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Cytotoxic Effect of Fruit of *Prunus mandshurica* on Human Monocytic Leukemia Cells

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Abstract

***Prunus mandshurica* var. *glabra* Nakai (Rosaceae) is widely distributed in South Korea and bears a fruit with a bitter and astringent taste. An ethyl acetate-soluble extract of *Prunus mandshurica* was found to exhibit significant cytotoxicity against human leukemia cell lines. Bioassay-directed fractionation of this extract using an MTT(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) cell proliferation assay as a monitor led to the isolation of the bioactive compounds. Two compounds, 1 and 2 were subsequently found to mediate cytotoxicity against U937, human monocytic leukemia cells. The 50% growth inhibitory concentrations (IC_{50}) of compounds 1 and 2 on U937 were 40 and 62 μg/mL, respectively.**

Key words: *Prunus mandshurica*, flavonoid, cytotoxicity, human monocytic leukemia cells

INTRODUCTION

Bioactive compounds in plant foods are considered to be critical for human health. There is overwhelming evidence, from experimental and epidemiological studies, that most common forms of human cancers are related to environmental and life-style factors. Diets rich in fruits and vegetables are associated with lower risk of several degenerative ailments. Natural plant products have had, and continue to have, very important roles as nutritious and medicinal agents, as purified isolates and extracts (1).

In general, cancer cells escape growth control and thus prohibit a normal functioning of the organ in which these cells are located. It has been proposed that flavonoids may have potential as cancer preventive and/or anticancer agents. Flavonoids are natural components in our diet and, with the burgeoning interest in alternative medicine, are increasingly being ingested by the general population.

There are 10 species in the genus *Prunus* which occurs in the regions of South Korea. Previous works of genus *Prunus* have been identified various types of bioactive phytochemicals with antioxidant, cytotoxicity, hypoglycemic and anti-hyperlipidemic effects (2-7).

Though *Prunus mandshurica* var. *glabra* Nakai (Rosaceae) is widely grown in South Korea, it has not been used as a beneficial crop because of its bitter and astringent taste.

As a part of our studies on the characterization of cytotoxic components from natural food sources (8-12), two bioactive cytotoxic flavonoids (1 and 2) were isolated from the ethyl acetate-soluble extract of fruit of *Prunus mandshurica* var. *glabra* Nakai (Rosaceae). It is the first phytochemical report to date on in this plant. From this result, we may suggest that fruit of *Prunus mandshurica* var. *glabra* Nakai can be a useful functional food crop of the genus *Prunus*.

MATERIALS AND METHODS

Plant material

Fruit of *Prunus mandshurica* var. *glabra* Nakai (Rosaceae) was collected at Gyeongbuk area in South Korea in June 2002. A voucher specimen has been deposited at Department of Forestry College of Agriculture and Life Sciences, Kyungpook National University, Daegu, Korea. The seeds from the fruits of the plant were removed and dried at room temperature for sample preparation.

Instrumental analysis

Melting points (mp) were determined using a Mitamura-Riken melting point apparatus and are uncorrected. Electron impact mass spectrometry (EI-MS) spectra were obtained on a Hewelett Packard Model 5985B Gas chromatography/Mass (GC/MS) system. The Ultraviolet /Visible and Infrared (IR) spectra were recorded on a Hitachi 3100 UV/Vis and JASCO Fourier transform (FT)-IR-5300 spectrophotometer, respectively. A Bruker AMX500 spectrometer was used to record nuclear magnetic resonance (NMR) spectra (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR) with tetramethylsilane (TMS), and

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DMSO-d_6 as an internal standard and NMR solvents, respectively.

General experiment

Thin-layer chromatographic (TLC) analysis was performed on silica gel (Kieselgel 60 F_{254}) and plates (0.25 mm layer thickness; Merck, Darmstadt, Germany), with compounds visualizied by spraying with 10% $FeCl_3$ followed by heating at 110°C on a hot plate. Silica gel (Merck 60 A, 230~400 mesh ASTM) and Sephadex LH-20 (25~100 m; Pharmacia Fine Chemicals, Piscataway, NJ) were used for column chromatography.

Chemicals

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were purchased from commercial sources and were of the highest purity available.

