Antioxidant Polyphenols from Tart Cherries (*Prunus cerasus*)

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Montmorency and Balaton tart cherries were lyophilized and sequentially extracted with hexane, ethyl acetate, and methanol. Methanolic extracts of dried Balaton and Montmorency tart cherries (*Prunus cerasus*) inhibited lipid peroxidation induced by Fe^{2+} at 25 ppm concentrations. Further partitioning of this methanol extract with EtOAc yielded a fraction that inhibited lipid peroxidation by 76% at 25 ppm. Purification of this EtOAc fraction afforded eight polyphenolic compounds, 5,7,4′-trihydroxyflavanone (**1**), 5,7,4′-trihydroxyisoflavone (**2**), chlorogenic acid (**3**), 5,7,3′,4′-tetrahydroxyflavonol-3-rhamnoside (**4**), 5,7,4′-trihydroxyflavonol 3-rutinoside (**5**), 5,7,4′-trihydroxy-3′methoxyflavonol-3-rutinoside (**6**), 5,7,4′-trihydroxyisoflavone-7-glucoside (**7**), and 6,7-dimethoxy-5,8,4′-trihydroxyflavone (**8**), as characterized by ^{1}H and ^{13}C NMR experiments. The antioxidant assays revealed that 7-dimethoxy-5,8,4′-trihydroxyflavone (**8**) is the most active, followed by quercetin 3-rhamnoside, genistein, chlorogenic acid, naringenin, and genistin, at 10 μM concentrations.

Keywords: *Prunus cerasus; Balaton; Montmorency; antioxidant; flavonoids, isoflavonoids*

INTRODUCTION

The Montmorency (*Prunus cerasus*) variety of cherries constitutes >95% of tart cherry cultivations in Michigan and the United States. However, Balaton tart cherry (*P. cerasus*), a new tart cherry cultivar, is being planted to replace Montmorency in several Michigan orchards. This cherry has higher anthocyanin contents and was regarded as a better variety (Iezzoni, personal communication). Anthocyanin contents of Montmorency and Balaton tart cherries have been reported (Wang et al., 1997; Chandra et al., 1993). However, a detailed investigation of other phenolic compounds in Balaton tart cherry has not been carried out before.

Flavonoids, a group of polyphenolic compounds, are widely distributed and have been reported to act as antioxidants in biological systems (Morel et al., 1993). Flavonoids are considered to have antioxidant activity similar to that of α-tocopherol, vitamin E. It is one of the most common and active naturally occurring antioxidant compounds used in food because of its activity in both hydrophilic and lipophilic systems (Kühnau, 1976).

Kaempferol-3-rutinoside and kaempferol-3-glycoside were reported in the fruits of Montmorency cherries (Schaller and Von Elbe, 1970). Geissman (1956) indicated the presence of quercetin 3-glucoside in the leaves of *P. cerasus*. Also, various kaempferol and quercetin glucosides were identified from Montmorency cherry (Shrikhande and Francis, 1973). From the bark of *P. cerasus*, tectochrysin 5-glucoside and genistein 5-glucoside, pinostrobin, naringenin, prunin, sakuranetin, sakuranin, dihydrowogonin 7-glucoside, chrysin, tectochrysin, genistein, prunetin, and prunetin 5-glucoside were reported (Geibel, 1995; Geibel and Feucht, 1991; Geibel et al., 1990). Isomers of caffeoylquinic acid, *p*-coumaroylquinic acids, and caffeic and ferulic acids were characterized from Montmorency tart cherry (Schaller and Von Elbe, 1970). Similarly, Schwab et al. (1990) reported benzyl-β-D-glucoside, 6-hydroxy-2,6-dimethylocta-2(*E*),7-dienyl β-D-glucoside, and 2-methoxy-4-(2-propenyl)phenyl β-D-glucoside from Montmorency cherry pulp.

Recently, meat products containing tart cherries have become available to consumers. Researchers have found that cooled low-fat ground beef with ∼12% of tart cherries had less rancidity development (Crackel et al., 1988). Also, the addition of cherry fruits to ground beef before frying significantly inhibited the formation of heterocyclic aromatic amines (Britt et al., 1998). The mechanism of this protective action may be involved in the potential antioxidant flavonoids and other polyphenolics present in cherries. Until now, researchers have not investigated the antioxidant compounds in Balaton and Montmorency tart cherries. In this paper, the isolation, identification, and efficacy of antioxidant polyphenolic compounds from Balaton and Montmorency tart cherries are described.

