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Anti-UV Activity of Lignin–Carbohydrate Complex and Related Compounds

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Abstract. *Background: We recently reported that an alkaline extract of the leaves of Sasa senanensis Rehder (SE) and Lentinus edodes mycelia extract (LEM), exhibiting lignin–carbohydrate complex (LCC)-like activity, protected cells from UV-induced injury (referred to as anti-UV activity). We investigated whether LCC is the major active components responsible for anti-UV activity. Materials and Methods: Human oral squamous cell carcinoma HSC-2 cells were exposed to short UV irradiation in phosphate-buffered saline, containing different concentrations of LCC. After culturing for 48 h in fresh culture medium, the viable cell number was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. From the dose–response curve, the 50% cytotoxic concentration (CC_{50}) and the concentration that increased the viability of the UV-irradiated cells to 50% of the control value (EC_{50}) were determined. The selectivity index (SI) was determined by the following equation: $SI=CC_{50}/EC_{50}$. Results: LCCs (Fr. VI) of pine cones and seed shell, and sulfated LCC exhibited relatively high anti-UV activity (SI=7.1-38), compared with that of SE and LEM. LCCs with lower lignin content (Fr. VII) exhibited anti-UV activity, approximately one half that of Fr. VI. However, polysaccharides (laminarin, pullulan, dextran) introduced with dimethylaminoethyl- or sulfate groups with different substitution ratios were totally inactive (SI<1). The introduction of a sulfate group to LCC did not enhance the anti-UV activity of LCC. Sodium ascorbate and vanillin were the most active (SI=65), whereas gallic acid (SI=5), epigallocatechin gallate (SI=2.6), artrumeron (SI<1), and turmeric extract (SI<1) were much less active. Conclusion: The prominent anti-UV activity of SE and LEM seems to be generated by LCCs present in the extract.*

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Ultraviolet rays (UV) are invisible electromagnetic waves with wavelength raging from 1-400 nm, and are 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, whereas UVC cannot pass through the air due to absorption. UV exerts several favorable effects such as sterilization and disinfection (1), vitamin D synthesis (2), stimulation of the metabolism and skin resistance. However, excessive doses of UV produce 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 (4, 5).

High-dose of UV irradiation induced apoptotic cell death in human myelogenous leukaemia cell lines, but induced other types of cell death in human T-cell leukaemia, erythroleukaemia, glioblastoma (6), oral squamous cell carcinoma (OSCC) cell lines and human normal oral cells (gingival fibroblasts, pulp cells, periodontal ligament fibroblasts) (7). We recently established a method that can measure the activity of a substance in protecting the cells from UV-induced injury (referred to as anti-UV activity) (7, 8). Using this method, we previously reported that *Lentinus edodes* mycelia extract (LEM) (9) and alkaline extract of the leaves of *Sasa senanensis* Rehder (SE) (10) exhibited much higher anti-UV activity than green tea, black tea, Jasmine tea, oolong tea, barley tea and Kohki tea. The data of the anti-UV activity of natural products are limited, only available from our laboratories.

We recently found that both SE (11) and LEM (12) exhibited activities characteristic of several lignin–carbohydrate complex (LCC), such as high anti-HIV activity and synergistic

action with sodium ascorbate. Furthermore, *p*-coumaric acid was recently identified as an anti-UV substances present in SE (13). LEM contains several lignin precursors such as vanillic acid, syringic acid, *p*-coumaric acid, ferulic acid, vanillin, caffeic acid, but does not contain tannin precursors (12). These data suggest the possibility that LCC present in LEM and SE might be responsible for their high anti-UV activity. To test this possibility, we prepared several LCCs from pine cone and seed shell (14, 15), and polysaccharides to which positively charged dimethyaminoethyl (DMAE) group or negatively charged sulfate group was introduced (16), and investigated their anti-UV activity.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), (–)epigallocatechin gallate (EGCG): Sigma-Aldrich Inc., St. Louis, MO, USA; dimethyl sulfoxide (DMSO): Wako Pure Chemical Ind., Osaka, Japan; sodium ascorbate, gallic acid, alkali-lignin: Tokyo Chemical Industry Co., Ltd., Tokyo. Ar-Trumeron and turmeric extract and lignin sulfonate (P201, P252) (produced by sulfation of a by-product of paper manufacturing) was provided by Maruzene Pharmaceutical Co., Ltd., Tokyo, Japan.

