

## Anti-UV Activity of Kampo Medicines and Constituent Plant Extracts: Re-evaluation with Skin Keratinocyte System

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**Abstract.** *Aim:* In order to search for new biological activity of Kampo medicines and their constituent plant extracts, we investigated their ability to protect the cells from UV irradiation (referred to as 'anti-UV activity') using the human immortalised skin keratinocyte cell line HaCaT. *Materials and Methods:* Anti-UV activity was represented by the selectivity index (SI), defined as the ratio of the concentration that reduced the viable cell number by 50% to the concentration that increased the viability of UV-irradiated cells to 50%. *Results:* HaCaT cells were highly resistant to UV irradiation, approximately 20% of cells survived even when the exposure time was prolonged to 480 min. Sodium ascorbate, a popular antioxidant, used as positive control, had excellent anti-UV activity (SI=200). Among 10 Kampo medicines, Shosaikoto (SI=34) had the highest anti-UV activity, followed by Hangesyashinto (SI>28), Unseiin (SI>23) and Ninjinyoeito (SI=23), Saireito (SI>19), whereas another four Kampo medicines were much less active (SI<9.6). Among 25 constituent plant extracts, Scutellaria root had the highest anti-UV activity (SI=38), followed by Polyporus sclerotium (SI>26), Gardenia fruit (SI>23), Japanese Gentian (SI>20) and Saposhnikovia root (SI>20). Glycyrrhizin also had potent anti-UV activity (SI=36). The SI values determined with the present HaCaT system were generally one order higher than those obtained with previously reported HSC-2 human oral squamous cell carcinoma system, although there was good

correlation between these two systems ( $R^2=0.9118$ ). *Conclusion.* The present study highlights the improved sensitivity of anti-UV activity detection with the HaCaT system, and suggests the possible application of Kampo medicines as a component of sunscreens cosmetics.

Ultraviolet rays (UV) are invisible electromagnetic waves. Moderate doses of UV exert several favorable effects, such as sterilization and disinfection (1), induction of vitamin D synthesis (2), and stimulation of 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, a cause of mutation (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 or extract in protecting cells from UV-induced injury (referred to as 'anti-UV activity'), using human OSCC HSC-2 cells as target cells (7, 8). Using this method, we reported that alkaline extract of *Sasa senanensis* Rehder leaf (9-12), lignin-carbohydrate complex (13, 14), newly-synthesized water-soluble azulenes (15) and sodium ascorbate (vitamin C) (9-15) exhibited potent anti-UV activity. Their anti-UV activity was higher than that of hot water extracts of the leaves of green tea, oolong tea, black tea and barley tea, and their commercially available PET (polyethylene terephthalate) bottle drinks (13) and Kampo medicines and their constituent plant extracts (16). Alkaline extracts of green tea leaf, oolong tea leaf and orange flower exhibited significantly higher anti-UV activity

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than their corresponding hot water extract counterparts, possibly due to higher lignin–carbohydrate complex content of the alkaline extracts (17).

We previously reported that various Kampo medicines (18-20) and their constituents, such as glycyrrhizin (21) and flavones (22), inhibited cyclooxygenase-mediated prostaglandin E<sub>2</sub> production by lipopolysaccharide-activated mouse macrophage-like RAW264.7 cells, suggesting its possible anti-inflammatory activity. We thought anti-UV activity should be evaluated using skin-derived cells for the screening of candidate products for skincare. We re-examined the possible anti-UV activity of 35 Kampo medicines and their constituent plant extracts using the human immortalised skin keratinocyte cell line HaCaT. We also investigated whether there is a good correlation between this newly-established HaCaT system and a previous HSC-2 cell-based system (16).

## 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 powders, and dissolved in phosphate-buffered saline without calcium and magnesium [PBS (-)] prior to the experiments.

**Assay for anti-UV activity.** Spontaneously immortalized HaCaT keratinocytes, derived from adult human skin that remain highly related to their normal counterparts (CLS Cell Lines Service GmbH, Eppelheim, Germany) were inoculated at  $1 \times 10^4$  cells/0.1 ml in the inner 60 wells of a 96-microwell plate (Becton Dickinson Labware, Franklin Lakes, 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 24 hours, the attached cells were replenished with PBS(-) containing different concentrations of samples. The cells were then placed at 20.5 cm from a UV lamp (wavelength=253.7 nm), taking off the lid, and exposed to UV irradiation (6 J/m<sup>2</sup>/min) for 90 seconds, unless otherwise stated. The media were immediately replaced with fresh DMEM plus 10% FBS and cells were cultured for a further 24 hours at 37°C in an incubator with 5% CO<sub>2</sub> to determine the relative viable cell number by MTT method. In brief, the treated cells were incubated for another 4 hours in fresh culture medium containing 0.2 mg/ml MTT.