MATERIALS AND METHODS

Cherry Fruits. Pitted and frozen Montmorency and Balaton tart cherries were obtained from commercial growers (Traverse City, MI) through the Cherry Marketing Institute, Inc. (Okemos, MI). The cherries were flushed with nitrogen in freezer bags prior to their storage at −20 °C.

General Experimental Procedure. Silica gel (60 mesh size, 35−70 μm) was purchased from E. Merck. After development, TLC plates (GF uniplate, Analtech, Inc., Newark, DE) were viewed under UV light at 254 and 366 nm, respectively. For preparative HPLC (LC-20, Japan Analytical Industry Co.,

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Tokyo) purification, two JAIGEL-ODS, A-343-10 (20 mm × 250 mm, 10 μm, Dychrom, Santa Clara, CA) columns were used in tandem. Peaks were detected using a UV detector equipped with a model D-2500 Chromato-integrator (Hitachi, Tokyo). ^{1}H, ^{13}C, and double quantum filter correlation spectroscopy (DQFCOSY) and heteronuclear multiple quantum correlation (HMQC) NMR spectra were recorded on Varian UNITY 500 and INOVA 300 MHz spectrometers at 25 °C. All chemical shifts are given in parts per million relative to CD_3OD and DMSO-d_6 at 3.30 and 49.0 ppm and at 2.49 and 39.5 ppm, respectively. Fast atom bombardment mass spectra (FABMS) were obtained on a JEOL JMS-HX110 using a glycerol matrix, and electron ionization mass spectra (EIMS) were obtained on JEOL JMS-AX505 mass spectrometers.

Antioxidant Assay. A mixture containing 10 μM 1-stearoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine (Avanti Polar Lipids, Inc., Alabaster, AL) and 0.03 μM dimethylformamide (DMF) solution of the fluorescence probe 3-[*p*-(6-phenyl)-1,3,5-hexatrienyl]phenylpropionic acid (Molecular Probe, Inc., Eugene, OR) were dried under vacuum using a rotary evaporator. The resulting lipid film was suspended in 1000 μL of a solution containing NaCl (0.15 M), EDTA (0.1 mM), and 4-morpholinepropanesulfonic acid (MOPS) (0.01 M) and subjected to 10 freeze−thaw cycles using a dry ice/ethanol bath. The buffer solution was previously treated with chelating resin, Chelex 100 (Sigma, St. Louis, MO) (5 g/100 mL of buffer), to remove trace metal ions. The lipid−buffer suspension was then extruded 29 times through a Liposo-Fast extruder (Avestin, Inc., Ottawa, Canada) containing a polycarbonate membrane with a pore size of 100 nm to produce unilamellar liposomes. A 20 μL aliquot of this liposome suspension was diluted to 2 mL in Chelex 100-treated buffer containing a 100 mM NaCl/50 mM sodium phosphate buffer at pH 7 and incubated for 5 min at room temperature, followed by another incubation for 5 min in the thermostatic cuvette holder at 23 °C of the spectrofluorometer. The peroxidation was then initiated by the addition of 20 μL of 0.5 mM $FeCl_2$ stock solution to achieve a final concentration of 0.5 μM of Fe^{2+} in the absence or presence of test compounds. The control sample did not contain either Fe^{2+} or test compounds. The fluorescence intensity of these liposome solutions at an excitation wavelength of 384 nm was recorded every 3 min on a spectrofluorometer (SLM4800, Urbana, IL) over a period of 21 min. The decrease in relative fluorescence intensity with time indicated the rate of peroxidation. The percent inhibition of the lipid oxidation was calculated using the equation

$$\% \text{ inhibition} = \{[(F_{rel})_{Pl} - (F_{rel})_{Fe}]/[(F_{ref})_C - (F_{rel})_{Fe}]\} \times 100$$

where $(F_{rel})_{Pl}$ is the relative fluorescence for the Fe^{2+} and test samples at the end of 21 min, $(F_{rel})_C$ is the relative fluorescence for the control sample at 21 min, and $(F_{rel})_{Fe}$ is the relative fluorescence for the Fe^{2+}-containing sample at the end of 21 min (Arora and Strasburg, 1997).

Extraction of Cherries. Lyophilized (5 °C) Balaton tart cherries (200 g) were ground and extracted sequentially with hexane, ethyl acetate, and methanol (500 mL × 3), and the solvents were evaporated under reduced pressure at 40 °C to yield crude extracts of 0.42, 1.49, and 116.3 g, respectively. Similarly, dried Montmorency tart cherry yielded crude extracts of 0.29, 0.74, and 125.4 g, respectively.

