Arch. Pharm. Res. Vol. 17, No. 6, pp. 476-479, 1994 Note

Phenolic Compounds from *Duchesnea chrysantha* and their Cytotoxic Activities in Human Cancer Cell

Ihn Rhan Lee and Mi Young Yang

College of Pharmacy, Ewha Womans University, Seoul 120, Korea

(Received September 20, 1994)

Five phenolic compounds were isolated from 80% aq. acetone extract of *Duchesnea chrysantha*. Their cytotoxicities were screened by the colorimetric tetrazolium assay (MTT assay). Gallic acid, methyl caffeate, protocatechuic acid and pedunculagin mildly inhibited the survival of PC_{14} and MKN_{45} human cancer cell. Brevifolin carboxylic acid showed a strong cytotoxic activity.

Key words: *Duchesnea chrysantha*, Rosaceae, Phenolic compounds, Gallic acid, Brevifolin carboxylic acid, Methylcaffeate, Protocatechuic acid, Pedunculagin, Cytotoxicity

INTRODUCTION

Duchesnea chrysantha (Andr.) Fock (Rosaceae) has been used as a traditional medicine to treat congenital fever, toothache, pain of mensturation, bleeding, inflammation, fever and a tumor (Stuart G, 1991). Linoleic acid, β-sitosterol, n-hentriacontane, esculin, quercitrin and friedelin etc. were isolated (Mukherjee *et al.*, 1983). Recently it has been reported that hexane and ether soluble fractions showed antibiotic effect for *Staphylococcus aureus, Escherichia coli, Bacillus subtilis,* and *Aspergillus niger* (Lee *et al.*, 1987). Water soluble fraction fairly showed antitumor activity against Sarcoma 180 cells in ICR mice (Lee *et al.*, 1986). In the previous study, the polysaccharide and tannin fractions exhibited colony stimulation factor activity (Lee *et al.*, 1990).

We can expect that some special components of *Duchesnea chrysantha* affect the function of the immune cells.

This paper deals with isolation of tannin and phenolic compounds and screenning of cytotoxic activity against PC_{14} and MKN_{45} human cancer cell.

MATERIALS AND METHODS

Materials

Duchesnea chrysantha Herba was collected in the suburbs of Seoul in July, 1991.

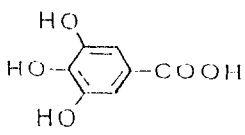
Correspondence to: Ihn Rhan Lee, College of Pharmacy, Ewha Womans University, Seoul 120, Korea

Instruments

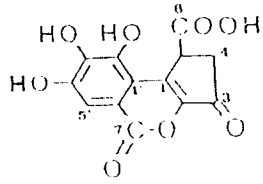
Melting point was recorded on a METTLER FP 62 melting point apparatus. UV was measured on a Shimadzu, UV visible recording spectrophotometer UV-240 Graphicord. The ^{1}H and ^{13}C-NMR spectra were recorded on Bruker AMX 500 spectrometer. The chemical shifts were reported in parts per million (δ, ppm) downfield from tetramethylsilane. IR spectra were obtained on Perkin-Elmer 1420 Ratio Recording Infrared Spectrophotometer. Mass spectra were taken by High Resolution Mass (positive FAB MS) VG 70 VSEQ. TLC was performed on precoated Kiesegel 60 F_{254} (Merck, 5553) and cellulose plate (Merck, 5552). HPLC was carried out on Waters system using μ-Bondapak C_{18} column (4 mm×300 mm).

Extraction and Isolation

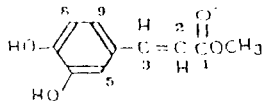
The dried herb (2.8 Kg) were extracted with 80% aqueous acetone at room temparature (for 24 hrs, 4 times). After removal of acetone in vacuo, the aq. solution was filtered. The filtrate was applied to Sephadex LH-20 column. Elution with H_2O, MeOH and acetone gradiently afforded 4 fractions (Fr. I-IV). Fr. II was rechromatographed on Sephadex LH 20, MCI gel CHP 20P and Toyopearl column with H_2O-MeOH-acetone, EtOH-MeOH-acetone gradiently to give compound **I** (89 mg), **II** (60 mg), **IV** (18 mg). Compound **III** (115 mg) was isolated on silica gel TLC preparatively. Com-