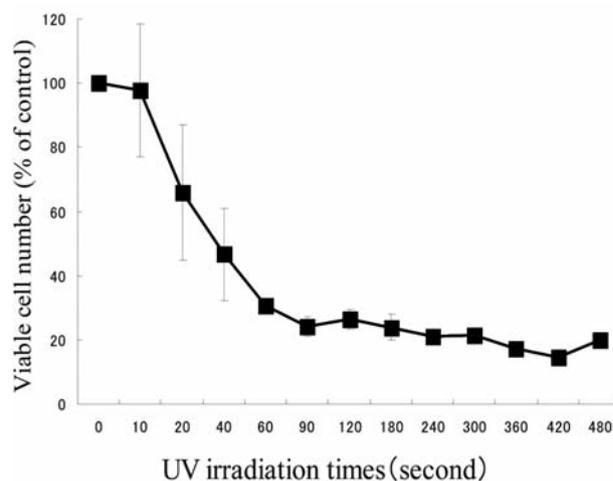


Figure 1. Effect of the time of exposure to UV irradiation on viable cell number. Near-confluent HaCaT cells were replenished with PBS(-) and then exposed to UV irradiation for the indicated periods. After incubation for 24 h in fresh culture medium, the viable cell number was determined as described in the Materials and Methods. Each value represents the mean±SD of three independent experiments.

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 Labssystem, Helsinki, Finland). From the dose–response curve, the 50% cytotoxic concentration against unirradiated cells and the concentration that increased the viability of UV-irradiated cells to 50% (EC<sub>50</sub>) were determined. The selectivity index (SI) was determined by the following equation:  $SI = CC_{50}/EC_{50}$  (7, 8).

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

## Results

**Optimum condition of UV irradiation.** We first determined the minimum exposure time required to induce maximum cytotoxicity. When HaCaT cells were exposed to UV irradiation in PBS(-), the viable cell number determined 24 h after incubation in fresh culture medium declined to the lowest level at 90 sec, where approximately 25% of the cells are still viable, and this level was maintained up to 480 sec (Figure 1). Based on this finding, we used an exposure time of 90 sec for the subsequent experiments.

**Anti-UV activity of Kampo medicines and their constituent plant extracts.** Sodium ascorbate (vitamin C), a popular antioxidant used as positive control, showed excellent anti-UV activity (SI=200) (Figure 4, Table I). Kampo medicines and their constituent plant extracts protected HaCaT cells from the UV-induced cytotoxicity to various extents (Figures 2-4).

