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Anti-UV/HIV Activity of Kampo Medicines and Constituent Plant Extracts

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Abstract. *Aim: In order to search for new biological activities of Kampo medicines and their constituent plant extracts, we investigated whether they protect the cells from the cytotoxicity induced by UV irradiation and human immunodeficiency virus (HIV) infection. Materials and Methods: Anti-UV/HIV activity (SI value) was evaluated as the ratio of the CC_{50} (concentration that reduced the viable cell number by 50%) to the EC_{50} (the concentration that increased the viability of UV-irradiated or HIV-infected cells to 50%): $SI=CC_{50}/EC_{50}$. The content of glycyrrhizin in each sample was determined by high performance liquid chromatography (HPLC). Caspase-3/-7 activity was assayed by cleavage of poly ADP ribose polymerase using western blot analysis. Results: Among 25 plant extracts, Gardenia fruit had the highest anti-UV activity (SI≥8.0), followed by Glycyrrhiza (SI=4.3), Coptis rhizoma (SI=1.5), Cimicifuga rhizoma (SI>1.4), Saposhnikovia root (SI>1.3) and Japanese Gentian (SI>1.1). Among ten Kampo medicines, Unseiin and Hangesyashinto (SI>4.9) had the highest anti-UV activity, followed by Shosaikoto (SI>4.3), Saireito (SI>3.4), Rikkosan (SI>1.2) and Kikyoto (SI=1.1). Glycyrrhiza inhibited UV-induced caspase-3/-7 activation. Only Polyporus sclerotium (SI>4.4), Gardenia fruit (SI>2.7), Atractylodes lancea rhizoma (SI>1.9), Cnidium rhizoma (SI>1.5) and Japanese Angelica root (SI>1.1) exhibited some anti-HIV activity. There was no apparent correlation of their anti-UV/HIV activity and content of glycyrrhizin, a major component of Glycyrrhiza, which exhibited much higher anti-UV activity (SI=20.6) and some anti-HIV activity (SI>2.0). Conclusion: The present study suggests the involvement of substances other than glycyrrhizin in the anti-UV/HIV activity of Kampo medicines and their constituent plant extracts.*

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Ultraviolet rays (UV) are invisible electromagnetic wave. Classified into UVA (400-315 nm), UVB (315-280 nm) and UVC (<280 nm). UVA and UVB pass through the ozonosphere and reach the ground earth's surface, whereas UVC cannot pass through the air due to absorption. Ninety nine percent of UV that reaches to the ground is UVA. Moderate doses of UV exert several favorable effects such as sterilization and disinfection (1), induction of vitamin D synthesis (2), and stimulation of the metabolism and skin resistance. However, an excessive dose of UV produces reactive oxygen species (ROS), which damage cellular DNA and proteins, leading to carcinogenesis (3). Guanine, the most susceptible DNA base, is oxidized to 7,8-dihydroxy-8-oxoguanine upon UV-irradiation, and triggers the transversion of G:C to T:A (5). High doses of UV irradiation induced apoptotic cell death in human myelogenous leukemia cell lines, but induced other types of cell death in human T-cell leukemia, erythroleukemia, glioblastoma (6), oral squamous cell carcinoma (OSCC) cell lines and human normal oral cells (gingival fibroblasts, pulp cells, periodontal ligament fibroblast) (7). We recently established a method that can measure the activity of compound/extract to protect cells from the UV-induced injury (referred to as 'anti-UV activity') (7, 8). Using this method, we previously showed that alkaline extract of *Sasa senanensis* Rehder leaf and vitamin C exhibited potent anti-UV activity (9, 10), and their activity is higher than that of commercially available tea extract (11).

We also reported that various Kampo medicines (12-14) and their ingredients such as glycyrrhizin (15), and flavone and its related compounds (16), inhibited cyclooxygenase (COX)-mediated prostaglandin E_2 (PGE_2) production by activated mouse macrophages. We investigated here whether a total of 35 Kampo medicines and their constituent plant extracts protect the cells from UV-induced damage, and if so, whether their anti-UV activity is correlated with glycyrrhizin content, and whether it is induced *via* apoptosis inhibition.