Preparation of LCCs. LCCs [fraction (Fr). VI and VII] from pine cone of *P. parviflora* Sieb. et Zucc., *P. densiflora* Sieb. et Zucc., *P. elliottii* var *Elliottii*, *P. taeda* L., *P. thunbergii* Parl., and pine seed shell of *P. parviflora* Sieb. et Zucc., were isolated by acid precipitation to obtain Fr. VI and ethanol precipitation of the resultant supernatant to obtain Fr. VII after washing with ethanol and hot water, as described previously (14, 15).

Chemical modification. DMAE and sulfate groups were introduced into various glucans (paramylon, laminarin, pullulan and dextran), as previously reported (16, 17). These products were kindly provided by Dr. Ichikawa, Tsukuba Research Laboratory, Harima Chemicals, Inc., Tsukuba, Ibaragi, Japan.

UV protection assay. The 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 calcium and magnesium-free phosphate-buffered saline [PBS (–)] to minimize the evaporation of water from the culture medium. After 48 h, the media of the near-confluent cells were replaced with PBS(–) containing different concentrations of LCC test samples. PBS(–) was used instead of DMEM plus 10% (FBS), since the latter contains radical scavenger(s) or UV absorbing substance(s) such as phenol red and proteins (9). The cells were then placed at 20.5 cm distance 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 the cells were cultured for a further 48 h at 37˚C in an incubator with 5% CO_2 and then the relative viable cell number was determined by the MTT method, as described previously (9). From the dose–response curve, the 50% cytotoxic concentration (CC_{50}) and the concentration that increased the viability of the UV-irradiated cells to 50% of the control value (EC_{50}) were determined. The selectivity index (SI) was determined by the following equation: $SI=CC_{50}/EC_{50}$ (9).

Results

LCC. When human oral squamous cell carcinoma HSC-2 cells were exposed to UV irradiation for 1 min in PBS(-), essentially no viable cells remained after 48 h of culture in the regular culture medium (open circles in Figure 1). However, the addition of the LCC fraction prepared from pine cones from different *Pinus species* or from seed shell of *Pinus parviflora* Sieb. et Zucc. in PBS(–), dose-dependently protected the cells from the cytotoxic effect of UV irradiation. We used two different LCC fractions: Fr. VI prepared by acid-precipitation of the alkaline extract, and Fr. VII prepared by ethanol precipitation of the acid-soluble fraction (14, 15).

LCC Fr. VI of pine cone of *P. elliottii* var. *Elliottii* (SI>38.1) had the greatest anti-UV activity, followed by that of pine cone of *P. parviflora* Sieb. et Zucc. (SI>37), seed shell of *P. parviflora* Sieb. et Zucc (SI=25.6), pine cone of *P. taeda* L. (SI=24.8), *P. densiflora* Sieb. et Zucc. (SI=>9.8) and *P. thunbergii* Parl. (SI=7.6) (upper panel, Figure 1, Exp. 1 in Table I).

Next, we investigated the anti-UV activity of LCC Fr. VII that contains relatively higher amounts of polysaccharides with lower amounts of lignin. LCC Fr. VII of seed shell of *P. parviflora* Sieb. et Zucc. had the highest anti-UV activity (SI=>21.1), followed by that of pine cone of *P. elliottii* E. (SI>12.3), *P. taeda* L. (SI>10.3), *P. parviflora* Sieb. et Zucc. (SI>7.8) and *P. thunbergii* Parl. (SI>7.1) (lower panel, Figure 1, Exp. 2 in Table I).

Chemically–modified glucans. DMAE glucans such as DMAE-laminarin, DMAE-pullulan and DMAE-dextran, paramylon sulfate (substitution ratio=0.07, 4 and 7.2%) exibited no anti-UV activity (SI<1), whereas sulfated lignin (P-201 and P-202) (SI>8.2 and >7.1) and sodium ascorbate (SI=89.3) had much higher anti-UV activity (Figure 2, Exp. 3 in Table I).

Antioxidants. Among the tested antioxidants, sodium ascorbate and vanillin exhibited the highest anti-UV activity (SI=64.2 and 63.8), followed by gallic acid (SI=5.4), epigallocatechin gallate (EGCG) (SI>2.6). On the other hand, ar-trumeron and turmeric were inactive (SI<1) (Figure 3, Exp. 4 in Table I).