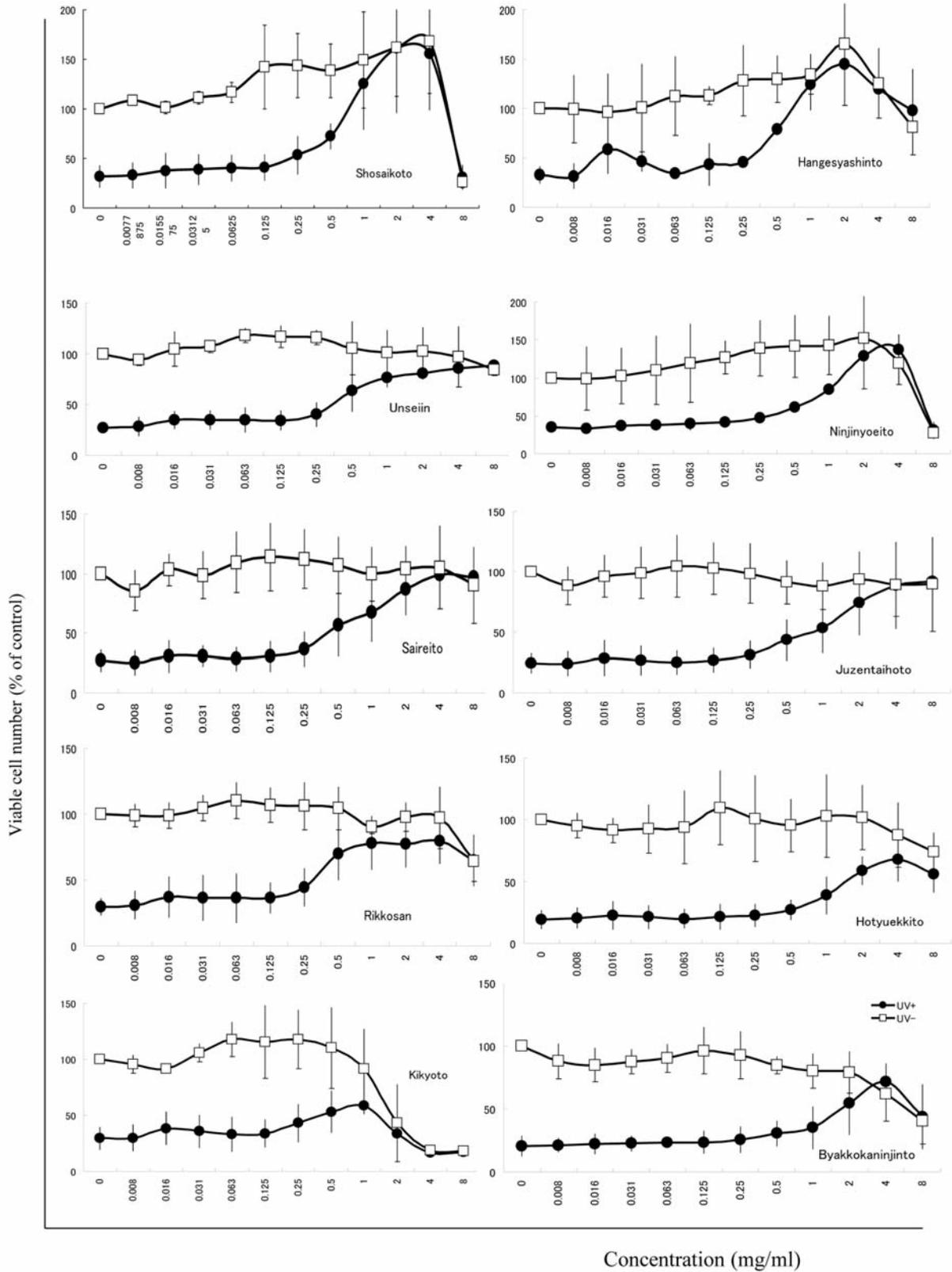


Figure 2. Anti-UV activity of 10 Kampo medicines. Near-confluent HaCaT cells were replenished with PBS(-) containing the indicated concentration of each Kampo medicine. The cells were then exposed to UV irradiation, and the viable cell number was determined after incubation for 24 h in fresh culture medium. Each value represents the mean±SD of three independent experiments.

