Inhibition of Matrix Metalloproteinase-2 Activity of Flavonol Glycosides from *Cedrela sinensis*

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Cedrela sinensis is a broadleaf tree that is widely cultivated in Korea and China. It was used for treating enteritis, dysentery, and skin itch in oriental medicine. In this study, three major flavonoids, kaempferol-3-O-rhamnoside (1), quercetin-3-O-rhamnoside (2), and quercetin-3-O-glucoside (3), were isolated from the leaf of *Cedrela sinensis*. The biological activities of these compounds were tested by inhibitory activity of matrix metalloproteinases-2 (Type IV collagenase) method together with a cytotoxicity and a apoptosis test against human cancer cell lines.

Key words – *Cedrela sinensis*, flavonol glycosides, cytotoxic activity, apoptosis, matrix metalloproteinases-2

Introduction

The majority of cancer patients who surrender to their disease die from metastasis[1,2]. Metastasis is the spread of cancer cells from a primary lesion to distant sites. Invasion of malignant tumor cells is required for the formation of metastatic colonies[3,4]. Invasive tumor growth and metastasis are complex processes[5]. The initial steps include the degradation of stromal architecture and basement membrane components, especially type IV collagen. Uncontrolled expression of type IV collagenases, matrix metalloproteinase-2 (MMP-2), is a critical part of the invasive potential of tumor cells and is affected by the balance between the enzymes and the inhibitors secreted by the cell[6,7]. MMP inhibitors block the action of a family of enzymes, the matrix metalloproteinases, used by metastatic cancer cells to break down and remodel tissue matrices during the process of metastatic spread[8]. It is hoped that these inhibitors will be relatively non-toxic since they are designed not to kill the cancer cell, but to modify its behavior[1].

Some medicinal plants are employed for a syndrome expressed in oriental medicine as chest paralysis and tumor are thought to be effective for angina pectoris. Therefore, we investigated the effects of an oriental medicinal plant, *Cedrela sinensis*. The leaves of *C. sinensis* are have been used as oriental medicine for treating enteritis, dysentery and itch and no irreversible side effects were observed after treatment[9-11]. In addition, Koreans have been consuming the young leaves as herb salad during early spring. This study was to find flavonoid derivatives as Matrix metalloproteinase-2 inhibitors from *C. sinensis*. Also we compared their inhibitory activities of etoposide-induced apoptosis and *in vitro* cytotoxic activities against human tumor cell lines.

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Materials and Methods

Plant material

C. sinensis leaves were collected from Oksan-ri, Munsan, Gyeongsangnam-do, Korea. The scientific name was determined by Prof. Myong Gi Chung of Gyeongsang National University. A voucher specimen (*S. W. Hwang & M. S. Yang 023*) was deposited at the herbarium of the university.

Instruments

For isolation, silica gel (MERCK, Germany) and Sephadex LH-20 (Pharmacia Co., Sweden) were used. All other chemicals used in this study were analytical grade. UV spectra were measured using a Beckman DU650 spectrophotometer. ^{1}H and ^{13}C NMR and 2D-NMR data were obtained on a Bruker AM 500 (^{1}H-NMR at 500 MHz,

^{13}C-NMR at 125 MHz) spectrometer. Molecular weight was measured with EI-MS (JEOL JMS-700) spectrometer.

Extraction and isolation

The air-dried leaves of *C. sinensis* (2.5 kg) were extracted with MeOH (10L×3) at room temperature for 72 hr. The MeOH solution was combined, concentrated, and dried under reduced pressure at temperature not higher than 45°C. The MeOH extract (320 g) was successively partitioned with $CHCl_3$, EtOAc, and H_2O fractions. The EtOAc extract (45 g) was chromatographed over silica gel (500 g; 70-230 mesh) using CH_2Cl_2-EtOH (19:1→1:1) gradient to give 12 fractions (F1-F12). Fraction F10 was submitted to a silica gel column chromatography eluted with $CHCl_3$-MeOH (19:1→1:1) gradient resulting in 10 subfractions. Subfractions 7~9 were rechromatographed on silica gel with $CHCl_3$-MeOH (9:1→1:1) gradient to yield compound **1** (98 mg). Fraction F11 was submitted to a silica gel column chromatography eluted with $CHCl_3$-MeOH (9:1→1:1) gradient resulting in 7 subfractions. Subfractions 5~6 were rechromatographed on silica gel with $CHCl_3$-MeOH (9:1→ 1:1) gradient to yield compounds **2** (120 mg) and **3** (42 mg).

