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Antiviral Triterpenes from *Prunella vulgaris*

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Abstract □ Two triterpenes 1 and 2 with antiviral activity against *Herpes simplex* virus type 1 *in vitro* were isolated from *Prunella vulgaris*. Each compound caused a significant reduction in viral cytopathic effect when *vero* cells were exposed to them for 72 hours after viral challenge. They were identified as *betulinic acid*(**1**) and 2α,3α-dihydroxyurs-12-en-28-oic acid(**2**) on the basis of their spectroscopic properties. The antiviral activity of them was estimated as EC_{50}=30 μg/m*l*(**1**) and 8 μg/m*l*(**2**), respectively by plaque reduction assay.

Keywords □ *Prunella vulgaris*, Labiatae, betulinic acid, 2α,3α-dihydroxyurs-12-en-28-oic acid, antiviral, *Herpes simplex*.

We have been searching for the new class of antiviral agents from natural resources especially from plant materials. For this purpose, more than fifty kinds of medicinal plants were screened for the antiviral activity against *Herpes simplex* virus type 1 (HSV-1) and 2 (HSV-2), *in vitro*[1]. Among these, chloroform extracts of the whole herb of *Prunella vulgaris* (Labiatae), of the fruit of *Forsythia koreana* (Oleaceae) and the root of *Sanguisorba officinalis* (Rosaceae) were observed to exhibit activity in our *in vitro* antiherpes bioassay, which were chosen as candidates for active component isolation studies.

Prunella vulgaris is a perennial herb which is widely distributed throughout Korea, Japan and China, and has been used in the folk medicine as a diuretic or an astringent. It was recently reported that aqueous extract of *Prunella vulgaris* showed antiviral activity against Human immunodeficiency virus (HIV-1)[2], and more recently, the prunellin which is a polysaccharide isolated from *Prunella vulgaris*, was reported to exhibit anti-HIV activity[3]. The present paper deals with the isolation and identification of active components of *Prunella vulgaris*, which showed significant anti-HSV-1 activities *in vitro*, by the activity-guided fractionation.

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EXPERIMENTAL

^{1}H-NMR spectra were run at 300 MHz and ^{13}C-NMR at 75 MHz and recorded by Bruker AM-300. MS (70 eV) were taken with a direct inlet and recorded by GC-MS QP-100 (Shimadzu) spectrometer.

In vitro *evaluation of anti-HSV-1 activity*

The anti-HSV-1 activity was evaluated by plaque reduction assay[4]. Confluent monolayers of *vero* cells in 24-well plates were inoculated with 0.2 m*l* of HSV-1 strain F at the M.O.I. (multiplicity of infection) of 100 PFU (plaque forming unit) per well. After 1 hour adsorption at 37℃ in CO_2 incubator, unadsorbed viruses were aspirated off and test material at various concentrations prepared in overlay-medium 〈Dulbecco's modified essential medium (Gibco): 2% heat inactivated fetal bovine serum (Gibco): 0.8% gum tragacanth: 4 μg/m*l* gentamycin〉 was applied in a volume of 0.5 m*l* per well in duplicate. In case of necessity, the test material was dissolved in small amount of dimethylsulfoxide (DMSO), but the final concentration of DMSO in

test material-medium mixture was not exceeded 1.0 %. Each plate contained duplicated cell control (no virus and no test material added) and virus control (no test material added). After 3 days incubation at 37℃ in CO_2 incubator, medium was removed and cells were fixed and stained with dye-fixer solution (5% formalin: 50% ethanol: 0.5% crystal violet: saline). Plaque numbers obtained in cultures containing test material at varying concentrations were compared with those of virus control which was considered as 100%. The 50% effective concentration (EC_{50}) was defined as the concentration of test material that caused 50% reduction of plaque number compared with that of virus control.

Extraction and isolation

The whole herb of *Prunella vulgaris* was purchased at market and 1.8 kg of dried material was extracted with methanol at room temperature for 2 weeks to give an extract 85g, which was suspended in water and extracted with chloroform to give a chloroform extract 38g. One part of chloroform extract (4g) was dissolved in $CHCl_3$/MeOH (10 : 1) solvent and subjected to pass through neutral *alumina* (Al_2O_3, activity 1, Merck) gel (200g), which were eluted with $CHCl_3$/MeOH (10 : 1) 5*l* and washed with additional 5*l* of MeOH. The eluate and wash were pooled up and evaporated to dryness to give Fr.N 2.4g. The alumina gel was further eluted with 2.5% NH_4OH/MeOH 5*l* and the eluate were collected and evaporated to dryness to give Fr.A 1.5g (see Fig. 1). The Fr.A was divided into five portions (Fr. a1~Fr.a 5) by SiO_2 column chromatography. Two of which, Fr.a2 and Fr.a4 were showed marked anti-HSV-1 activity. Fr.a2 was purified by repeated SiO_2 column chromatography using $CHCl_3$/MeOH as a gradient elution followed by activity monitoring to give 52 mg of compound **1**.