The methanol extract of Balaton tart cherries (116.3 g) was dissolved in water (300 mL) and extracted with ethyl acetate (300 mL × 3). The ethyl acetate extract was evaporated to dryness under reduced pressure to yield fraction I (5. 3 g). The aqueous layer was evaporated under reduced pressure to remove ethyl acetate and applied to an XAD-2 column (100 g, Amberlite resin, mesh size 20−50, Sigma Chemical Co., St. Louis, MO), which was prepared as described by Chandra et al. (1993). The column was then washed with distilled water (3 L) until the colorless washing gave a neutral pH. The adsorbed pigments were then eluted with methanol (500 mL). The red methanolic solution was concentrated at 40 °C, and the aqueous solution was then lyophilized to yield fraction II (3.5 g). Because the major components of this fraction were anthocyanins, similar to the components in the water extract of Montmorency and Balaton cherries (Wang et al., 1997), this fraction was not further purified. The crude solvent extracts from Montmorency and Balaton cherries and fractions I and II from the methanol extract of Balaton tart cherry were bioassayed for antioxidant activity (Figure 1).

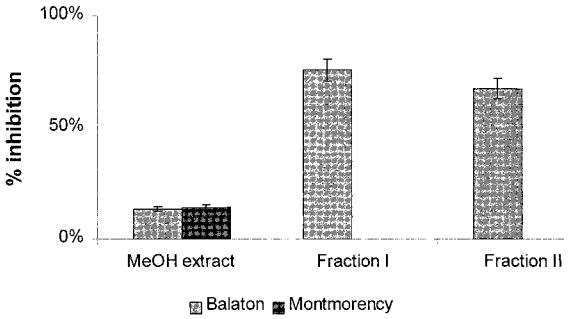

Figure 1. Inhibitory effects of methanol extracts from Balaton and Montmorency tart cherries and fractions I and II from Balaton cherry on Fe^{2+}-induced LUVs peroxidation at 25 ppm concentrations. Fraction I contains compounds **1**−**8**, and fraction II contains anthocyanins (Wang et al., 1997).

Purification of Fraction I. From the preliminary bioassay results, it was evident that fraction I from Balaton cherries contained the most active antioxidant compounds. Therefore, fraction I was further purified for antioxidant compounds. Fraction I (5.3 g) was chromatographed by medium-pressure liquid chromatography (MPLC) (200 g) using a solvent system of $CHCl_3$ and a methanol gradient starting with $CHCl_3$/MeOH (16:1, v/v, 1 L), $CHCl_3$/MeOH (8:1, v/v, 800 mL), $CHCl_3$/MeOH (4:1, v/v, 1 L), and finally MeOH (1 L). Sixteen fractions were collected and monitored by silica TLC plates using $CHCl_3$/MeOH (10:1) and $CHCl_3$/MeOH/HCOOH (4:1:0.1) as developing solvents. The fractions were combined to yield fractions A−F: 740, 2500, 466, 386, 418, and 370 mg, respectively. Fractions A and B showed only weak antioxidant activity and, hence, were not further purified for antioxidant compounds.

*Compounds **1** and **2***. Fraction C (427 mg) was further purified on preparative silica gel TLC plates (20 × 20 cm, 500 μm) and developed with $CHCl_3$/MeOH (15:1). The antioxidant band (26.2 mg), which showed very strong UV fluorescence at λ_{366} and λ_{254}, was repeatedly purified by preparative TLC using acetone/$CHCl_3$ (1:6) as the mobile phase. This yielded compounds **1** (R_f = 0.48, 2.4 mg) and **2** (R_f = 0.46, 2.4 mg).

*Compound **1**:* ^{1}H NMR (DMSO-d_6) δ 12.15 (1H, s, 5-OH), 10.80 (1H, s, 7-OH), 9.60 (1H, s, 4′-OH), 7.32 (2H, d, J = 8.5 Hz, H-2′, H-6′), 6.81 (2H, d, J = 8.5 Hz, H-3′, H-5′), 5.90 (2H, s, H-6, H-8), 5.43 (1H, dd, J = 12.7 Hz, 2.8 Hz, H-2), 3.26 (dd, J = 17.1 Hz, 12.7 Hz, H-3$_{ax}$), 2.69 (dd, J = 17.1 Hz, 2.8 Hz, H-3$_{eq}$); ^{13}C NMR (DMSO-d_6) δ 196.6 (C-4), 166.8 (C-7), 163.4 (C-5), 162.1 (C-9), 157.8 (C-4′), 129.4 (C-1′), 128.8 (C-2′, C-6′), 115.6 (C-3′,5′), 102.2 (C-10), 96.1 (C-6), 95.4 (C-8), 78.9 (C-2), 42.4 (C-3).