Inhibition of matrix metalloproteinase-2

The catalytic activity of MMP-2 was analyzed by peptide cleavage assay using a quenched fluorescent peptide, Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (Sigma, USA) as a substrate[12,13]. 20 ng of Pro-MMP-2 (Boehringer Manheim, Germany) was activated in the presence of a final concentration of 1 mM *p*-aminophenylmercuric acetate (Sigma, USA) at 37°C for 30 min. The activated MMP-2 (18 ng) was incubated in 40 μl of the TNBC buffer containing 1 μ M of the peptide in the presence of various concentrations of the test compounds at 37°C for 30 min. The reaction was stopped by the addition of 0.1 M sodium acetate (pH 4.0) at final concentration. The fluorescence was measured by a Perkin-Elmer LS-50B fluorometer at excitation wavelength of 328 nm and emission of 393 nm. The potency of inhibition was measured from the amount of substrate cleavage obtained using a range of test compound concentrations and, from the resulting dose-response curve, an IC_{50} value was calculated.

Inhibition of etoposide-induced apoptosis

The etoposide-induced caspase induction assay was conducted in the U 937 leukemia cells using pyrrolidine dithiocarbamate (PDTC) as a standard apoptosis inhibitor[13]. Etoposide (10 μM) was added to the U 937 cells in the presence or absence of various concentrations of the test compound. The cells were incubated for 7 hr at 37°C in a 5% CO_2-95% air atmosphere. After determining apoptosis cells by microscopy, the CPP32 protease activity was estimated from the cell lysate using DEVD-AFC as substrate[15].

Cytotoxic test

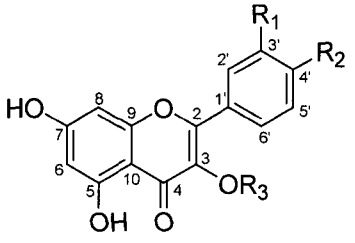
The isolated compounds were examined for their *in vitro* cytotoxic activity against human tumor cell lines such as HCT 15, LOX-IMVI, NCI H23, PC 3 and A 549. The growth inhibitory property was determined by *in vitro* treatment of the respective cell lines using the sulforhodamine B assay (SRB)[16].

Results and Discussion

Structure identification

We isolated and identified the biologically active compounds from the ethyl acetate extract as three flavonol glycosides, compound **1**, **2**, and **3** (Fig. 1). The structure of each compound was established by the following evidence.

Kaempferol-3-O-rhamnoside **(1)**; Yellow crystal; mp 209-210°C; UV λmax(MeOH) 221, 253, 265, 285, 435 nm; MS(EI, 70 eV, m/z, rel. int.): 284(M^+), 255, 227, 213, 167, 149; IRν(KBr, cm^{-1}) 3417, 1627, 1479 cm^{-1}; ^{1}H-NMR(500 MHz, CD_3OD, δ): 6.09(1H, d, J=1.3Hz, H6), 6.22(1H, d, J=1.3Hz, H8), 7.73(2H, d, J=8.7Hz, H2′ and H6′), 6.91(2H, d, *J* = 8.6Hz, H3′ and H5′), 5.35(1H, s, H1″), 4.21(1H, s, H2″), 3.72(1H, dd, J=7.9, 3.0 Hz, H3″), 3.31(1H, dd, J=3.5, 2.0 Hz, H4″), 3.36(1H, m, H5″), 0.94(3H, m, H6″); ^{13}C-NMR(125 MHz, CD_3OD, δ): Table 1.



Compound 1: R1=H, R2=OH, R3=rhamnose
Compound 2: R1, R2=OH, R3=rhamnose
Compound 3: R1, R2=OH, R3=glucose

Fig. 1. Structures of compound 1, 2 and 3.