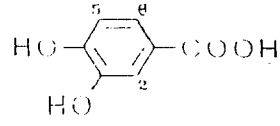
Compound I: gallic acid



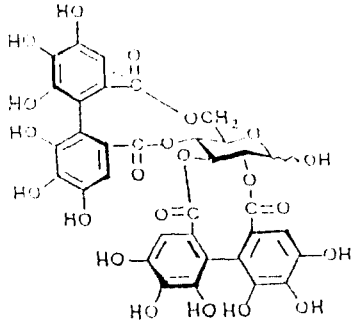
Compound II: brevifolin carboxylic acid



Compound III: methyl caffeate



Compound IV: protocatechuic acid



Compound V: pedunculagin

Fig. 1. The chemical structures of compound I to V isolated from *Duchesnea chrysantha*.

pound **V** (653 mg) was isolated from Fr. III in the same way as described for compound **I**, **II** and **IV**. The chemical structures of each compound were listed on Fig. 1.

Compound I: white chrystal, mp: 270-271℃, TLC: Rf=0.77 (Benzene : Ethyl formate : Formic acid=1 : 7 : 1), $FeCl_3$: blue ; ^{1}H-NMR (acetone-d6) δ: 7.15 (s)

Compound II: yellow christal, mp >300℃, TLC; Rf=0.44 (Benzene : Ethyl formate : Formic acid=1 : 7 : 1), $FeCl_3$: blue, UV λ_{max}^{MeOH} nm: 278; IR ν_{max} cm^{-1}: 2900 (broad -COOH), 1720, 1100 (-COO-), 1600, 1400 (-C=C-, olefine, aromatic), 1300; ^{1}H-NMR (DMSO-d_6) δ (ppm): 2.50 (1H, d, J=19 Hz, CH-$\underline{CH_2}$-C=O), 3.05 (1H, dd, H=7, 19 Hz, CH-$\underline{CH_2}$-C=O), 4.45 (1H, d, J=7 Hz, $\underline{CH}$-CH_2-C=O), 7.35 (1H, s, aromatic H); ^{13}C-NMR (DMSO-d_6) δ (ppm): 37.38 (C-4), 40.76 (C-5), 108.02 (C-5'), 113.03 (C-1'), 115.13 (C-6'), 138.76 (C-3'), 140.09 (C-1), 143.72 (C-2'), 145.57 (C-4'), 149.42 (C-2), 160.38 (C-7'), 173.15 (C-6), 192.99 (C-3); MS (positive FAB-MS): 293 $(M+H)^+$, 185.

Compound III: yellowish amorphous powder, TLC: Rf=0.54 (Benzene : Ethyl formate : MeOH Formic acid =1 : 7 : 1), $FeCl_3$: blue; UV λ_{max}^{MeOH} nm: 330, 300 (shoulder); IR ν_{max} cm^{-1}: 3000 (OH), 1700 (C=O), 1600 (olefine, aromatic), 1400, 1250, 1170, ^{1}H-NMR (CD_3OD+D_2O) δ (ppm): 3.39 (3H, s, COO-$\underline{CH_3}$), 6.35 (1H, d, J=15 Hz, aromatic ring-$\underline{CH}$=CH-COO-), 6.75 (1H, d, J=6 Hz, aromatic H), 7.07 (1H, d, J=2 Hz, aromatic H), 7.58 (1H, d, J=15Hz, aromatic ring-CH =CH-COO-); ^{13}C-NMR (D_2O+DMSO-d_6) δ: 42.45 (-OCH_3), 117.95 (C-8), 118.84 (C-5), 119.29 (C-9), 126.13 (C-4), 130.59 (C-2), 147.72 (C-6), 149.68 (C-3), 150.54 (C-7), 170.20 (C-1).

Compound IV: yellow, $FeCl_3$-EtOH: dark blue, TLC: Rf=0.80 (Benzene : Ethyl formate : Formic acid=1 : 7 : 1), UV λ_{max}^{MeOH} nm: 330; ^{1}H-NMR (acetone-d_6) δ: 6.84 (1H, d, J=6 Hz, aromatic H), 7.43 (1H, dd, J=2,6 Hz, aromatic H), 7.48 (1H, d, J=2 Hz, aromatic H).

