstrated COX-2 enzyme inhibitory activities of 49% and 45%, respectively. The standards Aspirin, Celebrex, and Vioxx at 180, 1.67 and 1.67 ppm, showed 69%, 82% and 85% of COX-2 inhibition, respectively. The acetylated derivatives of compounds **4** and **5** did not exhibit COX enzyme inhibitory activities.

The in vitro COX-1 and COX-2 inhibitory activities of compounds **7** and **8**, and **9** demonstrated weak anti-inflammatory activity, as shown in Fig. 3. The mixture of compounds **7** and **8**, and compound **9** at 100 ppm demonstrated 4% and 16% inhibition of COX-1. Similarly, mixture of compounds **7** and **8**, and compound **9** showed 6% and 15% COX-2 inhibition, respectively. Compound **10** did not show any COX inhibitory activities. Compounds **7** and **8**, a mixture of ursolic acid and oleanolic acid, did not show much COX inhibitory activities at the test concentration in our studies. Previous investigation showed that ursolic and oleanolic acids were good anti-inflammatory compounds and preferentially inhibited COX-2 activity at higher concentrations (Ringbom, Segura, Noreen, Perera, & Bohlin, 1999). The flavonoid, compound **9**, showed weak COX-1 and COX-2 inhibitory activities, but compound **10** was inactive. This is the first report of COX enzyme inhibitory activity of compounds **9**.

In conclusion, six compounds were isolated from ethyl acetate and methanol extracts of *A. canadensis* fruits. Two tryglycerides, 1,3-dilinoleoyl-2-olein (**1**) and 1,3-dioleoyl 2-linolein (**2**) were previously reported from *A. canadensis*. Two other compounds, 5-hydroxymethyl-2-furfural (**3**) and 5-(α-D-glucopyranosyloxymethyl)-2-furancarboxaldehyde (**6**), are reported for the first time from this plant. The compounds from *A. arborea*, mixture of oleanolic acid (**7**) and ursolic acid (**8**), kaempferol-3-*O*-α-L-rhamnosylrhamnoside (**9**), and kaempferol-3-*O*-α-L-rhamnoside (**10**), are reported for the first time from this plant. This is the first report of COX inhibitory activities of compounds **3**–**6**. Compounds **4** and **5** are novel.

Lipid peroxidation is linked to various diseases, such as cancer and cardiovascular disorder. The findings of these bioactive compounds in *A. canadensis* and *A. arborea* substantiated that consumption of *Amelanchier* fruits might be beneficial to health. The widespread dis-

tribution of the genus, its presence in the nursery and landscape industry and the current use of *Amelanchier* spp. in crop production provide the basis for its further expansion as a food crop and investigations into its beneficial contribution to human health. In vivo and clinical studies are required to determine an effective dose of the *Amelanchier* fruits, or its extracts, prior to its consumption as a supplement with health benefits.

Acknowledgement

This research was supported in part by the Agricultural Experiment Station and the Center for Plant Products and Technologies at Michigan State University.

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