Isolation and Characterization of a New Alkaloid from the Seed of *Prunus persica* L. and Its Anti-inflammatory Activity

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Persicaside has been isolated as a new alkaloid natural compound from a methanol (EtOA)-soluble extract of *Prunus persica* seed. It was purified by a combination of chromatographic techniques and recrystallization. The structure of Persicaside was determined by extensive NMR experiments and mass ppectroscopic data. It inhibited nitric oxide (NO) and prostaglandin E_2 (PGE_2) production *via* suppression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2 expression in rat osteoblast sarcoma cells (ROS 17/2.8) in concentration-dependent manner whereas it spares the COX-1 enzyme activity.

Key Words : Persicaside, Nitric oxide, Prostaglandin E2, Inducible nitric oxide synthase, Cyclooxygenase-2

Introduction

Prunus persica (L.) BATSCH (Rosaceae) seeds are well known as a traditional folk medicine (Persicae Semen; Tounin, Taoren in Chinese) in China, Korea, and other Asian countries.[1] They are frequently used as an ingredient in a variety of Chinese medicine prescriptions, particularly those used to treat women's diseases.[2-6]

The chemical constituents of the herb include the cyanogenic glycosides, amygdalin and prunasin as major components along with glycerides, sterols, and emulsin.[7] Recently, glycosides from this plant seeds have been reported for the anti-tumor activity promoting Epstein-Bar virus activity in early antigen-infected lymphoblastoid cells.[8] Amygdalin is also abundant in the seeds of bitter almond and apricots of the *Prunus* genus, and other rosaceous plants. Amygdalin extracted from *Persicae semen* was studied for anticancer activity recently.[9] Arichi *et al.* reported anti-inflammatory activity of aqueous extraction of *Persicae semen* based on carrageenin-induced hind paw edema in rats, in which two proteins PR-A and PR-B were fractionated and assumed to be responsible for the anti-inflammatory activity. However, little is known concerning the other chemical constituents of *Prunus persica* about anti-inflammatory effects.

Although causes of autoimmunity remain largely unknown, control of inflammation is apparently critical in pathogenic treatments.[10] For instance, bone formation that is essential for skeletal growth, remodeling and repair involves the synthesis and deposition of mineralizing extracellular matrix by osteoblasts, and bone resorption by osteoclasts.[11,12] The differentiation and proliferation of osteoblasts can be affected by numerous extracellular factors such as hormones, growth factors and cytokines.[13] Anti-inflammatory drugs would be potent for immune regulation to avoid a possible autoimmune diseases such as various sclerosis which are considered to be etiogenic to rheumatism and tumors along with oncogenes. Bone tumors are eventually developed into microscopic metastases in diagnosis.[14] Lung is the most common metastatic site, and fatality grows abruptly after crossing this phase. Patients need to respond to systemic chemotherapy plus and/or radiation under their weakened physical state for survival. Recurrent patients are, however, particularly resistant to repeated chemotherapy, leaving few treatment options. Thus, as in the design of non-steroid anti-inflammatory drugs (NSAIDs),[15,16] new drugs of long lasting but adverse effects are necessary to be found.

In this work, we report isolation of a new natural compound extracted from *Prunus persica* seed and its anti-inflammatory activities on rat osteoblast sarcoma cells (ROS 17/2.8).

Experimental Section

General Experimental Procedures. Reagents were purchased from Aldrich and Sigma, and used without further purifications. Solvents from Fisher were dried and purified with standardized procedures. HPLC separations were accomplished on symmetry Prep C_{18} column (2.0 × 30 cm; 7-μm particle size; flow rate of 4 mL/min). NMR spectra were obtained in acetone-d6 using a JEOL Eclipse-500 MHz spectrometer and referenced relative to residual solvent resonances at δ2.04 for ^{1}H and 29.8 for ^{13}C signals. Purified compound was identified by Micro Mass Quatro LC to obtain the ESIMS Data..