*Compound **2**:* ^{1}H NMR (DMSO-d_6) δ 12.98 (1H, s, 5-OH), 10.92 (1H, s, 7-OH), 9.60 (1H, s, 4′-OH), 8.26 (1H, s, H-2), 7.38 (2H, d, J = 8.2 Hz, H-2′, H-6′), 6.82 (2H, d, J = 8.2 Hz, H-3′, H-5′), 6.39 (1H, d, J = 1.95 Hz, H-8), 6.21 (1H, d, J = 1.95 Hz, H-6); ^{13}C NMR (DMSO-d_6) δ 180.6 (C-4), 164.4 (C-7), 163.6 (C-5), 158.1 (C-9), 157.5 (C-4′), 154.4 (C-2), 130.6 (C-2′,6′), 122.8 (C-3), 121.7 (C-1′), 115.5 (C-3′, C-5′), 104.9 (C-10), 99.3 (C-6), 94.2 (C-8).

*Compound **3**:* Fraction D (155 mg) was purified by HPLC using CH_3CN/H_2O (25:75) as the mobile phase at a flow rate of 4 mL/min to yield active compound **3** (27.2 mg, R_t = 52 min): ^{1}H NMR (DMSO-d_6) δ 7.45 (1H, d, J = 15.9 Hz, H-7′), 7.00 (1H, d, J = 2.0 Hz, H-2′), 6.95 (1H, dd, J = 8.4 Hz, 2.0 Hz, H-6′), 6.75 (1H, d, J = 8.4 Hz, H-5′), 6.18 (1H, d, J = 15.9 Hz, H-8′), 5.16 (1H, m, H-5), 3.85 (1H, m, H-3), 3.53 (1H, m, H-4), 2.02−1.83 (4H, m, H-2, H-6); ^{13}C NMR (DMSO-d_6) δ 176.1 (COO^-), 166.1 (C-9′), 148.2 (C-3′), 145.6 (C-4′), 144.4 (C-7′), 125.7 (C-1′), 121.1 (C-6′), 115.8 (C-5′), 115.1 (C-8′), 114.6

(C-2′), 72.9 (C-4), 71.2 (C-1), 71.0 (C-3), 67.3 (C-5), 38.8 (C-2), 35.1 (C-6).

*Compounds **4**−**6** and **7***: Fraction E (418 mg) was purified by HPLC using CH_3CN/H_2O (30:70) as mobile phase at flow rate of 4 mL/min to yield compounds **4** (R_t = 64 min, 11 mg), **5** (R_t = 64 min, 8.6 mg), **6** (R_t = 71 min, 13 mg), and **7** (R_t = 84 min, 3.8 mg), respectively.

*Compound **4***: ^{1}H NMR (DMSO-d_6) δ 7.62 (1H, d, 2.2, H-2′), 7.58 (1H, dd, 8.6, 2.2, H-6′), 6.70 (1H, d, 8.6, H-5′), 6.10 (1H, d, 2.0, H-8), 5.96 (1H, d, 2.0, H-6), 4.96 (1H, s, H-1″), 3.82−3.22 (H-2‴H-5″), 1.15 (3H, d, 6.1, H-6″).

*Compound **5***: ^{1}H NMR (DMSO-d_6) δ 7.94 (2H, d, J = 8.8 Hz, H-2′, H-6′), 6.85 (2H, d, J = 8.8 Hz, H-3′, H-5′), 6.34 (1H, s, H-8), 6.12 (1H, s, H-6), 5.24 (1H, d, 7.3, H-1″), 4.35 (1H, s, H-1‴), 3.51 (d, 10.5 Hz, H-6″), 3.37 (1H, m, H-4″), 3.28 (1H, m, H-5″), 3.27 (1H, m, H-3‴), 3.22 (1H, dt, J = 9.3 Hz, 6.0 Hz, H-5‴), 3.22 (1H, m, H-3″), 3.13 (1H, m, H-4‴), 3.13 (1H, d, J = 5.1 Hz, H-2‴), 3.01 (1H, dd, J = 9.6 Hz, 7.3 Hz, H-2″), 0.94 (3H, d, J = 6.0 Hz, H-6‴); ^{13}C NMR (DMSO-d_6) δ 177.2 (C-4), 161.0 (C-7), 159.9 (C-5), 156.9 (C-9), 156.8 (C-2), 156.8 (C-4′), 133.4 (C-3), 131.1 (C-2′), 131.1 (C-6′), 121.3 (C-1′), 115.2 (C-3′), 115.2 (C-5′), 103.6 (C-10), 101.7 (C-1″), 100.9 (C-1‴), 99.4 (C-6), 94.2 (C-8), 76.4 (C-3″), 75.8 (C-5″), 74.2 (C-2″), 71.8 (C-4‴), 70.6 (C-3‴), 70.4 (C-2‴), 70.0 (C-4″), 68.4 (C-5‴), 67.1 (C-6″), 17.9 (C-6‴); FABMS m/z 594 $[M + H]^+$, 617 $[M + Na]^+$; EIMS m/z (% rel intensity) 286 (100).