Plant extracts such as lignin–carbohydrate complex (LCC) (17) and oligomeric hydrolyzable tannins (18) were found to exhibit showed potent anti-HIV activity. Therefore, we also investigated whether these Kampo Medicines and constituent plant extracts have any detectable anti-HIV activity.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Glycyrrhizin, Wako Pure Chem. Ind., Osaka, Japan; Dulbecco's modified Eagle medium (DMEM) (Invitrogen Corp, Carlsbad, CA, USA), fetal bovine serum (FBS), Gemini Bio-Products, Woodland, CA, USA; 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO), Sigma Chem. Ind., St. Louis, MO, USA; Alisma rhizoma and *Asiasarum* root, *Astragalus* root, *Atractylodes lancea* rhizoma, *Bupleurum* root, *Cimicifuga* rhizoma, *Cinnamon* bark, *Cnidium* rhizoma, *Coptis* rhizoma, *Gardenia* fruit, ginger, ginseng, *Glycyrrhiza*, Japanese Angelica root, Japanese Gentian, Jujube, Peony root, *Phellodendron* bark, *Pinellia* tuber, *Platycodon* root, *Polyporus sclerotium*, *Poria* sclerotium, *Rehmannia* root, *Saposhnikovia* root, *Scutellaria* root, Byakkokaninjinto, Hangesyashinto, Hotyuekkito, Juzentaihoto, Kikyoto, Ninjinyoeito, Rikkosan, Saireito, Shosaikoto and Unseiin were obtained from Tsumura Corp., Tokyo, Japan. Kampo medicines were supplied as dried powers, and dissolved in phosphate-buffered saline without calcium and magnesium [PBS(–)] prior to the experiments.

Determination of glycyrrhizin. The concentration of glycyrrhizin in the plant extracts and Kampo medicines was determined by high performance liquid chromatography (HLPC). The HPLC system comprised a JASCO PU-980 pump, a JASCO UV-970 UV/VIS detector and a column of Inertsil ODS-3 (4.6 mm i.d. ×150 mm, 5 μm; GL Sciences Inc., Tokyo, Japan). The detection wavelength was set at 254 nm and the sample was injected manually. The mobile phase used was acetonitrile: 2.5% acetic acid (40: 60), with a flow rate of 1.2 ml/min.

Assay for anti-UV activity. Cells were inoculated at 3×10^3 cells/0.1 ml in the inner 60 wells of a 96-microwell plate (Becton Dickinson Labware, NJ, USA). The surrounding 36 exterior wells were filled with 0.1 ml of PBS(–) to minimize the evaporation of water from the culture medium. After 48 hours, the attached cells were replaced with PBS(–) containing different concentrations of samples. The cells were then placed at 20.5 cm from a UV lamp (wavelength=253.7 nm) and exposed to UV irradiation (6 J/m^2/min) for 1 min. The media were replaced with fresh DMEM plus 10% FBS and cells were cultured for a further 48 hours at 37˚C in a CO_2 incubator to determine the relative viable cell number by MTT method. In brief, the treated cells were incubated for another 4 h in fresh culture medium containing 0.2 mg/ml MTT. Cells were then lysed with 0.1 ml of dimethyl sulfoxide (DMSO), and the absorbance at 540 nm of the cell lysate was determined using a microplate reader (Biochromatic Labsystem, Helsinki, Finland). From the dose–response curve, the 50% cytotoxic concentration (CC_{50}) and the concentration that increase the viability of UV-irradiated cells to 50% (EC_{50}) were determined. The selectivity index (SI) was determined by the following equation: $SI=CC_{50}/EC_{50}$ (7, 8).