Compound **1** white needles (in MeOH). mp.> 300°, $[\alpha]_D = +10.2$(c=0.5, $CHCl_3$), MS (**1b**, 70 eV): *m/z* (rel. int.); 470(M^+, missing), 411 (M^+-$COOCH_3$, 16%), 262 (65%), 207 (72%), 189 (100%). ^{1}H-NMR (**1b**; $CDCl_3$): δ 0.72, 0.80, 0.90, 0.95 and 0.96 (each 3H, s, 23, 24, 25, 26 and 27-C$\underline{H}_3$), 1.65 (3H, brs, 30-C$\underline{H}_3$), 2.22 (1H, dd, *J*=8.8, 4.4 Hz, 18-H), 2.95 (1H, *m*, 19-H), 3.15 (1H, dd, *J*=11.0, 5.2 Hz, 3-H), 3.58 (3H, s, COOC$\underline{H}_3$), 4.58 and 4.70 (each 1H, *m*, 29-H). ^{13}C-NMR (**1b**; $CDCl_3$): δ 38.9 (1-C), 27.5 (2-C), 79.1 (3-C), 38.8 (4-C), 55.2 (5-C), 18.2 (6-C), 34.2 (7-C), 40.8 (8-C), 50.5 (9-C), 37.2 (10-C), 20.8 (11-C), 25.7 (12-C), 38.2 (13-C), 42.2 (14-C), 29.9 (15-C), 32.2 (16-C), 56.5 (17-C), 49.4 (18-C), 47.0 (19-C), 150.5 (20-C), 30.7 (21-C), 36.8 (22-C), 28.0 (23-C), 15.5 (24-C), 16.0 (25-C), 15.9 (26-C), 14.9 (27-C), 176.5 (28-C), 109.5 (29-C), 19.5 (30-C), 51.4 (-COO$\underline{C}$H$_3$).

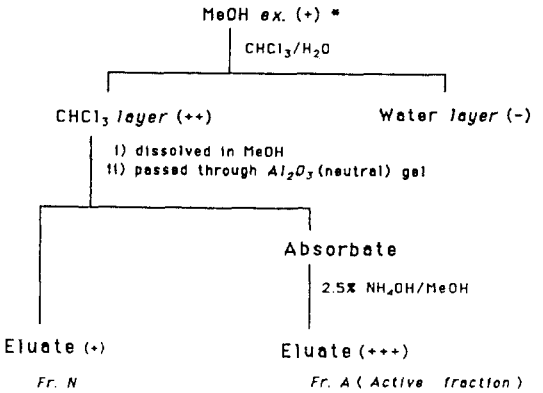


Fig. 1. Fractionation scheme of the MeOH extract of *Prunella vulgaris*.
*: Antiviral activity aginst HSV-1 *in vitro*; + + +, EC_{50}<50 μg/m*l*, + +, EC_{50}<100 μg/m*l*, +, EC_{50}<200 μg/m*l*, −, EC_{50}>200 μg/m*l*

Fr.a4 was also repeated SiO_2 column chromatography using $CHCl_3$/MeOH as gradient elution to give compound **2** 43 mg.

Compound **2** white needles (in MeOH). mp (**2b**): 186-188°, $[\alpha]_D = +55$ (c=0.2, $CHCl_3$), MS (**2b**; 70 eV): *m/z* (rel. int.); 486 (M^+, 1), 427 (M^+-COOMe, 4), 262 (100), 223 (15), 203 (68). ^{1}H-NMR (**2b**, $CDCl_3$): δ 0.72 (3H, s, 26-C$\underline{H}_3$), 0.83 (3H, s, 24-C$\underline{H}_3$), 0.86 and 0.91 (each 3H, d, *J*=7.5 and 8.2 Hz, 29 and 30-C$\underline{H}_3$), 0.93 (3H, s, 25-C$\underline{H}_3$), 1.00 (3H, s, 24-C$\underline{H}_3$), 1.06 (3H, s, 27-C$\underline{H}_3$), 2.22 (1H, d, *J*=11 Hz, 18-H), 3.40 (1H, d, *J*=2.6 Hz, 3-H), 3.58 (3H, s, -COOC$\underline{H}_3$), 3.98 (1H, *m*, 2-H), 5.22 (1H, *m*, 12-H). ^{13}C-NMR (**2b**; $CDCl_3$): δ 41.7 (1-C), 66.5 (2-C), 78.9 (3-C), 39.0 (4-C)*, 48.1 (5-C), 18.0 (6-C), 32.7 (7-C), 39.6 (8-C)*, 47.3 (9-C), 38.2 (10-C), 23.3 (11-C), 125.3 (12-C), 138.2 (13-C), 42.1 (14-C), 27.9 (15-C), 24.2 (16-C), 48.1 (17-C), 52.6 (18-C), 38.6 (19-C), 38.3 (20-C), 30.6 (21-C), 36.6 (22-C), 28.5 (23-C), 21.6 (24-C), 16.4 (25-C), 17.0 (26-C), 23.7 (27-C), 178.1 (28-C), 16.9 (29-C), 21.2 (30-C), 51.4 (-COOC$\underline{H}_3$). *: Assignments may be reversed.