Compound V: colorless crystal, TLC: Rf=0.51 (Benzene : Ethyl formate : Formic acid=1 : 5 : 2), $FeCl_3$; blue; UV λ_{max}^{MeOH} nm: 230; IR ν_{max} cm^{-1}: 3420 (-OH), 1730 (C=O), 1610 (aromatic), 1500, 1440, 1320, 1200 (C-O-C), 1040, ^{1}H-NMR (CD_3OD) δ: 3.78 (1H, dd, J=8, 13 Hz, β-glucose-H-6), 3.85 (1H, dd, H=8, 13 Hz, α-glucose-H-6), 4.16 (1H, m, α-glucose-H-5), 4.60 (1H, m, β-glucose-H-5), 5.15-5.35 (8H, m, α,β-glucose-H-1,2, 4), 5.45 (2H, t, α,β-glucose-H-3), 6.33, 6.35, 6.51, 6.55, 6.60, 6.61, 6.67, 6.68 (8H, each s, HHDP-H), ^{13}C-NMR (CD_3OD) δ: 64.12, 67.72, 69.98, 70.38, 72.37, 72.99, 76.18, 76.38, 78.10, 78.71, 92.12 (α-C-1), 95.65 (β-C-1), 107.67-108.6 (m), 114.81-116.54 (m), 125.88-126.58 (m), 137.04-137.46 (m), 144.54-145.83 (m), 169.04, 169.08, 169.57, 169.70, 170.18, 170.33, 170.75, 170.79 (-COO-).

HPLC of compound V: When the mobile phase was CH_3CN/H_2O (60/40, pH=4.66 with HAc), two peak appeared. The two peak were separated by the semipreparative HPLC. The separated fraction was applied on the analytical HPLC with the same above condition. It was divided two peaks (column: μ-Bondapak C_{18} (4 mm×300 mm, 10 μm)).

Acid hydrolysis of compound V: When Compound **V** was hydrolysed by 5% H_2SO_4, yellow crystal was produced. The aglycone was identified with ellagic acid on the basis of TLC (Benzen : Ethyl formate : Formic acid=1 : 5 : 2). The sugar part was done CO-TLC with each sugar samples.

Cell cytotoxicity: Human lung adenocarcinoma cell (PC_{14}) and human gastric adenocarcinoma cell (MKN_{45}) were used as the cell line for cytotoxicity. The medium was RPMI-1640 containing 5% fetal bovine serum, streptomycin and penicillin. Single cell suspension of PC_{14} cell and MKN_{45} cell (concentration: 3.5×10^4 cell/ml) was added 135 μl to 96 well flat-bottom microplate. After addition of 15 μl each compound samples (dose: 1 μg/ml, 10 μg/ml, 100 μg/ml), it was incu-

bated for 4 days, 37℃, 5% CO_2 incubator. MTT solution was added 15 μl and incubated for 4 hours, 37℃, 5% CO_2 incubator. 0.04 N-isopropanol was added 150 μl. Optical density was measured by UV spectrophotometer at 540 nm. The cell cytotoxic activity was indicated as percent survival (%).

$$\text{Percent survival} = \frac{\text{Optical density of samples}}{\text{Optical density of control}} \times 100$$

RESULTS AND DISCUSSION

In previous study, we found that 80% aqueous acetone extract of *Duchesnea chrysantha* showed antibacterial and antitumor activity. Thus the isolation of the active principles from the extract was conducted using Sephadex LH-20 and MCI gel CHP 20P chromatography. The solvent systems of water - 100% MeOH - acetone, EtOH - MeOH - acetone were used as a eluent. Five tannin compounds which were positive in $FeCl_3$ reagent were obtained. Their cytotoxicities were screened by MTT assay.

Analysis of Compounds

Compound I: It was presumed phenolic compound because it was positive in $FeCl_3$ and singlet peak showed at δ 7.15 in its ^{1}H-NMR spectrum. It had the same Rf value with the authentic sample of gallic acid. So compound I was identified as gallic acid which had been isolated previously.

Compound II: The positive reaction in $FeCl_3$ and UV spectrum suggested that compound II was phenolic compound. Its ^{1}H-NMR spectrum showed a one proton peak at 7.35 ppm which was corresponded to aromatic proton. The peak at 4.45 ppm was thought to have carboxyl group and double bond closely. Two peaks of 3.05, 2.56 ppm were assignable to nonequivalent protons with geminal coupling. Its ^{13}C-NMR showed signals due to carbonyl carbon at 192.99 ppm, carboxyl carbon at 173.15 ppm, ester carbon at 160.04 ppm. The peaks at 149.42, 140.09 ppm of the aromatic region were assignable to the double bond which was bonded to aromatic ring. Its MS spectrum showed m/z 293 as $(M+H)^+$ peak. It showed the same Rf value with the authentic sample of brevifolin carboxylic acid at CO-TLC test. All above data were coincided with the spectrum data of brevifolin carboxylic acid of the literature (Von Otto *et al.*, 1967) and led to the conclusion that compound II was brevifolin carboxylic acid.