Assay for caspase-3/-7 activation. HSC-2 cells were exposed to UV irradiation (6 J/m^2/min, 1 min) or not in PBS containing 0 (control) or 4 mg/ml of *Glycyrrhiza*. Cells were replenished with fresh culture medium (DMEM plus 10% FBS) and incubated for a further 6 h. The caspase-3/-7 activity was then assayed by measuring the production of cleaved product of poly ADP ribose polymerase (PARP) with western blot analysis, using Promega PARP (Asp 214) human specific antibody (distributed by Cell Signaling Technology, Inc. Boston, MA, USA). In brief, cells were washed in ice-cold PBS, scraped, collected in lysis buffer [20 mM HEPES pH 7.4, 1% Triton X-100, 150 mM NaCl, 1.5 mM $MgCl_2$, 12.5 mM β-glycerophosphate, 2 mM EGTA, 10 mM NaF, 2 mM dithiothreitol (DTT), 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF) plus 1 × protease inhibitor]. The cell lysates were applied to 8% polyacrylamide gel electrophoresis (SDS-PAGE) and the protein bands in the gels were transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% (w/v) nonfat dry milk, incubated with primary antibody [anti-cleaved PARP1 (Cell Signaling Technology), anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, USA)], and then with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (19).

Assay for HIV activity. MT-4 cells were infected with HIV-1IIIB at a multiplicity of infection (m.o.i.) of 0.01. Samples (10 mg) was dissolved or suspended in 0.5 ml physiological saline, and heated for 3 min at 100˚C. The supernatant was recovered after the centrifugation. HIV- and mock-infected (control) MT-4 cells were incubated for 5 days with different concentrations of the plant extract/ Kampo medicines, and the relative viable cell number was determined by MTT assay. The CC_{50} and EC_{50} were determined from the dose–response curve for mock-infected and HIV-infected cells, respectively (18). All data represent the mean values of triplicate measurements. The anti-HIV activity was evaluated by SI as above.

Statistical analysis. Results are presented as the mean±standard deviation (SD) of triplicate assays.

Results

Anti-UV activity. Kampo medicines and their constituent plant extracts protected the HSC-2 cells from the UV-induced cytotoxicity to various extents (Figures 1 and 2). Among 25 plant extracts, *Gardenia* fruit exhibited the highest anti-UV activity (SI≥8.0), followed by *Glycyrrhiza* (SI=4.3) *Coptis* rhizoma (SI=1.5), *Cimicifuga* rhizoma (SI>1.4), *Saposhnikovia* root (SI>1.3) and Japanese Gentian