Compound **3** 380 mg and compound **4** 8 mg were yielded from Fr.a3 and Fr.a1, respectively by re-

peated SiO_2 column chromatography, which were identified as an *ursolic acid* (**3**) and an *3-O-Acetylursolic acid* (**4**) by their spectroscopic data (H-NMR and ^{13}C-NMR) and co-TLC with authentic samples.

For the purpose of studying on the structure-activity relation of **1** and **2**, derivatives **1a**, **2a**, **1b**, **2b**, **3b**, **1c**, and **2c** and were prepared by the acetylation of corresponding parent compound with pyridine/acetic anhydride and by the methylation with diazomethane, and an analogue of *betulinic acid* (**1**), *betulin* (Lup-20[29]-ene-3β,28-diol) was purchased from *Sigma* (see Fig. 2).

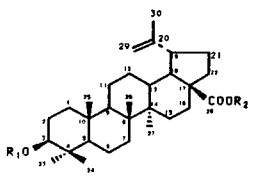
RESULTS AND DISCUSSION

The bioassay guided fractionation of the MeOH extract of *Prunella vulgaris* yielded two triterpenes **1** and **2** as active principles of its anti-HSV-1 property *in vitro*. **1** was identified as a *betulinic acid* by the direct comparison (co-TLC and spectral data[5]) with the authentic compound and **2** as a *2α,3α*-dihydroxyurs-12-en-28-oic acid by the comparison of spectral properties with reported ones[6].

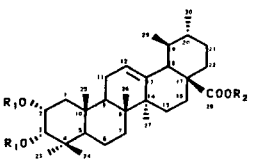
Anti-HSV-1 activity of the isolated active compounds were summarized in Fig. 2, together with that of their derivatives (**1a-2c**), which were prepared by the simple modification of functional groups of corresponding parent compounds. None of these derivatives examined showed significant activity, compared by the parent compound. The most active one was compound **2**, which has a *1,2-diol* moiety in A ring and a carboxylic group at D/E ring of *α-amyrin* skeleton. These two functional groups of compound **2** were considered to be responsible for its strong anti-HSV-1 activity.

It has been reported that some triterpenes such as aescin[7], gymnemic acid (polyoxytriterpene glycoside)[8], dammar resin[9], protoprimulagenin[10], and glycyrrhetinic acid[11,12] were observed to exhibit antiviral activity *in vitro* and in some cases, *in vivo*, especially against HIV and HSV. Interestingly, one of the active compounds in the present paper, the *betulinic acid* (**1**) had been noted to inhibit the replication of Epstein-Barr virus (EBV) which is considered to be responsible for the promotion of some tumor[13].

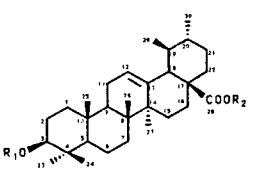
Recently, H. Tabba[3] had reported that the prunellin, a sulfated polysaccharide, which was isolated from aqueous extract of *Prunella vulgaris* was responsible for the anti-HIV property of *Prunella vulgaris* and H. S. Chen[14] suggested that isoflavones were active ingredients of *Prunella vulgaris* by the reason of their strong inhibitory activity against HIV reverse transcriptase (RT) *in vitro*. However, our present study revealed that the anti-HSV-1 property of *Prunella vulgaris* was predominantly due to compound **1** and **2**, which belonged to a totally different class from isoflavones or polysaccharide *etc.* It is probably due to the difference of the target virus and the difference of bioassay systems which they had employed.



	R_1	R_2	EC_{50}(μg/ml)
1	−H	−H	30
1a	−Ac	−H	>100
1b	−H	$-CH_3$	90
1c	−Ac	$-CH_3$	>100
Betulin			>100



	R_1	R_2	EC_{50}(μg/ml)
2	−H	−H	8
2a	−Ac	−H	80
2b	−H	$-CH_3$	80
2c	−Ac	$-CH_3$	>100



	R_1	R_2	EC_{50}(μg/ml)
3	−H	−H	80
4(3a)	−Ac	−H	>100
3b	−H	$-CH_3$	>100

Fig. 2. Anti-HSV-1 activity of isolates and derivatives *in vitro*.

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