Quercetin-3-O-rhamnoside **(2)**; Yellow powder; mp 174 -176°C; UV λ_{max}(MeOH) 259, 304, 352 nm; (+$AlCl_3$) 278, 306, 335 nm; (+NaOAc) 275, 324, 373; ($AlCl_3$/HCl) 273, 304, 354; (+NaOAc/H_3Bo_3)263, 303, 369; MS(EI, 70 eV, m/z, rel. int.): 448, 447, 355, 283, 255; IRν(KBr, cm^{-1}) 3432, 1660, 1445 cm^{-1}; ^{1}H-NMR(500 MHz, CD_3OD, δ): 6.15(1H, s, H6), 6.31(1H, s, H8), 7.33(1H, s, H2′), 6.91(1H, d, J = 6.5 Hz, H5′), 7.27(1H, d, J=7.5 Hz, H6′), 5.34(1H, s, H1″), 4.22(1H, s, H2″), 3.76(1H, brd, J=7.5 Hz, H3″), 3.31(1H, dd, J=3.5, 2.0 Hz, H4″), 3.43(1H, m, H5″), 0.94(3H, m, H6″); ^{13}C-NMR(125 MHz, CD_3OD, δ): Table 1.

Quercetin-3-O-glucoside **(3)**; Yellow powder; mp 234-236°C; UV λ_{max}(MeOH) 258, 357 nm; (+$AlCl_3$) 275, 434 nm; (+NaOAc) 270, 383; ($AlCl_3$/HCl) 269, 360, 400; (+NaOAc /H_3Bo_3) 262, 382; MS(EI, 70 eV, m/z, rel. int.): 464, 367, 183, 125; IRν(KBr, cm^{-1}) 3345, 1662, 1498 cm^{-1}; ^{1}H-NMR(500 MHz, CD_3OD, δ): 6.15(1H, s, H6), 6.26(1H, s, H8), 7.83(1H, s, H2′), 6.85(1H, d, J = 6.5 Hz, H5′), 7.56(1H, d, J=7.5 Hz, H6′), 5.05(1H, s, H1″), 3.70(1H, s, H2″), 3.59(1H, m, H3″), 3.30(1H, dd, J=3.5, 2.0 Hz, H4″), 3.57 (1H, m, H5″), 1.17(3H, m, H6″); ^{13}C-NMR(125 MHz, CD_3OD, δ): Table 1.

Table 1. ^{13}C-NMR spectral data of compound 1, 2 and 3 (CD_3OD, 125MHz)

Position	1	2	3
2	159.2	146.9	156.3
3	136.3	136.4	133.3
4	179.6	179.7	177.4
5	163.3	163.4	161.2
6	101.4	101.1	98.6
7	169.8	168.7	164.1
8	96.0	95.7	93.5
9	159.5	150.3	156.3
10	105.3	105.6	104.0
1′	123.1	123.4	121.6
2′	132.2	117.3	115.2
3′	117.0	159.1	144.8
4′	162.0	159.4	148.4
5′	117.0	116.8	116.2
6′	132.2	123.2	121.6
Rhamnosyl			
1″	103.9	103.9	
2″	72.3	72.3	
3″	72.4	72.4	
4″	73.6	73.7	
5″	72.5	72.5	
6″	18.0	18.0	
Glucosyl			
1″			100.9
2″			74.1
3″			76.5
4″			70.0
5″			77.5
6″			61.0

Inhibition of matrix metalloproteinase-2

The flavonol glycosides isolated from the *C. sinensis* were tested for their ability to inhibit MMP-2. A synthetic anti-metastatic agent, N-carbobenzoxy-Pro-Leu-Gly hydroxamate (Sigma), was used as positive control. The addition of three flavonol glycosides and positive control to the reaction mixture dose-dependently inhibited MMP-2. As shown in Table 2, all tested samples exhibited desirable activity. The 50% inhibitory concentration (IC_{50}) values of following kaempferol-3-O-rhamnoside (1), quercetin-3-O-rhamnoside (**2**), quercetin-3-O-glucoside (**3**), and CBZ-PLG-NHOH (N-carbobenzoxy-Pro-Leu-Gly hydroxamate) were 3.24, 2.42, 1.61, and 1.52 μg/mL, respectively. Quercetin-3-O-glucoside (**3**) exhibited the highest inhibitory activity with similar IC_{50} value of CBZ-PLG-NHOH. Although the activity of compounds **1** and **2** were lower than that of CBZ-PLG-NHOH, they still exhibited significant activities. Compounds **2** and **3** have the same aglycone as quercetin, but compound **3** was more effective than compound **2** due to the conjugate of glucose rather than rhamnose. This results showed that the glucose was much more important than the rhamnose in MMP-2 inhibition. Although, the present study demonstrated that three flavonol glycosides are potent inhibitors of the MMP-2.