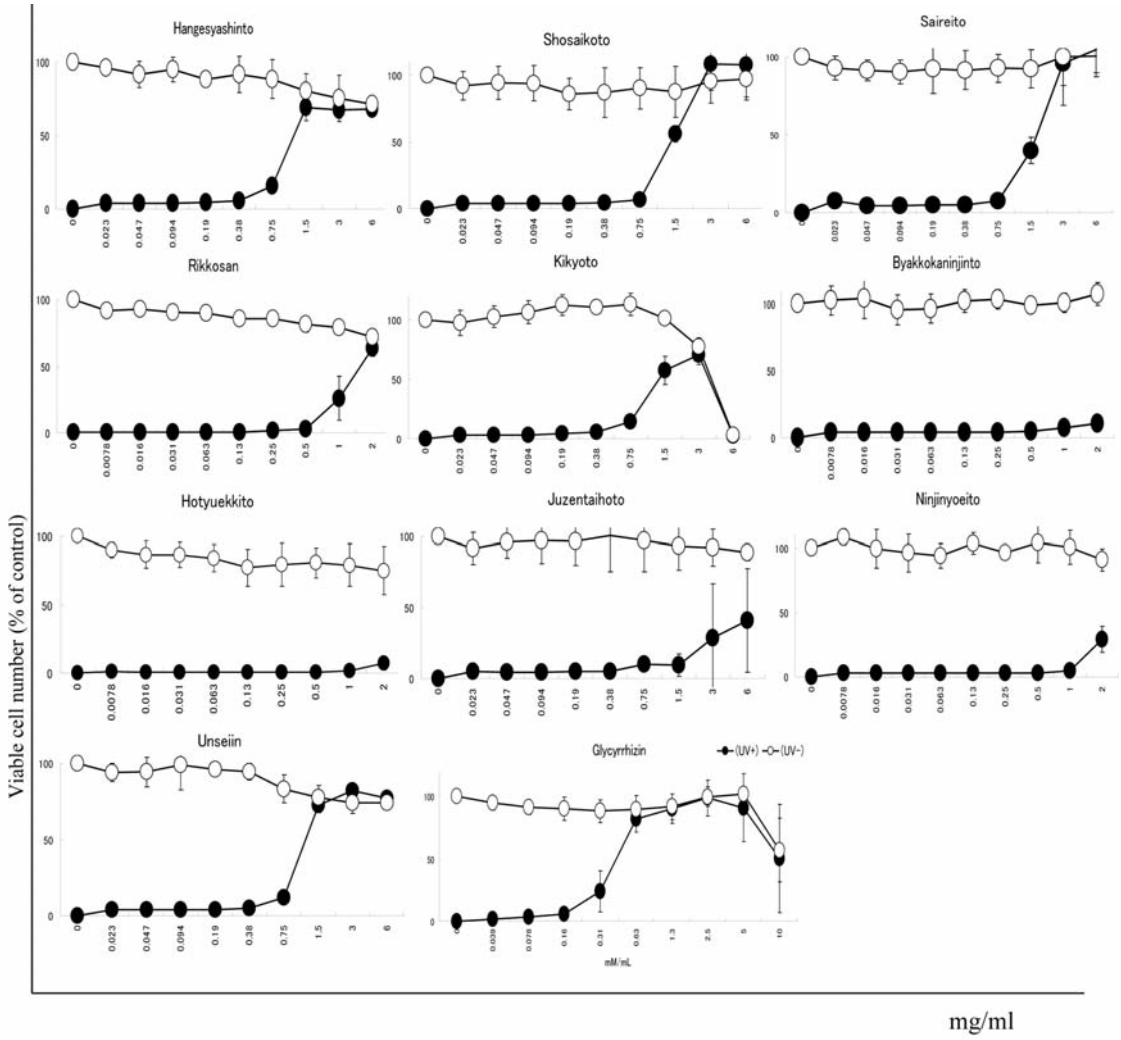

Figure 1. *Effect of 10 Kampo medicines on UV-induced cytotoxocity. Near confluent HSC-2 cells were replaced with PBS(–) containing different concentrations of Kampo medicines. The cells were then exposed to UV irradiation, and the viable cell number was determined as described the Materials and Methods. Each value represents the mean±SD of three independent experiments.*

(SI>1.1), whereas other 19 extracts were much less active (SI<1.0) (Figure 1). Among 10 Kampo medicines, Unseiin and Hangesyashinto (SI>4.9) had the highest anti-UV activity, followed by Shosaikoto (SI>4.3), Saireito (SI>3.4), Rikkosan (SI>1.2) and Kikyoto (SI=1.1), whereas another four Kampo Medicines were much less active (SI<1.0) (Figure 1) (Table I).

We next investigated the mechanism by which *Glycyrrhiza* induced anti-UV activity. UV irradiation induced the production of cleaved PARP, indicating the activation of caspase-3/-7. Although *Glycyrrhiza* itself slightly induced the production of cleaved PARP, it more clearly inhibited the UV-induced production of cleaved PARP (Figure 3). This result suggests that *Glycyrrhiza* contains both apoptosis inducer(s) and inhibitor(s) of UV-induced apoptosis.