*Compound **6***: ^{1}H NMR (DMSO-d_6) δ 7.81 (1H, d, J = 2.0 Hz, H-2′), 7.48 (1H, d, J = 8.4 Hz, 2.0, H-5′), 6.88 (1H, d, J = 8.4 Hz, H-6′), 6.34 (1H, s, H-8), 6.12 (1H, s, H-6), 5.39 (1H, d, J = 7.3 Hz, H-1″), 4.38 (1H, s, H-1‴), 3.82 (OCH_3), 3.67 (1H, d, J = 10.5, H-6″), 3.37 (1H, m, H-4″), 3.31 (1H, dd, J = 6.4 Hz, 5.1 Hz, H-3‴), 3.28 (1H, m, H-5″), 3.22 (1H, m, H-3″), 3.22 (1H, dt, J = 9.3, 6.0 Hz, H-5‴), 3.13 (d, 5.1, H-2‴), 3.13 (1H, m, H-4‴), 3.05 (1H, dd, J = 9.6 Hz, 7.3 Hz, H-2″), 0.95 (3H, d, J = 6.0 Hz, H-6‴); ^{13}C NMR (DMSO-d_6) δ 177.2 (C-4), 161.0 (C-7), 159.9 (C-2), 159.9 (C-5), 156.7 (C-9), 133.2 (C-3), 121.2 (C-1′), 113.4 (C-2′), 99.3 (C-6), 94.2(C-8), 103.6 (C-10), 149.4 (C-3′), 147.0 (C-4′), 115.3 (C-5′), 122.4 (C-6′), 101.5 (C-1″), 101.1 (C-1‴), 76.4 (C-3″), 76.0 (C-5″), 74.3 (C-2″), 71.8 (C-4‴), 70.6 (C-3‴), 70.4 (C-2‴), 70.2 (C-4″), 68.5 (C-5‴), 67.1 (C-6″), 55.9 (OCH_3), 17.9 (C-6‴); FABMS, m/z 624 $[M + H]^+$, 647 $[M + Na]^+$; EIMS (% rel intensity) m/z 316 (100).

*Compound **7***: ^{1}H NMR (DMSO-d_6) δ 8.41 (1H, s, H-2), 7.40 (2H, d, J = 8.7 Hz, H-2′, H-6′), 6.82 (2H, d, J = 8.7 Hz, H-3′, H-5′), 6.72 (1H, d, J = 2.1 Hz, H-8), 6.46 (1H, d, J = 2.1 Hz, H-6), 5.05 (1H, d, J = 7.5 Hz, H-1″), 3.91−3.30 (5H, H-2″-6″); ^{13}C NMR (DMSO-d_6) δ 181.7 (C-4), 164.2 (C-7), 163.8 (C-5), 158.4 (C-9), 155.8 (C-2), 155.8 (C-4′), 131.3 (C-2′, C-6′), 123.7 (C-3), 123.7 (C-1′), 116.3 (C-3′, C-5′), 101.0 (C-10), 100.7 (C-1″), 100.1 (C-6), 95.9 (C-8), 78.4 (C-5″), 77.6 (C-3″), 74.2 (C-2″), 70.7 (C-4″), 61.8 (C-6″).