Compound III: It became green in the reaction with $FeCl_3$. In its UV spectrum, a shoulder was observed at 300 nm which was a distinctive feature of caffeic acid groups (U Won Sik, 1989). The singlet peak at 3.34 ppm in ^{1}H-NMR spectrum was presumed as the methyl proton of $COO-CH_3$. The other peaks in ^{1}H-NMR were similar to the data of caffeic acid in the literature (Von Otto *et al.*, 1967). Compound III was identified as methyl caffeate.

Compound IV: It became dark green in reaction with $FeCl_3$. It was confirmed as protocatechuic acid by comparison with the reported ^{1}H-NMR data of authentic sample. It showed the same Rf value of the authentic sample. So compound IV was identified as protocatechuic acid.

Compound V: It showed deep blue in reaction with $FeCl_3$. Its IR spectrum showed strong absorbance peak at 3400 cm^{-1}. It suggested it to be phenolic compound. In ^{13}C-NMR spectrum, eight carbon peak of carboxyl group showed between 169.04 ppm and 170.79 ppm and two anomeric carbon peaks of sugar at 92.12 and 95.65 ppm sugesting a mixture of anomeric isomer. In ^{1}H-NMR spectrum the multiplet near 3.7 ppm could be interpreted as two overlapping doublet signals of α-H-6 and β-H-6. It was presumed as ellagic acid glycoside because of the eight proton peaks over 6.00 ppm. The shifts of the hydrogens of the bridging HHDP groups always appear upfield from the galloyl hydrogens (Margaret K. *et al.*, 1970). The bond position of ellagic acid and sugar was suggested 2, 3 and 4, 6 position of sugar. The sugar protons were appeared under 5.40 ppm indicating the anomeric hydroxy to be free. Whether or not the anomeric hydroxy was aroylated could generally be determined by a prominent downfield signal over 6.00 ppm for H-1, for the

Table I. Effect of samples on human lung adenocarcinoma cell line (PC_{14}) & human gastric adenocarcinoma cell line (MKN_{45})

compound	dose (μg/ml)	PC_{14} (1.629)	MKN_{45} (1.431)
gallic acid	1	88.8%	90.3%
	10	77.5	85.9
	100	62.1	74.1
brevifolin carboxylic acid	0.5	88.5	89.5
	5	45.6	53.8
	50	32.2	40.2
methyl caffeate	1	95.3	98.1
	10	90.5	89.8
	100	35.9	57.4
protocatechuic acid	1	99.4	97.2
	10	90.9	87.1
	100	34.7	33.6
pedunculagin	1	94.4	91.8
	10	69.9	74.8
	100	38.1	43.9

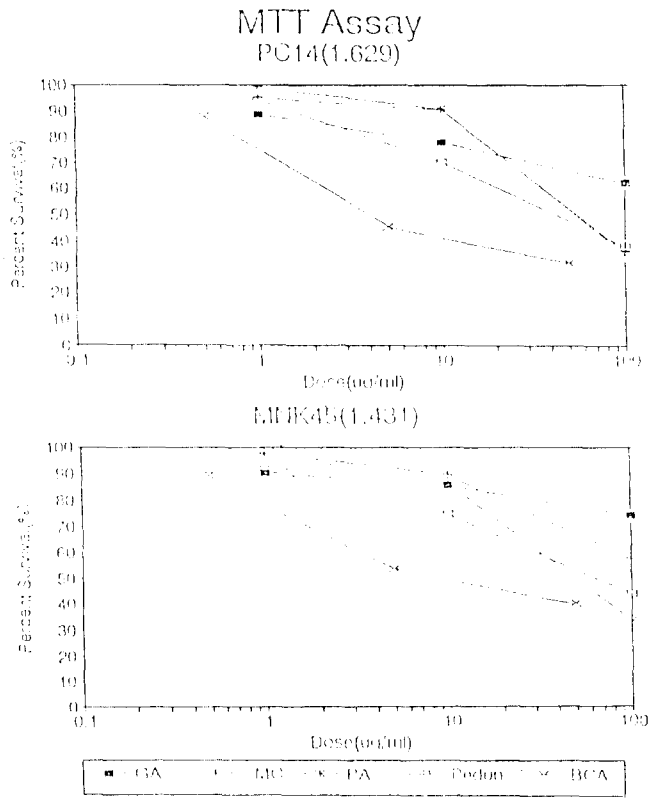


Fig. 2. IC_{50} graph of *Duchesnea chrysantha* in PC_{14} and MKN_{45} cells.