Relationship between anti-UV activity and glycyrrhizin content. Glycyrrhizin, a major component of *Glycyrrhiza*, was found to exhibit very high anti-UV activity (SI=20.6). This urged us to investigate whether the anti-UV activity of Kampo medicines and constituent plant extracts relates to their content of glycyrrhizin. Twenty-five plant extracts, except for *Glycyrrhiza* (175.4 mg/g), did not contain detectable amounts of glycyrrhizin. On the other hand, 10 Kampo medicines (Byakkokaninjinto, Hangesyashinto, Hotyuekkito, Juzentaihoto, Kikyoto, Ninjinyoeito, Rikkosan, Saireito, Shosaikoto, Unseiin) contained up to 50.3 mg/g of glycyrrhizin, possibly due to the inclusion of *Glycyrrhiza* (Table I). However, there was no clear-cut relationship between the anti-UV activity and glycyrrhizin content of Kampo medicines and constituent plant extracts (left panel vs. middle panel, Table I).

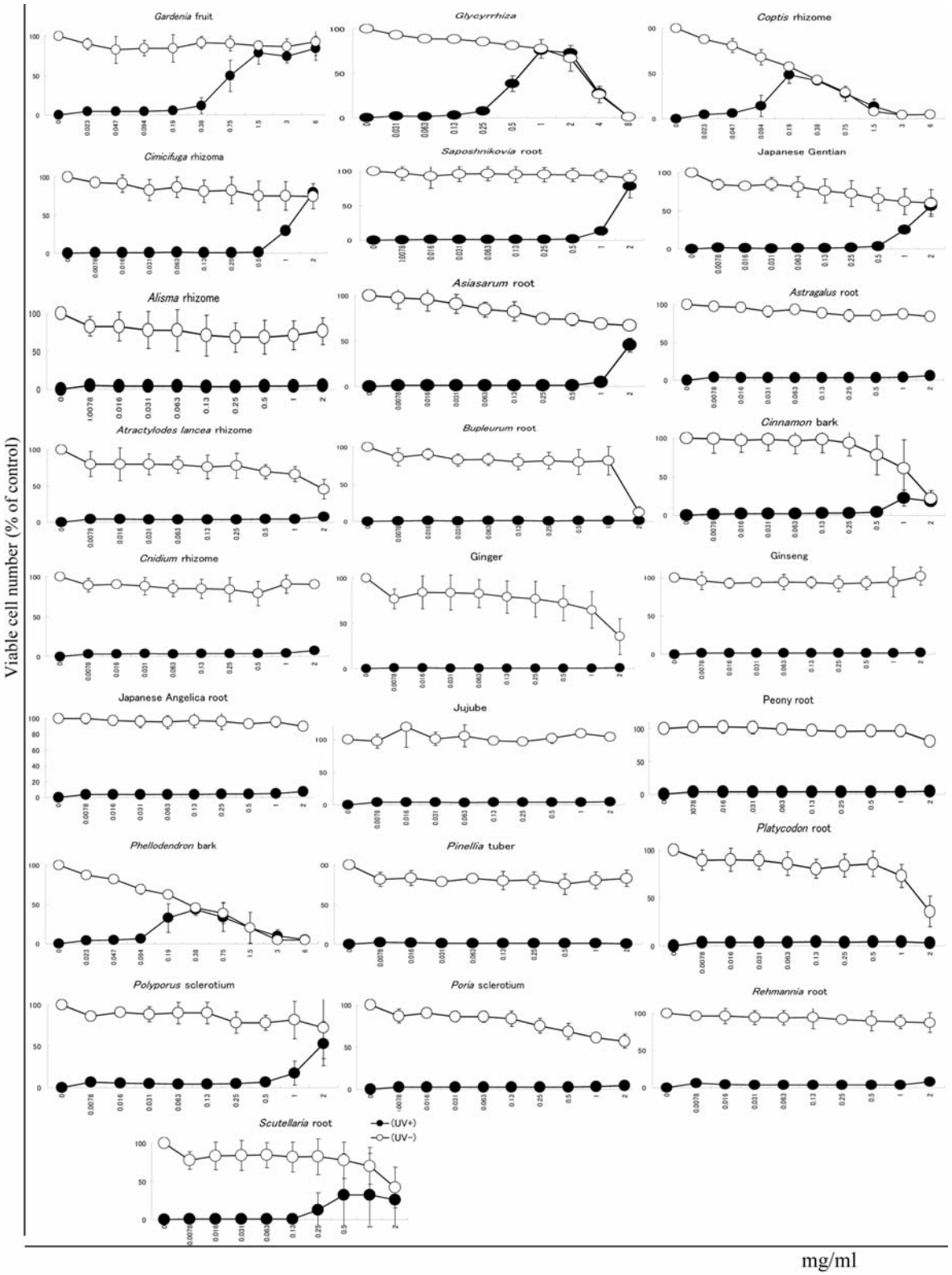

Figure 2. *Effect of 25 Kampo medicines constituent plant extracts on UV-induced cytotoxocity. Near confluent HSC-2 cells were replaced with PBS(−) containing different concentrations of plant extracts. The cells were then exposed to UV irradiation, and the viable cell number was determined as described the Materials and Methods. Each value represents the mean±SD of three independent experiments.*

Table I. *Anti-UV and -HIV activity and glycyrrhizine content of Kampo Medicines and constituent plant extracts*.

	Anti-UV activity				Anti-HIV activity		
	CC_{50} (mg/ml)	EC_{50} (mg/ml)	SI	Glycyrrhizin Content (mg/g)	CC_{50} (μg/ml)	EC_{50} (μg/ml)	SI
Constituent plant extracts							
Alisma rhizoma	>2	>2	><1.0	0	>1000	>1000	><1.0