Plant Materials. The seeds of *Prunus persica* were purchased from the herbal medicine co-operative association of Jeonbuk Province, Korea, in October 2003. A voucher specimen (no. PP 777) was deposited at the Herbarium of the College of Oriental Medicine, Wonkwang University (Korea).

Extraction and Isolation. The seeds of *Prunus persica* (ca 600 g) was chopped to pieces and extracted with

methanol for one week at room temperature to obtain 17.3 g of extract. Then, the dried residue was suspended into deionized water and was extracted with *n*-hexane (2.1 g), and subsequently ethyl acetate solvent (1.3 g).

The ethyl acetate fraction was placed in round bottom flask and evaporated under reduced pressure followed by being dissolved by 5 ml of methanol. The methanolic solution was applied on a glass-column chromatography (30 mmID) packed with 60 g of silica gel, and eluted with stepwise gradient mixtures of the methanol-dichloromethane solvent system. From this chromatography, the eleven fractions were divided as follows: fraction 1 (94.4 mg), fraction 2 (29.2 mg), fraction 3 (140.3 mg), fraction 4 (232.2 mg), fraction 5 (83.2 mg), fraction 6 (141.4 mg), fraction 7 (121.4 mg), fraction 8 (61.3 mg), fraction 9 (33.8 mg), fraction 10 (29.2 mg), fraction 11 (44.9 mg).

Fraction 4 which showed a desired physiological activity was purified on reverse phased HPLC with a gradient elution of 0 to 100% aqueous CH_3CN over 60 min. to yield four compounds. Among them, a compound (6.0 mg) at a retention time of 39 min. was identified as a new compound with physiological activities and was named as percicaside.

Osteoblast Sarcoma Cell Line Culture. Rat osteoblast sarcoma cell line ROS 17/2.8 was obtained from American Type Culture Collection (ATCC, TIB 71, Maryland, USA). The cells were maintained at 1×10^6 cells/mL culture in complete RPMI 1640 medium supplement with 10% heat-inactivated fetal bovine serum, 1% l-glutamine, 1% non-essential amino acids, 1% antibiotic/antimycotic (100 U/mL of penicillin, 25 μg/mL of amphotericin D, and 100 μg/mL of streptomycin), 1.5% sodium bicarbonate, and 1% minimal essential vitamins at 37 °C in a humidified 5% CO_2 atmosphere.

Measurement of Nitrite Concentration. Accumulated nitrite, an oxidative product of NO, was measured in the culture medium by Griess reaction. Briefly, 100 μL of cell culture medium were mixed with 100 μL of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydorchloride/2.5% phosphoric acid) and incubated at room temperature for 10 min, then the absorbance at 540 nm in a microplate reader. Fresh culture medium was used as the blank in all experiments. The nitrite levels in the samples were calculated from a nitrite standard curve freshly prepared in culture medium.

Measurement of PGE_2 Concentration. Cells (1×10^6/mL) were pre-incubated 2 h with Persicaside and further cultured 6 h or 18 h with CM (100 U/mL IL-1β, 200 U/mL IFN-γ, and 500 U/mL TNF-α) in 24-well plates. Supernatants were removed at the allotted times and PGE_2 levels were quantified by immunoassay kits according to the manufacture's protocols (R&D System, Minneapolis, MN, USA).