*Compound **8***: Fraction F (370 mg) was purified by preparative TLC (20 × 20 cm, 500 μm) using $MeOH/CHCl_3/H_2O$ (1:2:0.1, v/v) as the mobile phase. Six bands were collected and eluted with MeOH to yield bands I−VI: 9.6, 5.8, 14.5, 55.6, 131.6, and 56.2 mg, respectively. The active band, VI (56.2 mg), was further purified by preparative TLC using $MeOH/CHCl_3$ (1:8, v/v) as the mobile phase and yielded compound **8** (R_f = 0.62, 17 mg): UV λ_{max} (MeOH) 209, 222 (sh), 290 (sh), 303 nm; UV λ_{max} (MeOH + $AlCl_3$) 209, 235, 229, 321, 360 nm; UV λ_{max} (MeOH +$AlCl_3$ + HCl) 209, 233, 289, 329, 355 nm; UV λ_{max} (MeOH + NaOAc) 210, 305 nm; ^{1}H NMR (CD_3OD) δ 7.95 (2H, d, J = 8.5 Hz, H-2′, H-6′), 6.92 (2H, d, J = 8.5 Hz, H-3′, H-5′), 6.64 (1H, s, H-3), 4.02 (3H, s, OCH_3), 3.91 (3H, s, OCH_3); ^{13}C NMR (CD_3OD) δ 184.8 (C-4), 166.7 (C-2), 162.9 (C-4′), 149.2 (C-7), 146.4 (C-9), 142.9 (C-5), 137.8 (C-6), 132.2 (C-8), 129.8 (C-2′, C-6′), 123.3 (C-1′), 117.0 (C-3′, C-5′), 107.9 (C-10), 103.5 (C-3), 62.0 (OCH_3), 61.3 (OCH_3).

RESULTS AND DISCUSSION

Our preliminary antioxidant assay revealed that the percent inhibitions of Fe^{2+}-induced lipid peroxidation of hexane, EtOAc, and MeOH extracts of Balaton tart cherry were 9.9, 28.3, and 13.6%, respectively. Similarly, the percent inhibitions of hexane, EtOAc, and MeOH extracts of Montmorency tart cherry were 11, 26.3, and 14.3%, respectively. The percent inhibitions of fractions I and II from the methanol extract of Balaton tart cherry were 75.7 and 67.3%, respectively. Because Montmorency and Balaton cherry extracts gave similar chromatographic profiles, only Balaton extracts were further studied for antioxidant compounds. This was mainly due to larger quantities of the extracts available from Balaton compared to Montmorency. Also, Balaton is the new variety of tart cherry grown commercially in several Michigan cherry orchards.

Purification of fraction I by MPLC, TLC, and HPLC afforded compounds **1**−**8**. Compounds **1** and **2** gave ^{1}H and ^{13}C NMR spectral data identical to those of naringenin and genistein, respectively (Harborne, 1994; Agrawal, 1989). The spectral data of **3** were identical to the ^{1}H and ^{13}C NMR spectral data of an authentic sample of chlorogenic acid. Similarly, compounds **4** and **7** were confirmed to be quercetin 3-rhamnoside and genistein 7-glucoside, respectively, by comparison of their ^{1}H and ^{13}C NMR spectral data (Kosuge et al., 1985; Ohta et al., 1980).

The FABMS and EIMS revealed a molecular formula of $C_{27}H_{30}O_{15}$ for compound **5**. The ^{1}H NMR spectrum of compound **5** gave signals for two anomeric protons that appeared at δ 5.24 and 4.35, respectively. These were assigned to anomeric protons of glucose and rhamnose, respectively. The 7.3 Hz coupling constant for the anomeric proton at δ 5.24 indicated a β-linkage of a glucose moiety to the aglycon. The doublet appearing at 0.94 ppm was assigned to a methyl group of a rhamnose sugar moiety. Therefore, the doublet at β 4.35 corresponded to the anomeric proton of an L-rhamnopyranose. Also, the small coupling constant of <1 Hz for this proton indicated an α-glycosidic linkage. The DQF-COSY spectrum of compound **5** helped to confirm the assignment of all protons in **5**. The HMQC spectrum was used to assign the carbon signals in compound **5** and further confirmed glucose and rhamnose moieties in **5**. The appearance of C-6 at 67.1 ppm for the glucose moiety, which was ∼5 ppm further downfield than the normal chemical shift value of C-6 in glucose, indicated that the rhamnose moiety was attached to the C-6 of the glucose moiety. The ^{1}H and ^{13}C NMR spectra of **5** indicated a kaempferol aglycon functionality (Markham et al., 1978). The FABMS of **5** gave a molecular ion at m/z 594 and an ion at m/z 617, indicating an $[M + Na]^+$. The EIMS gave a base peak at m/z 286, which corresponded to a kaempferol moiety. Therefore, compound **5** was assigned as kaempferol 6″-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside (Figure 2).