Asiasarum root	>2	>2	><1.0	0	675	>1000	<1.0
Astragalus root	>2	>2	><1.0	0	>1000	>1000	><1.0
Atractylodes lancea rhizoma	1.8	>2	<0.78	0	>1000	531	>1.9
Bupleurum root	1.5	>2	<0.73	0	>1000	>1000	><1.0
Cimicifuga rhizoma	>2	1.42	>1.4	0	453	>1000	<1.0
Cinnamon bark	1.3	>2	<0.64	0	370	>1000	<1.0
Cnidium rhizoma	>2	>2	><1.0	0	>1000	687	>1.5
Coptis rhizoma	0.29	0.19	1.5	0	17	>1000	<1.0
Gardenia fruit	>6	0.75	>8.0	0	>1000	365	>2.7
Ginger	1.5	>2	<0.75	0	>1000	>1000	><1.0
Ginseng	>2	>2	><1.0	0	>1000	>1000	><1.0
Glycyrrhiza	2.8	0.65	4.3	175.4	188	>1000	<1.0
Japanese Angelica root	>2	>2	><1.0	0	>1000	934	>1.1
Japanese Gentian	>2	1.8	>1.1	0	>1000	>1000	><1.0
Jujube	>2	>2	><1.0	0	>1000	>1000	><1.0
Peony root	>2	>2	><1.0	0	416	>1000	<1.0
Phellodendron bark	0.33	>6	<0.054	0	28	>1000	<1.0
Pinellia tuber	>2	>2	><1.0	0	>1000	>1000	><1.0
Platycodon root	1.6	>2	<0.8	0	>1000	>1000	><1.0
Polyporus sclerotium	>2	1.9	>1.04	0	>1000	226	>4.4
Poria sclerotium	>2	>2	><1.0	0	>1000	>1000	><1.0
Rehmannia root	>2	>2	><1.0	0	>1000	>1000	><1.0
Saposhnikovia root	>2	1.6	>1.3	0	>1000	>1000	><1.0
Scutellaria root	1.7	>2	<0.86	0	90	>1000	<1.0
Kampo medicines							
Byakkokaninjinto	>2	>2	><1.0	7.2	587	>1000	<1.0
Hangesyashinto	>6	1.2	>4.9	16.2	268	>1000	<1.0
Hotyuekkito	>2	>2	><1.0	0.2	>1000	>1000	><1.0
Juzentaihoto	>6	>6	><1.0	7	958	>1000	<1.0
Kikyoto	4.1	3.9	1.1	50.3	457	>1000	<1.0
Ninjinyoeito	>2	>2	><1.0	3.5	>1000	>1000	><1.0
Rikkosan	>2	1.6	>1.2	24.1	462	>1000	<1.0
Saireito	>6	1.8	>3.4	7	501	>1000	<1.0
Shosaikoto	>6	1.4	>4.3	9.2	496	>1000	<1.0
Unseiin	>6	1.2	>4.9	0	263	>1000	<1.0
Glycyrrhizin	10	0.49	20.6		>1000	498	>2.0
AZT (μM)					264	0.015	17850