Analysis of iNOS, COXs, and β-actin Protein Expression. Cellular proteins were extracted from control and m-2-treated ROS 17/2.8 cells. The washed cell pellets were resuspended in cold lysis buffer (10 mM Tris-base, 5 mM EDTA, 50 mM NaCl, 1% triton X-100, 5 mM phenylmethylsulfonyl fluoride, 2 mM sodium orthovanadate, 10 μg/mL leupeptin, 25 μg/mL aprotinin) and incubated with 30 min at 4 °C. Nuclei and cell debris were removed by microcentrifugation, followed by quick freezing of the supernatants. 30 μg of cellular proteins from treated and untreated cell extracts were electroblotted onto nitrocellulose membrane following separation on a 10% sodium dodecyl sulfate (SDS)-polyacylamide gel electrophoresis. The immunoblot was incubated overnight with blocking solution (5% skim milk) at 4 °C, followed by incubation for 4 h with appropriate dilutions of primary antibodies (against rabbit anti-iNOS, rabbit anti-COX-1, and rabbit anti-COX-2). Blots were washed 2 times with PBS and incubated with a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG for 1h at room temperature. Blots were again washed three times in tween 20/Tris-buffered saline (TTBS) and the developed with 10 mL of a 1:1 mixture of solutions of ECL detection system for 1 min, dried quickly, and exposed to a film for 2-20 min. Protein concentration was determined by Bio-Rad protein assay reagent according to the manufacture's instruction.

Results and Discussion

Persicaside (**1**), a white powder separated and recrystallized from the seed of *Prunus persica*, was characterized by 1D and 2D NMR spectra, and mass spectroscopy. Table 1 listed the ^1H and ^{13}C NMR spectral data of for this compound.

Persicaside was supposed to possess aromatic ring and β-glucopyranosyl group on the basis of chemical shifts and coupling constants in the ^1H and ^{13}C NMR spectra. And the chemical shifts at δ 171.8 and 165.8 indicated the presence of two carbonyl carbons in this molecule. The correlations of

Table 1. ^{13}C-NMR, ^1H-NMR spectral data and HMBC correlations of Persicaside (**1**)

No	δ_C	δ_H (int., mult., *J* in Hz)	HMBC ($^1H \rightarrow {}^{13}C$)
1	100.3	4.23 (1H, d, 7.8)	2', 2
2	74.9	3.42 (1H, dd, 8.7, 7.8)	3
3	77.4	3.38 (1H, dd, 9.2, 8.7)	1, 2
4	71.5	3.49 (1H, m)	3, 5, 6
5	75.2	3.48 (1H, m)	1, 4, 6
6	64.9	4.44 (1H, dd, 11.9, 6.0) 4.69 (1H, dd, 11.9, 1.8)	1, 5, 1
1'	172.6		
2'	79.4	5.18 (1H, s)	1, 1', 4'(8')
3'	137.7		
4'(8')	129.0	7.30 (2H, m)	2', 4'(8')
5'(7')	128.8	7.43 (2H, m)	3', 5'(7')
6'	129.1	7.30 (1H, m)	4'(8')
1"	166.6		
2"	131.3		
3" (7")	130.3	8.09 (2H, dd, 8.3, 1.4)	1", 3" (7")
4" (6")	129.5	7.56 (2H, dd, 8.3, 7.3)	2", 4" (6")
5"	133.9	7.67 (1H, tt, 7.3, 1.4)	3" (7")

COSY spectrum confirmed the presence of β-glucopyranosyl unit. The assigned anomeric proton at δ 4.23 showed the correlation with the methine carbon (C-2') at δ 79.4 from the HMBC experiment, indicating the connectivity by a β-linkage. The singlet proton bonded directly to C-2' also exhibited the HMBC correlations with four neighboring carbons at δ 100.3, 129.7, 137.7 and 172.6. Further analysis induced this partial structure to be 2-phenyl-2-hydroxyl acetic acid or 2-phenyl-2-hydroxyl acetamide.

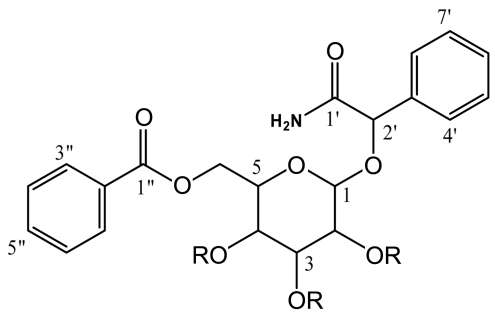
On the other hand, the methylene protons at the position of C-6 in β-glucopyranosyl unit also gave the HMBC correlations with the other carbonyl carbon at δ 166.6, which was connected to the other phenyl group at the terminal of the compound. This evidence was observed by HMBC correlations of two protons at δ 8.09 with the carbonyl carbon(C-2").