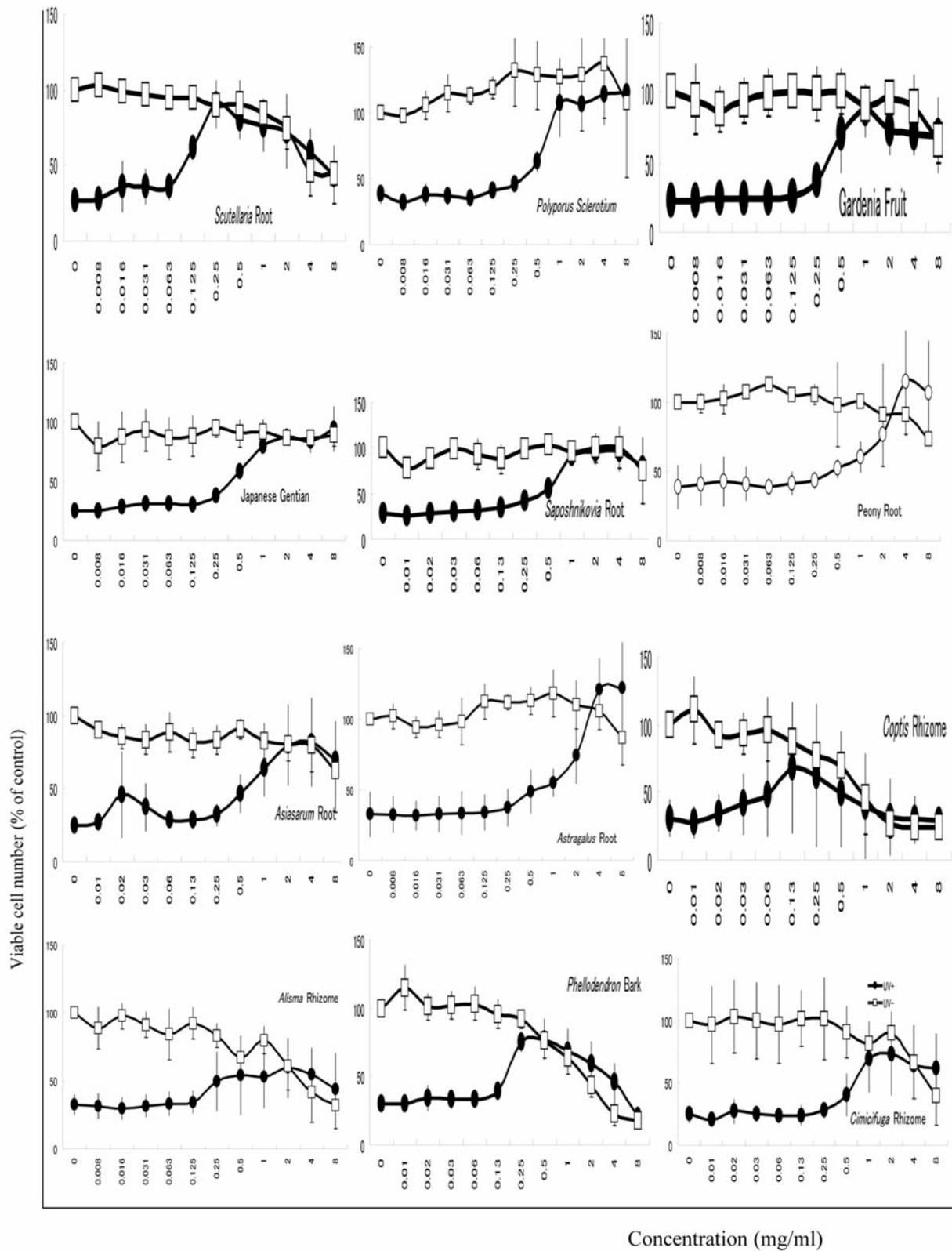


Figure 3. Anti-UV activity of 12 Kampo medicine constituent plant extracts. Near-confluent HaCaT cells were replenished with PBS(-) containing the indicated concentration of constituent plant extracts. The cells were then exposed to UV irradiation, and the viable cell number was determined after incubation for 24 h in fresh culture medium. Each value represents the mean±SD of three independent experiments.