AZT, 3'-azido-2',3'-dideoxythidine; CC_{50}, the 50% cytotoxic concentration; EC_{50}, the 50% effective concentration; SI, selectivity index.

Anti-HIV activity. Among 25 plant extracts, only Polyporus Sclerotium (SI>4.4), *Gardenia* Fruit (SI>2.7), *Atractylodes lancea* Rhizoma (SI>1.9), *Cnidium* Rhizoma (SI>1.5) and *Japanese Angelica* Root (SI>1.1) showed weak anti-HIV activity, whereas other twenty one extracts were inactive (SI<1.0). All 10 Kampo Medicines showed no apparent anti-HIV activity (Table I). There was no clear-cut relationship between their anti-HIV activity and glycyrrhizin content (middle panel vs right panel, Table I).

Discussion

The present study demonstrated for the first time that several but not all Kampo medicines and theirs constitutional plant extracts exhibited some anti-UV activity (SI=1.1-8.0 and anti-HIV activity (SI=1.1-4.4). Both Kampo medicines and plant extracts are extracted by hot water according to the traditional prescription method. The relatively low SI values of these materials may be due to interfering actions of

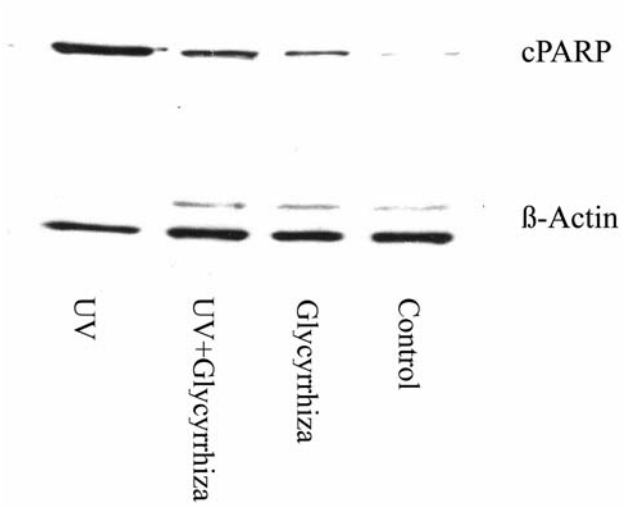

Figure 3. *Inhibition of caspase-3/-7 activation by Glycyrrhiza. HSC-2 cells were exposed to UV irradiation (6 J/m^2/min, 1 min) or not in PBS containing 0 (control) or 4 mg/ml of Glycyrrhiza. Cells were incubated for a further 6 hours in fresh culture medium without Glycyrrhiza, and caspase-3/-7 activity of the cell lysates was determined by the production of cleaved product of PARP, using western blot analysis.*

cytotoxic substance(s) that are extracted by hot water. Removal of the cytotoxic substances by solvent extraction or column chromatography may enhance both activities. We found LCCs, extracted by alkaline solution, had extremely high anti-UV activity (SI=24.8-38.1) (11) (unpublished data) and anti-HIV activity (SI=7-311) (17, 20-25). Therefore, it is possible that the lower SI values of Kampo medicines and plant extracts may be due to a lack of LCC that are poorly extracted by hot water; this remains to be determind.

The present study also demonstrated that the anti-UV/HIV activity of Kampo medicines and constituent plant extracts was not correlated with their glycyrrhizin content. This suggests that components other than glycyrrhizin may be involved in anti-HIV activity. Further purification is necessary to test this possibility.

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