Accordingly, the completion of the planar structure of this compound is dependent on the functional group attached at the C-1'. The molecular peak at *m*/*z* 417 in the ESI-MS led the aglycon part to be 2-phenyl-2-hydroxyl acetamide and furthermore the ^{1}H and ^{13}C NMR spectra of **2** obtained on acetylation of **1** exhibited three additional methyl and carbonyl signals corresponding to three hydroxyl groups, respectively. Therefore, the structure of Persicaside. (**1**) was determined as 6-*O*-benzoyl-β-glucopyranosyloxy-2-phenyl-acetonitrile.

It has been well known that NO, PGE_2 and pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 are involved in the development of inflammation. First, we investigated the effects of Persicaside on the release of two inflammatory mediators, NO and PGE_2.

The survival ratio of murine macrophases was unaffected within the range of 40 μg/mL by the dosage of presicaside. That is, the IFN-γ and LPS stimulated murine cell showed over 95% of survival ratio between 5 μg/mL and 40 μg/mL dosage of presicaside (Fig. 2). This implies that presicaside is a safe secondary metabolite for the mature cells. The IFN-γ/LPS-induced NO accumulation was suppressed by presicaside in a concentration dependent manner. There was more than 50% reduction of NO evolving with 40 μg/mL of Persicaside as compared to the untreated one (Fig. 3).

To elucidate the inhibitory mechanism, the effect of Persi-



R = H persicaside (1)
= Ac persicaside acetylation (2)

Figure 1. Chemical structure of Persicaside (**1**) and its acetylation (**2**) isolated from *Prunus persica*

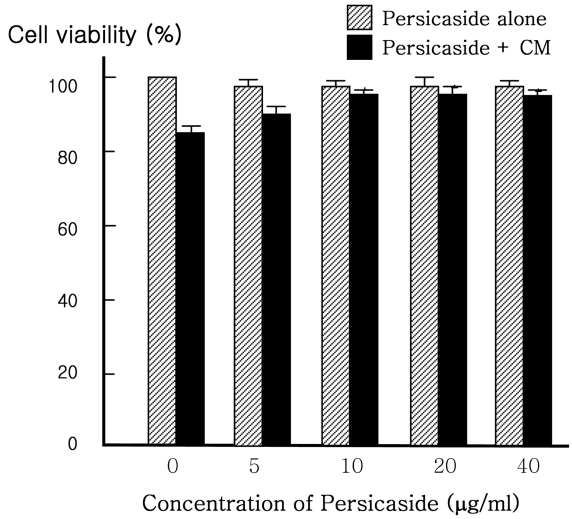


Figure 2. Effects of Persicaside on the cell viability of in ROS 17/2.8 cells. Cells (2.5×10^5/mL) were incubated with or without CM (100 U/mL IL-1β, 200 U/mL IFN-γ, and 500 U/mL TNF-α) for 24 h in the presence or absence of Persicaside at indicated concentrations. The cell viability was determined by MTT assay as described in Materials and Methods. Each column represents the mean ± S.D. from three independent experiments. $^{*}P < 0.01$ indicate significant differences CM treated group.