Extraction and isolation of bioactive compounds

Dried fruit of *Prunus mandshurica* var. *glabra* Nakai (2.0 kg) was extracted with ethyl alcohol (EtOH) three times in a water bath for three hours. The combined extracts were partitioned between chloroform ($CHCl_3$) and water, with the more polar layer then partitioned with ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH). The EtOAc-soluble extract exhibited significant cytotoxic activity in the human leukemia cells (IC_{50} value of 37.9 μg/mL). The dried EtOAc-soluble extract (11.4 g) was adsorbed onto silica gel and separated over additional silica gel by open column silica gel chromatography, using a gradient of 2~18% MeOH in $CHCl_3$, and elutes containing constituents with similar TLC profiles were combined to provide 7 pooled fractions. Fractions 3 and 5 (eluted with $CHCl_3$-MeOH, 94:6 and 92:8, respectively) exhibited cytotoxic activity against human leukemia cells using MTT assay (IC_{50} values of 39.1 and 49.2 μg/mL, respectively). Further chromatography of active fractions using Sephadex LH-20 size-exclusion chromatography (MeOH) yielded two further pure flavonoids 1 and 2.

Cell culture

U937 (human monocytic leukemia cells) were cultured at 37°C in 5% CO_2 in RPMI 1640 medium containing 2 mM glutamine, 10% heat-inactivated fetal calf serum, penicillin (100 units/mL), and streptomycin (100 g/mL).

Cell viability assay

The effects of plant fractions and compounds on the viability of U937 cells were determined by an MTT assay (13,14). Cells at the exponential phase were collected and transferred into each well (approximately $10^4 \sim 10^5$ cells in 180 μL/ well). The cells were incubated for 96 hrs in the presence of various amounts of fractions and pure compounds 1 and 2 (0~200 μg/mL) in a total reaction volume of 200 μL; 50 μL of 2 mg/mL MTT solution was then added to each well (0.1 mg/well). After incubating for 4 hrs, the plates were centrifuged at 800 g for 5 min and supernatants were aspirated. The formazan crystals in each well were dissolved in 150 μL of dimethylsulfoxide (DMSO) and the A_{540} was read on a scanning multi-well spectrophotometer (Molecular Device Co., Sunnyvale, CA). The IC_{50} was determined as the concentration that inhibited cell growth by 50% using the MTT assay. Data are expressed as mean±standard error (SE).

RESULTS AND DISCUSION

Isolation and identification of compounds

The dried fruit of *Prunus mandshurica* var. *glabra* Nakai was extracted with EtOH and partitioned between $CHCl_3$ and water, with the more polar layer then partitioned with EtOAc and *n*-BuOH. The EtOAc-soluble extract of *Prunus mandshuria* fruit, which showed significant cytotoxicity with an IC_{50} value of 37.9 μg/mL on U937 human monocytic leukemia cells, was loaded onto a silica gel column for a series of activity-guided chromatographic fractionation steps using a $CHCl_3$-MeOH gradient to give 7 sub-fractions monitoring TLC patterns on a UV lamp, $FeCl_3$ and H_2SO_4 spray reagents. Of these, fractions 3 and 5, which possessed cytotoxic activity with IC_{50} values of 39.1 and 49.2 μg/mL for U937 cells, were further chromatographed on a Sephadex LH-20 column by elution with MeOH in order to give pure compounds. Compounds 1 and 2 exhibited characteristic flavonoids color reactions (purplish brown with $FeCl_3$, yellow with NaOH, yellowish orange with Mg-HCl, pink with Zn-HCl). The UV, MS, ^1H NMR and ^{13}C NMR spectral data of the aromatic parts of compounds were characterized by major bands that resembled those of isoflavone and flavonol (15). The structures of compounds 1 and 2 (Fig. 1) were identified by comparing instrumental spectra with published data (15,16). The isolated cytotoxic compounds from *Prunus mandshurica* var. *glabra* Nakai were determined to be genistein (1) and kaempferol (2), and detailed data are described as follows.

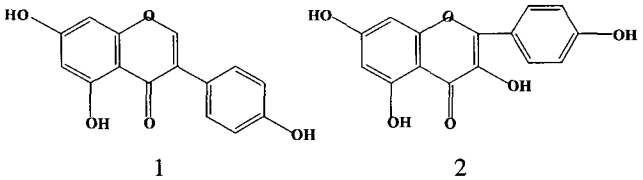

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Fig. 1. Structures of genistein (1) and kaempferol (2) isolated from the fruit of *Prunus mandshurica* var. *glabra* Nakai.