so-called aroylated H-1 or under 5.40 ppm for the non-aroylated H-1 (Margaret K. *et al.*, 1970). In HPLC, two peaks of the ratio of three to seven were shown (mobile phase: $CH_3CN/H_2O=60/40$, pH=4.66 with HAc). To confirm that the anomeric OH was free, we separated the fraction of two peaks by use of semipreparative HPLC. When the separated fraction was applied to HPLC, the single peak was separated to two peaks of the same ratio. It was caused by mutarotation of sugar. So compound V was shown to have the free anomeric OH. Compound V was hydrolysed with 5% H_2SO_4 to give yellow crystal. It showed blue in reaction with $FeCl_3$ and had same Rf value with ellagic acid on the TLC. It showed singlet peak at 7.44 ppm in ^{1}H-NMR. Ellagic acid was produced by hydrolysis of ellagitannin. Sugar part of compound V was identified as D-glucose. All the spectral data led to the conclusion that compound V was 2,3-4,6-di(s) HHDP-D-glucose. It was pedunculagin and confirmed by comparison with the spectral data of pedunculagin reported in literature (Takashi Tanaka *et al.*, 1986).

Cancer cell cytotoxicity of isolated compounds I-V

The cytotoxicities of the five compounds isolated from *Duchesnea chrysantha* was listed in Table I. Gallic acid, methyl caffeate, protocatechuic acid and pedunculagin showed relatively lower cytotoxicity. Brevifolin carboxylic acid showed 32.2% percent survival for PC_{14} cell line at the 50 μg/ml and 40.2% for MKN_{45} cell line. As shown in Fig. 2, the IC_{50} value of brevifolin carboxylic acid for PC_{14} was 3.95 μg/ml, which was almost similar to the IC_{50} value (4 μg/ml) against mouse leukemia cell. Brevifolin carboxylic acid strongly inhibited survival of PC_{14} cells.

ACKNOWLEDGEMENTS

This paper was supported by the Korean Cultural Research Institution of Ewha Womans University.

REFERENCES CITED

Ihn Rhan Lee, Seung Won Wee, Yong Nam Han, Studies on the Pharmacological Actions and Biologically Active Components of Korean Traditional Medicone (VI). Tannins from *Duchesnea indica*. *Kor. J. Pharmacogn.*, 20(2), 110-116 (1989).

Ihn Rhan Lee, Seung Hye Hahn, Young Nam Han, Studies on the pharmacological actions and biologically active components of Korean traditional medicines. Anticancer macromolecular substances from *Duchesnea chrysantha* herba. *Saengyak Hakhoechi*, 21(2), 186-92 (1990).

Ihn Rhan Lee, Young Hee Kim, Studies on the antitumor activity of *Duchesnea chrysantha* herba. *Arch. Pharm. Res*, 9(1), 1-4 (1986).

Ihn Rhan Lee, Young Hee Kim, Gwang Ja Jeong, Studies on the pharmacological actions and biological active components of Korean traditional medicine. *Yakhak Hoeji*, 31(4), 230-235 (1987).

Margaret K. Seikel and W. E. Hillis, Hydrolysable Tannins of *Eucalyptus delegatensis* Wood. *Phytochemistry*, 9, 1115-1128 (1970).

Mukherjee K. S., Bhattacharya M. K., Ghosh P. K., Phytochemical investigation of *Aesculus indica* Linn. and a2Fragaria indica Andr. *J. Indian Chem. Soc.*, 60(5), 507-508 (1983).

Stuart G., Chinese Materia Medica, Vegitable Kingdom, Shanghi, 178 (1991).

Takashi Tanaka, Gen-Ichiro Nonaka and Itsuo Nishioka. Tannins and Related Compounds. XI Revision of the Structures of Punicalin and Punicalagin, and Isolation and Characterization of 2-O-Galloylpunicalin from the Bark of *Punica granatum* L. *Chem. Pharm. Bull.*, 34 (2), 650-655 (1986).

Von Otto Th, Schmidt, Rudolf Eckert, Brevifolin carbonsaure, ihre optisch aktiven Formen und ihre Bindung an Glucose im Algarobina, einer neuen kristallisieten Verbindung aus Algarobilla. Eugenia Giinther und Hartmut Fiesser; *Liebig Ann. Chem.*, 706, 804-212 (1967).

Won Sik Woo, Reserch method of natural product, p. 65, Mineumsa, 65 (1989).