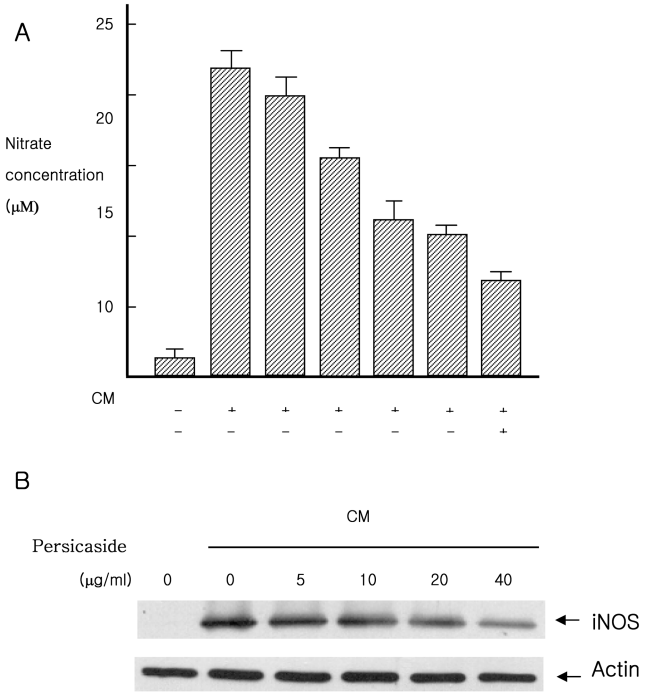


Figure 3. Effects of Persicaside on NO production (A) and iNOS protein expression (B) in ROS 17/2.8 cells. Cells (2.5×10^5/mL) were incubated 24 h (for NO assay) or 18 h (for iNOS Western blot) with medium, CM (100 U/mL IL-1β, 200 U/mL IFN-γ, and 500 U/mL TNF-α), or CM plus M-2 (1-40 μg/mL). NO concentration was determined by Griess reagent as described in Materials and Methods. Each column represents the mean ± S.D. from three independent experiments. $^{*}P < 0.01$ indicate significant differences CM treated control group. For the determination of intracellular iNOS protein, Western immunoblot analysis was carried out as described in Materials and Methods.

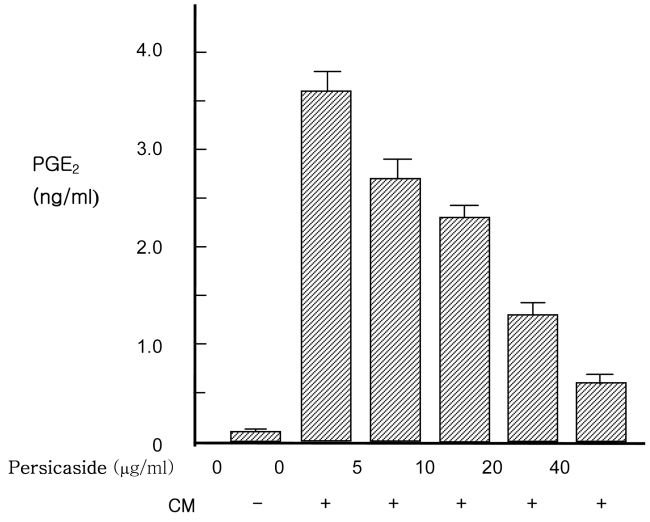


Figure 4. Efects of Persicaside on PGE_2 production in ROS 17/2.8 cells. Cells (1×10^6/mL) were incubated with or without CM (100 U/mL IL-1β, 200 U/mL IFN-γ, and 500 U/mL TNF-α), or CM plus Persicaside (1-40 μg/mL) for 18 h in the presence or absence of Persicaside at indicated concentrations. PGE_2 production in the culture medium was determined as described in Materials and Methods. Each column represents the mean ± S.D. from three independent experiments. $^*P < 0.01$ indicate significant differences LPS treated control group.