Genistein (*1*) : Pale yellow needles from MeOH; mp 294~295°C; UV λ_{max} (MeOH) (log ε) 260 (4.70), 315 (sh, 4.34), 368 (4.73) nm; λ_{max} (MeOH+NaOH) (log ε) : 271 (4.92), 319 (sh, 4.55), 426 (4.80) nm; λ_{max} (NaOAc) (log ε) : 275 (4.86), 306 (4.49) nm; λ_{max} (NaOAc+H_3BO_3) (log ε) : 267 (4.56), 315 (sh, 4.36), 368 (4.20) nm; λ_{max} ($AlCl_3$) (log ε) : 272 (4.80), 303 (sh, 4.34), 350 (4.21) nm; λ_{max} ($AlCl_3$+HCl) (log ε) : 272 (sh, 4.71), 270 (4.80), 304 (sh, 4.41), 424 (4.90) nm; IR (KBr) λ_{max} 3440 (OH), 1658 (α,β-unsaturated C=O), 1612, 1512 (aromatic C=C), 1258 (aromatic C-O) cm^{-1}; EI-MS (70 eV) *m/z* (relative intensity %): 304 $[M+1]^+$ (27.0), 286 $[M-CO]^+$ (12.5), 293 $[M-HCO]^+$ (23.4), 247 $[M-(CO+HCO)]^+$ (32.4), 171 $[A_1+H]^+$, (100.0), 139 $[B_2]^+$ (11.0); ^1H NMR and ^{13}C NMR data were consistent with those in the literature (15,16), described as Table 1.

Kaempferol (*2*) : Yellow amorpous from MeOH; mp 273~275°C; UV λ_{max} (MeOH) (log ε) : 267 (4.72), 325 (sh, 4.54), 368 (4.73) nm; λ_{max} (MeOH+NaOH) (log ε) : 281 (4.89), 319 (sh, 4.65), 426 (4.82) nm; λ_{max} (NaOAc) (log ε) : 275 (4.86), 306 (4.59), 387(4.78) nm; λ_{max} (NaOAc+H_3BO_3) (log ε) : 267 (4.76), 315 (sh, 4.56), 368 (4.80) nm; λ_{max} ($AlCl_3$) (log ε) : 270 (4.80), 303 (sh, 4.34), 350 (4.41), 420 (4.90) nm; λ_{max} ($AlCl_3$+HCl) (log ε): 258 (sh, 4.72), 270 (4.85), 304 (sh, 4.47), 424 (4.91) nm; IR (KBr) λ_{max} 3425 (OH), 1655 (α,β-unsaturated C=O), 1610, 1510 (aromatic C=C), 1256 (aromatic C-O) cm^{-1}; EI-MS (70 eV) *m/z* (relative intensity %): 286 $[M+1]^+$ (100.0), 258 $[M-CO]^+$ (32.5), 275 $[M-HCO]^+$ (13.4), 229 $[M-(CO+HCO)]^+$ (12.1), 153 $[A_1+H]^+$, (6.7), 121 $[B2]^+$ (21.5); ^1H NMR and ^{13}C NMR data were consistent with those in the literature (15,16), described as Table 1.

Cytotoxicity assessed by cell viability assay

Cultured cell-based assays were used to evaluate the cytotoxic potential of the extracts, EtOAc-soluble fraction, and isolated compounds against human leukemia cell lines, U937. Compounds 1 and 2 showed cytotoxic activity in a dose dependent pattern (Fig. 2). The IC_{50} of the two flavonoids for U937 cells were 40 and 62 μg/mL,

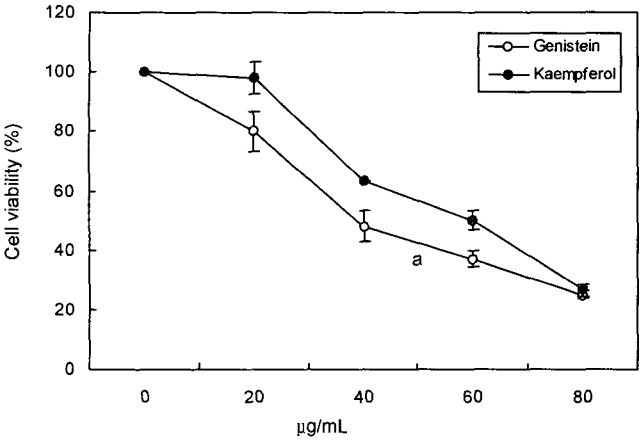

Fig. 2. Dose dependency of cytotoxic activity of genistein and kaempferol. U937 cells were incubated for 96 hrs in the presence of various amounts of compounds, and then cell viability was detected using MTT test and values were expressed as mean±standard error (SE).