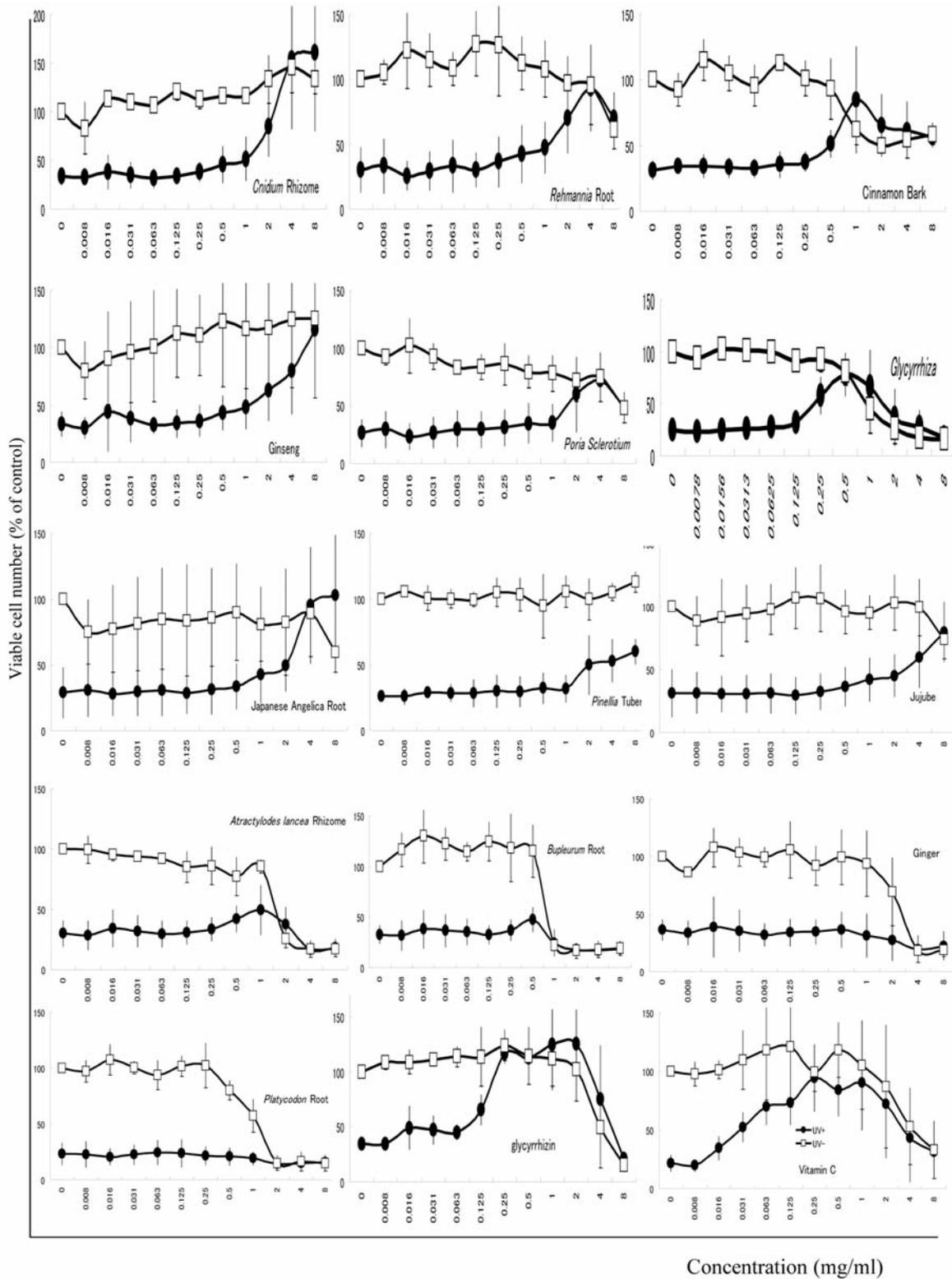


Figure 4. Anti-UV activity of 13 Kampo medicine constituent plant extracts and vitamin C. Near-confluent HaCaT cells were replenished with PBS(-) containing the indicated concentration of each constituent plant extract glycyrrizin, or sodium ascorbate (vitamin C). The cells were then exposed to UV irradiation, and the viable cell number was determined after incubation for 24 h in fresh culture medium. Each value represents the mean±SD of three independent experiments.

Table I. Anti-UV activity of *Kampo* medicines and constituent plant extracts against HSC-2 and HaCaT cells.

Name	HSC-2			HaCaT		
	CC <sub>50</sub> (mg/ml)	EC <sub>50</sub> (mg/ml)	SI	CC <sub>50</sub> (mg/ml)	EC <sub>50</sub> (mg/ml)	SI
<i>Alisma</i> rhizome	>2	>2	><1.0	3.1	0.28	11
<i>Asiasarum</i> root	>2	>2	><1.0	>8	0.6	>13
<i>Astragalus</i> root	>2	>2	><1.0	>8	0.6	>13
<i>Atractylodes lancea</i> rhizome	1.78	>2	<0.78	1.6	>8	<0.075
<i>Bupleurum</i> root	1.45	>2	<0.73	0.85	>8	<0.11
<i>Cimicifuga</i> rhizome	>2	1.42	>1.4	6.4	0.67	9.7
<i>Cinnamon</i> bark	1.27	>2	<0.64	2	0.27	7.1
<i>Cnidium</i> rhizome	>2	>2	><1.0	>8	0.87	>9.2
<i>Coptis</i> rhizome	0.286	0.1875	1.5	0.9	0.068	13
Gardenia fruit	>6	0.75	>8	>8	0.35	>23
Ginger	1.49	>2	<0.75	2.8	>8	<0.34
Ginseng	>2	>2	><1.0	>8	1.1	>7.1
<i>Glycyrrhiza</i>	2.8	0.65	4.3	0.92	0.21	4.4
Japanese Angelica root	>2	>2	><1.0	>8	2	>3.9
Japanese Gentian	>2	1.8	>1.1	>8	0.4	>20
Jujube	>2	>2	><1.0	>8	2.7	>3
Peony root	>2	>2	><1.0	>8	0.44	>18
Phellodendron bark	0.325	>6	<0.054	1.7	0.16	10
Pinellia tuber	>2	>2	><1.0	>8	2.1	>3.7
Platycodon root	1.62	>2	<0.8	1.2	>8	<0.15
<i>Polyporus sclerotium</i>	>2	1.92	>1.04	>8	0.3	>26
<i>Poria sclerotium</i>	>2	>2	><1.0	7.7	1.6	4.8
<i>Rehmannia</i> root	>2	>2	><1.0	>8	1.1	>7.1
<i>Saposhnikovia</i> root	>2	1.57	>1.27	>8	0.4	>20
<i>Scutellaria</i> root	1.71	>2	<0.855	3.7	0.1	38
Byakkokaninjinto	>2	>2	><1.0	6.2	1.8	3.5
Hangesyashinto	>6	1.23	>4.88	>8	0.28	>28
Hotyuekkito	>2	>2	><1.0	>8	1.6	>5.1
Juzentaihoto	>6	>6	><1.0	>8	0.83	>9.6
Kikyoto	4.1	3.9	1.05	1.9	0.42	4.4
Ninjinyoeito	>2	>2	><1.0	7	0.3	23
Rikkosan	>2	1.64	>1.22	>8	1.2	>6.8
Saireito	>6	1.77	>3.39	>8	0.42	>19
shosaikoto	>6	1.4	>4.29	7.3	0.21	34
Unseiin	>6	1.22	>4.9	>8	0.35	>23
Glycyrrhizin	10	0.49	20.6	4	0.11	36.4
Vitamin C	8	0.189	44	3.5	0.017	200