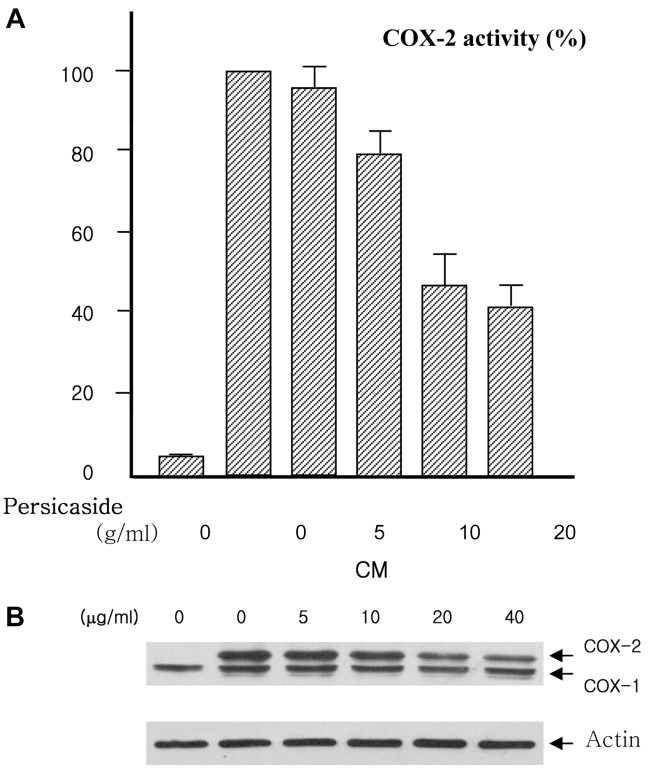


Figure 5. Effects of Persicaside on COX activity (A) and COX protein expression (B) in ROS 17/2.8 cells. Cells (1×10^6/mL) were incubated with or without CM (100 U/mL IL-1β, 200 U/mL IFN-γ, and 500 U/mL TNF-α) for 18 h in the presence or absence of Persicaside at indicated concentrations. COX activity in the culture medium was determined as described in Materials and Methods. Each column represents the mean ± S.D. from three independent experiments. Western immunoblot analysis was carried out as described in Materials and Methods.

caside on iNOS and COX-2 expression levels was investigated. The COX-2 selectivity has been considered important in the development of coumarine and non-steroidal anti-inflammatory drugs (NSAIDs) because of the well known bleeding complication.[16] Concurrently, ideal drugs must spare the COX-1 enzyme in gastrointestinal mucosa as well as in platelets.[17,18,19] Dosing of 40 μg/mL of Persicaside in to the murine cells reduced the evolution of PEG_2 down to 5 μg/mL whereas untreated cells produced 36 μg/mL of PEG_2. These findings indicate that Persicaside has the inhibition effect on IFN-γ/LPS-induced NO and PGE_2 generation in RAW 264.7 cells by suppressing iNOS and COX-2 protein expression.

In addition, the cytotoxic effect of Persicaside was evaluated in the absence or presence of IFN-γ/LPS. When treated alone, Persicaside did not affect the cell viability at the concentration used (1-50 μg/mL) (data not shown). However, when the treated rat osteoblast sarcoma cells being activated with IFN-γ/LPS, Persicaside increased cell viability as compared with the result with only IFN-γ/LPS activated cells (Fig. 4, 5).

Conclusions

To our best knowledge, Persicaside (**1**) isolated from the seed of *Prunus persica* is a new alkaloid compound. Interpretation of ^{1}H- and ^{13}C NMR spectra along with ESI-MS data could determine the planar structure of Percicaside (**1**) and its acetylation (**2**) confirmed the presence of acetamide moiety.

The IFN-γ and LPS stimulated rat osteoblast sarcoma cells showed over 95% of survival rate between 5 μg/mL and 40 μg/mL dosage of Presicaside. The IFN-γ/LPS-induced NO accumulation was suppressed by Presicaside in a concentration dependent manner. Over 50% reduction against NO evolution was observed with 40 μg/mL of Persicaside as compared to the untreated one.

iNOS and COX-2 expression levels was investigated. Dosing of 40 μg/mL of Persicaside in to the murine cells reduced the evolution of PEG_2 down to 5 μg/mL whereas untreated cells produced 36 μg/mL of PEG_2.

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