Table 1. ^1H NMR and ^{13}C NMR data for compounds 1 and 2[1] from *Prunus mandshurica* var. *glabra* Nakai

Position	δ ^1H[1] (*mult.*, *J* Hz)		^{13}C[2]	
	1	2	1	2
2	7.97 (s)		98.1	156.2[3]
3	6.14 (d, *J*=2.2)		156.2[3]	116.7
4			178.0	176.4
5		6.18 (d, *J*=2.1)	162.2	161.2
6	6.39 (d, *J*=2.2)		98.5	98.8
7		6.40 (d, *J*=2.1)	164.1	164.0
8			95.0	93.6
9			156.1[3]	156.1[3]
10			103.8	103.9
1'		8.05 (d, *J*=8.9)	119.3	120.1
2'	7.98 (d, *J*=9.0)	6.92 (d, *J*=8.9)	131.0	130.4
3'	6.94 (d, *J*=8.9)		115.0	114.9
4'		6.92 (d, *J*=8.9)	160.1	160.0
5'	6.94 (d, *J*=8.9)	8.05 (d, *J*=8.9)	115.5	115.6
6'	7.98 (d, *J*=9.0)	12.44 (br s)	129.7	129.8
OH	13.05 (br s)	10.58		
	10.51	10.15		
	10.09	10.08		

[1]TMS was used as the internal standard; chemical shifts are shown in the δ scale with *J* values in parentheses; measured at 500 MHz in DMSO-d_6.
[2]Measured at 125 MHz in DMSO-d_6.
[3]Chemical shifts within a column can be reversed.

Table 2. Values of IC_{50} μg/mL of compounds 1 and 2 isolated from *Prunus mandshurica* var. *glabra* Nakai on U937 human monocytic leukemia cells

Compounds	Yield (%)	IC_{50} μg/mL[1)]
Genistein	7.63×10^{-6}	40±2.5
Kaempferol	2.48×10^{-5}	62±0.7
Cisplatin[2)]		1.5±0.01

[1)]50% inhibitory concentration (IC_{50}) was measured by MTT assay after 96 hr incubation and values were expressed as mean ±standard error (SE).
[2)]Positive control.

respectively, based on the MTT cell proliferation assay (Table 2). The glycosides of compounds 1 and 2 did not have efficacy on U937 cells, since cell viability of its glycosides on U937 did not affect at highest tested dose (80 μg/mL), suggesting that the cytotoxic activity is due to the aglycone moieties. The structure-activity relationship revealed that either the C4-hydroxy structure in the B ring and certain structures in the A and C rings of the flavonoids are necessary for the protective activities.

It is well known that many flavonoids are effectively cytotoxic to various human cell lines. Long-term consumption of flavonoid-rich diets may suppress and/or inhibit experimental tumor cell growth (17-23). In general, cancer cells impair the normal function of the organ in which these cells are located. Apoptosis, also known as programmed cell death, plays a very important role in many normal biological processes. Screening of plant extracts and their solvent fractions for bioactive components that could effectively induce apoptosis has yielded some promising preventive and/or therapeutic candidates for degenerative diseases. Many cytotoxic agents and/or DNA damaging agents arrest the cell cycle to ensure that the cells repair the damaged DNA and then induce apoptotic cell death in case of irreparable cells. Apoptosis can also be induced in cells by imposition of external stresses such as bacterial toxins, heat shock, radiation, and oxidative stress (24-26). Recently, it has been reported that genistein and kaempferol inhibit the growth of rat and human tumor cells *in vivo* and *in vitro* (27-34).

Our results suggest that fruit of *Prunus mandshurica* var. *glabra* Nakai may inhibit proliferation of human monocytic leukemia cell lines *in vitro*. Further study on apoptosis mechanism is needed to confirm this hypothesis.

At the onset of the present study, fruits of *Prunus mandshurica* var. *glabra* Nakai was considered of potential importance for providing a source of ingredients with functional properties and an alternate source of income for the farm economy.

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