The molecular formula of compound **6** was determined as $C_{31}H_{32}O_{16}$ by FABMS and EIMS, respectively. ^{1}H and ^{13}C NMR spectra indicated that compound **6** contained sugar moieties and linkages identical to those in compound **5**. The only difference was that compound **6** showed the presence of a methoxy group. As in **5**, H-8 and H-6 in compound **6** appeared at δ 6.34, 6.12, respectively. The chemical shifts at δ 7.81, 7.48, and 6.88 were assigned to protons in the B ring and indicated that one methoxy group was at the 3′- or 4′-position. The HMBC spectrum of **6** suggested that this methoxy group was attached to the 3′-position. The FABMS of **6** gave a molecular ion at m/z 624 and an ion at m/z 647, indicating an $[M + Na]^+$. Also, the EIMS

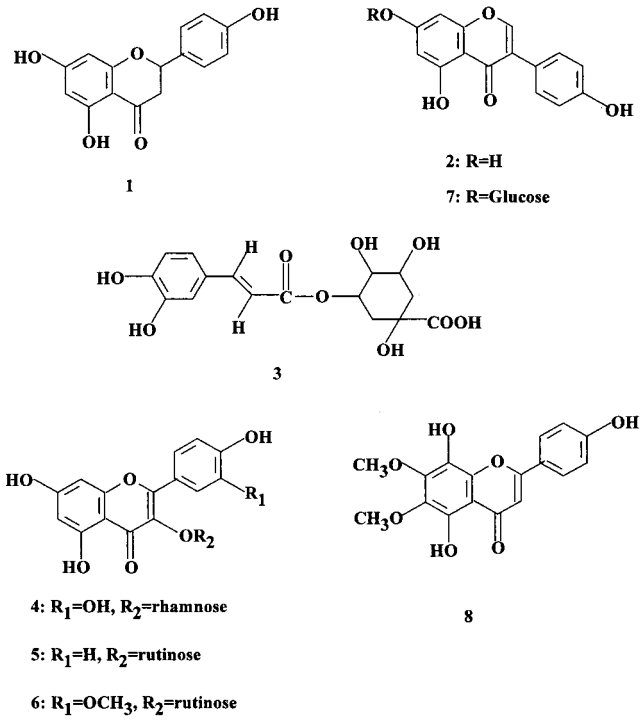

Figure 2. Structures of compounds **1**−**8**.

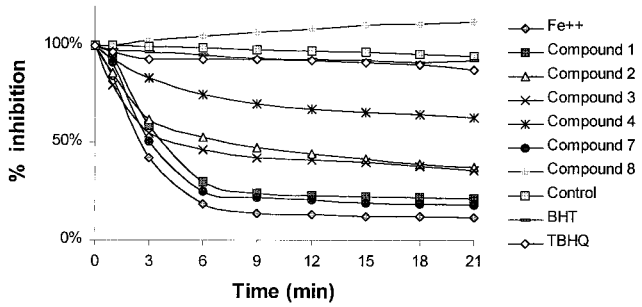

Figure 3. Antioxidant activities of compounds **1**−**4**, **7**, and **8** and commercial antioxidants TBHQ and BHT at 10 μM concentrations. Data represent the means of duplicate experiments.

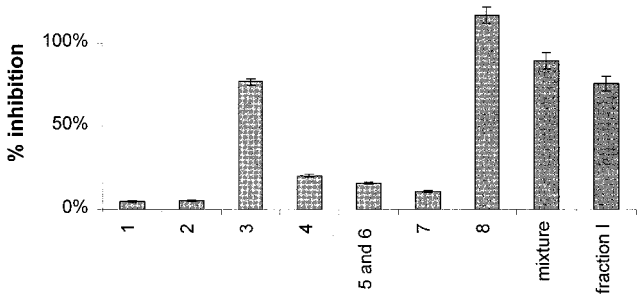

Figure 4. Percent inhibition of compounds **1**−**8** and their mixture on Fe^{2+}-induced LUVs peroxidation. The mixture of compounds **1**−**8** was prepared according to the ratio of their weight extracted from fraction I. The mixture and fraction I were tested at 25 ppm concentrations. The mixture at 25 ppm concentration contained compounds **1**−**8** at 0.6, 0.6, 14, 2.3, 1.1, 2.6, 0.8, and 3.5 ppm, respectively. Similarly, compounds **1**−**8** were assayed independently at 0.6, 0.6, 14, 2.3, 1.1, 2.6, 0.8, and 3.5 ppm concentrations, respectively. Data presented are the means of duplicate experiments.

showed a base peak at *m*/*z* 316, which corresponded to a 3′-methoxykaempferol moiety. Therefore, compound **6** was assigned as rhamnazin 6″-*O*-α-L-rhamnopyranosyl-*β*-D-glucopyranoside. This is the first report of the isolation of this compound from tart cherries (Figure 2).