CC<sub>50</sub>, 50% Cytotoxic concentration; EC<sub>50</sub>, 50% effective concentration; SI, selectivity index. Data of HSC-2 cells were derived from ref. 16.

Among 10 *Kampo* medicines, Shosaikoto (SI=34) showed the highest anti-UV activity, followed by Hangesyashinto (SI>28), Unseiin (SI>23), Ninjinyoeito (SI=23) and Saireito (SI>19), whereas other four *Kampo* medicines were much less active (SI<9.6) (Figure 2, Table I).

Among 25 plant extracts, *Scutellaria* root exhibited the highest anti-UV activity (SI=38), followed by *Polyporus sclerotium* (SI>26), *Gardenia* fruit (SI>23), *Japanese Gentian* (SI>20) and *Saposhnikovia* root (SI>20) (Figure 3 and 4). *Glycyrrhizin* also exhibited potent anti-UV activity (SI=36) (Figure 3, Table I).

*Correlation of anti-UV activity between the present and previous systems.* The SI values determined with the present HaCaT system were generally one order of magnitude higher than those determined by the previous HSC-2 system, although there was good correlation of these two systems (R<sup>2</sup>=0.9118) (Figure 5).

### Discussion

The present study demonstrates that *Kampo* medicines and their constituent plant extracts exhibit some extent of anti-UV activity. The present HaCaT system may be suitable for

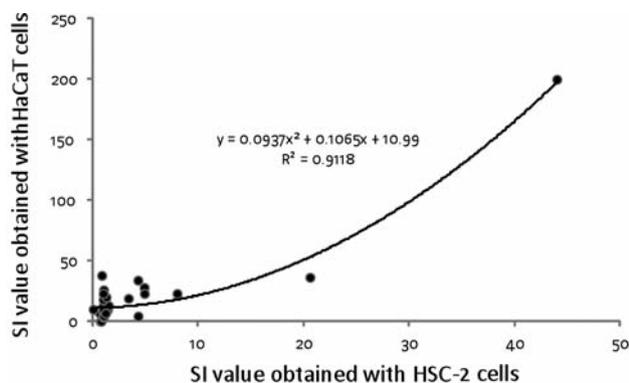


Figure 5. Correlation of anti-UV activity [evaluated as selectivity index (SI) value] between the present HaCaT and previous HSC-2 systems. Each point was derived from the data of Table I.

the first detection of anti-UV activity, since the SI value (reflecting the anti-UV activity) determined by the present HaCaT system was one order higher than that predicted by previous method with HSC-2 cells.

The HaCaT cells were found to be much more resistant to UV irradiation (approximately 20-25% cells were viable upon exposure to 6 J/m<sup>2</sup>/min for 480 sec) compared to HSC-2 cells (fewer than 1% viable upon exposure for only 60 seconds) (7-15, 17), elevating the background level of the viability of UV-irradiated HaCaT cells. This suggests that HaCaT cells should possess a strong UV protection system. However, higher baseline viability (20-35% in Figures 2-4) in the HaCaT system may make it difficult to accurately determine the EC<sub>50</sub> values (see large deviation of the samples with lower SI values from the asymptotic line in Figure 5). Such deviating samples should be re-checked with the HSC-2 system for the accurate determination of the SI value.

In conclusion, the present study demonstrates the improved sensitivity of the HaCaT anti-UV activity detection system, and suggests the possible application of Kampo medicines as sunscreens components in cosmetics.

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