Inhibition of etoposide-induced apoptosis

The etoposide-induced caspase induction assay was conducted in the U 937 leukemia cells using pyrrolidine dithiocarbamate (PDTC) as a standard apoptosis inhibitor. As shown in Table 3, all tested compounds inhibited the U 937 cell apoptosis induced by etoposide dose-dependently, but did not show significant inhibition values compared with the PDTC.

Table 2. Inhibiton of the isolated compounds and CBZ-PLG-NHOH on the matrix metalloproteinase-2

Compounds	IC_{50} (μg/mL)
Compound 1	3.24 ± 0.34
Compound 2	2.42 ± 0.28
Compound 3	1.61 ± 0.03
CBZ-PLG-NHOH*	1.52 ± 0.03

*N-carbobenzoxy-Pro-Leu-Gly hydroxamate.

Table 3. Inhibiton of the isolated compounds and PDTC on the etoposide-induced apoptosis[b]

Compounds	IC_{50} (μg/mL)
Compound 1	85.7 ± 0.8
Compound 2	25.0 ± 0.8
Compound 3	20.1 ± 0.6
PDTC	8.0 ± 0.5

[a] Pyrrolidine dithiocarbamate.
[b] Etoposide-induced caspase 3 induction in U937.

Cytotoxic test

All the three compounds were evaluated *in vitro* cytotoxic activity against human tumor cell lines, comprising HCT 15, LOX-IMVI, NCI H23, PC 3, and A 549 by sulforhodamine B (SRB) assay method. Three flavonol glycosides did not show a significant activity even at a concentration of 100 μg/mL (data not shown).

As flavonoids, numerous previous studies have been focused on potential direct effects on cancer cells in growth, survival signaling, and cell cycle regulation[17]. Here, we evaluated the effects of three flavonol glycosides on the MMP-2 inhibition.

Conclusively, the flavonol glycosides in *C. sinensis* showed pronounced MMP-2 inhibition but did not show other cancer related bioactivity. There are only a few effective antimetastatic chemotherapeutic agents currently available for clinical use, and most of them have life-threatenening adverse effects. The results in this study suggest that they might be new antimetastatic agents and that the *C. sinensis* may a useful herb.

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초록 : 참죽나무에서 분리한 flavonol glycoside의 금속단백분해효소-2 억제 활성

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참죽나무는 멀구슬과 나무로서 한국과 중국에 널리 분포하고 있다. 한방에서는 장염 (腸炎), 이질 (痢疾), 개선 (疥癬) 등의 치료에 이용되고 있다. 본 연구는 참죽나무 잎에서 3종의 flavonol glycosides를 분리하여 NMR을 통해 구조를 확인한 결과, kaempferol-3-*O*-rhamnoside (**1**), quercetin-3-*O*-rhamnoside (**2**)와 quercetin-3-*O*-glucoside(**3**)로 구조 동정되었다. 분리된 화합물들의 생리활성은 matrix metalloproteinase -2 저해 활성과 인체암 세포주에 대한 세포독성과 apoptosis 실험을 통하여 항암효과를 조사하였다. 그 결과 인체암 세포주에 대한 세포독성과 apoptosis 저해활성은 나타내지 않았으나, MMP-2 활성저해 조사에서는 강한 저해 효과를 나타내었다. 특히 quercetin-3-*O*-glucoside는 암세포에 직접적인 독성을 보이는 것이 아니라 암의 침윤과 전이에 특이적으로 작용하는 물질로 보여진다.