A flavonoid structure was revealed for compound **8** from its ^{1}H and ^{13}C NMR spectral data. Compound **8** showed a carbon signal at δ 184.8. This indicated that it is a flavone with a hydroxyl group at C-5 (Agrawal, 1989). The ^{1}H NMR spectrum of **8** showed the presence of two aromatic protons each at δ 7.95 and 6.92, respectively, which were assigned to H-2′,H-6′ and H-3′,H-5′, respectively. In addition, there were two OCH_3 groups at 62 and 61.3 ppm, respectively. The UV spectra of **8** in methanol before and after addition of aluminum chloride followed by HCl were comparable to the published value of 5,8-dihydroxy-6,7-dimethoxyflavone (Barberán et al., 1985). From further comparison with published ^{1}H and ^{13}C NMR spectral data of related compounds (Horie et al., 1995), compound **8** was assigned to be 6,7-dimethoxy-5,8,4′-trihydroxyflavone. Like compound **6**, this is the first report of compound **8** from tart cherries (Figure 2).

Compounds **1**−**4**, **7**, and **8** were assayed at 10 μm concentrations for antioxidant activity. The inhibitory effect of flavonoids on Fe^{2+} lipid peroxidation was attributed due to their ability to chelate Fe^{2+} with the formation of inert complexes that are unable to initiate peroxidation (Afanas'ev et al., 1989). Additionally, the Fe^{2+} complexes of flavonoids are considered to retain their free radical scavenging activities and, therefore, can scavenge the free radical intermediate in lipid peroxidation. Also, flavonoids can act as free radical scavengers. The antioxidant activity of compound **8** was superior to the antioxidant activities of **1**, **2**, **4**, and **7** at 10 μm concentrations studied (Figure 3).

Earlier reports suggested that the presence of *o*-dihydroxyl groups on the B ring (Bors et al., 1990), a hydroxyl group at position 3 on the C ring (Afanas'ev et al., 1989; Mora et al., 1990), and a double bond at C_2−C_3 in conjugation with a 4-oxo functional group (Bors et al., 1990) is considered to be essential for effective radical scavenging by the flavonoids. Even though compound **8** does not possess a 3-hydroxyl group and has only one hydroxyl group on the B ring, the antioxidant activity of **8** is higher than that of quercetin 3-rhamnoside. Quercetin 3-rhamnoside contains an *o*-dihydroxyl group in the B ring in addition to a 3-hydroxyl group and a double bond at C_2−C_3 in conjugation with a 4-oxo functional group. The enhanced antioxidant activity of compound **8** was probably due to the hydroxyl and two methoxy groups in ring A. Arora et al. (1997) reported that 7,8-dihydroxyflavone showed antioxidant activity similar to that of quercetin, although it lacked any substitution on the B ring and at the 3-position. Watanabe (1998) compared the antioxidant activities of (±)-catechin and (±)-epicatechin with those of rutin and quercetin on the basis of the inhibition of lipid oxidation. The peroxyl radical-scavenging activities of these compounds were investigated by measuring the inhibition of hydroperoxidation of methyl linoleate initiated by a radical initiator, 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN). The results indicated that (±)-catechin and (±)-epicatechin showed antioxidant activities similar to that of quercetin, even though they have a C_2−C_3 saturated bond and no 4-oxo functional group (Rice-Evans et al., 1996).

To evaluate which component contributed to the highest antioxidant activity in fraction I, a mixture of compounds **1**−**8** was prepared, according to the ratio of

their weight extracted from fraction I. The antioxidant activities of this mixture were determined at 25 ppm concentrations, which contained compounds **1**−**8** at 0.6, 0.6, 14, 2.3, 1.1, 2.6, 0.8, and 3.5 ppm, respectively (Figure 4). The antioxidant activities of individual components at 0.6, 0.6, 14, 2.3, 1.1, 2.6, 0.8, and 3.5 ppm concentrations, respectively, were also measured (Figure 4) and compared with those of the mixture at 25 ppm concentrations. Results indicated that the mixture of compounds **1**−**8** gave 89.6% inhibition on Fe^{2+}-induced lipid peroxidation, whereas compounds **1**−**8** showed 5.1, 5.4, 76.7, 20.5, 16, 11, and 110% inhibition, respectively. Compounds **8** and **3** were the most active components in the mixture and probably in fraction I. Interestingly, the sum of the antioxidant activities of the individual compounds is higher than that of the mixture of these eight compounds. This suggested that in our assay system some of the purified compounds are more effective inhibitors of lipid peroxidation when tested alone.

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