Combination effect of alkali-lignin and sodium ascorbate. Alkali-lignin alone had potent anti-UV activity (SI>61.5) (Figure 4A). The addition of 100 μg/ml (=0.5 mM) sodium ascorbate further enhanced its anti-UV activity (Figure 4B). The addition of 300 or 1000 μg/ml (1.5 or 5 mM) sodium ascorbate

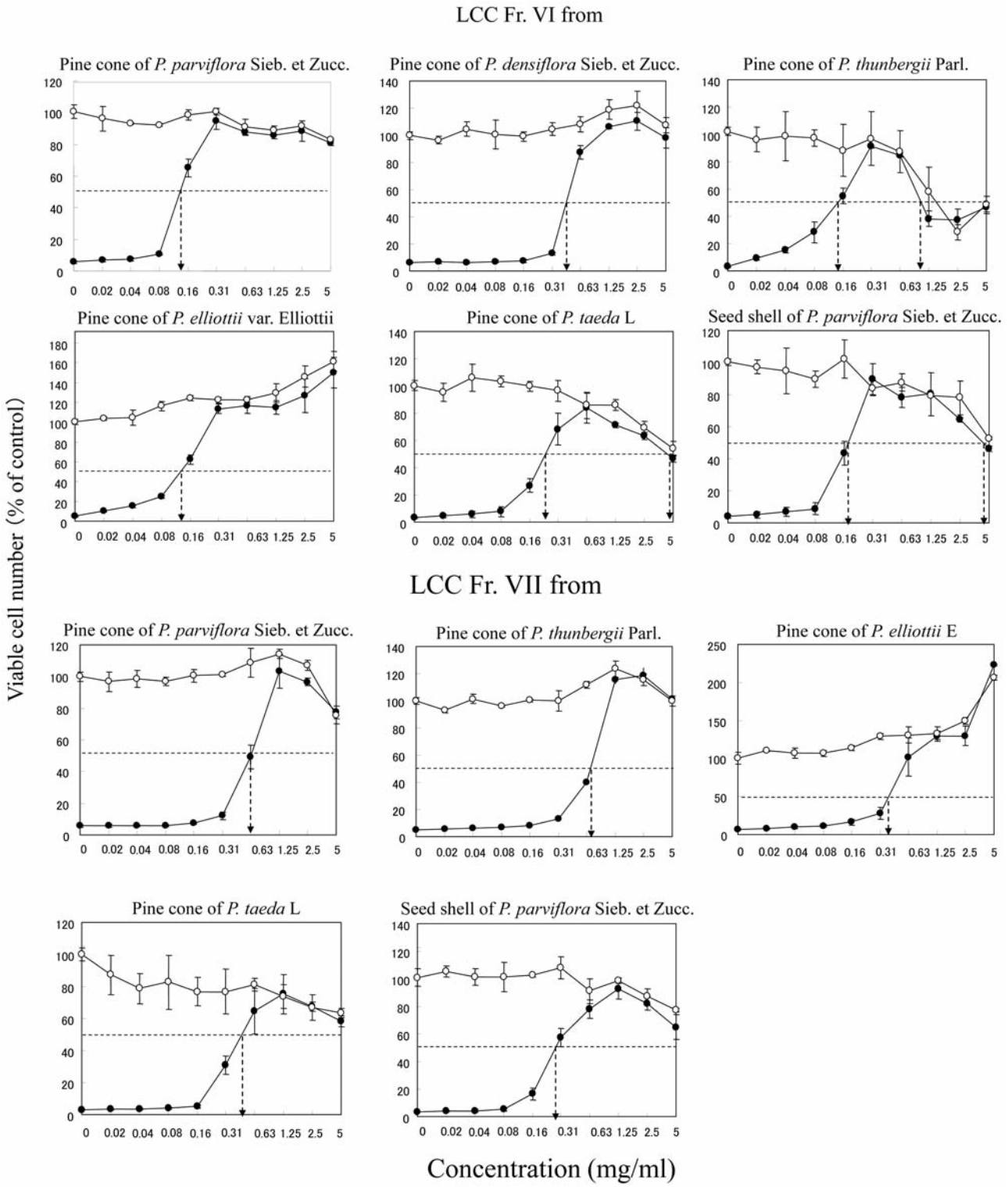

Figure 1. *Effect of lignin–carbohydrate complexes (LCCs) from various Pinus species on UV-induced cytotoxocity. Near confluent HSC-2 cells were replaced with PBS(–) in the absence (control) or presence of different concentrations of LCCs prepared by acid precipitation of the alkaline extract of the indicated plants (Fr. VI) (upper panel) and those prepared by 50%-ethanol precipitation of the Fr. VI-free supernatant (Fr. VII) (lower panel). The cells were then exposed to UV irradiation (6 J/m^2/min) for 1 min. The media were replaced with fresh fetal bovine serum (FBS) and cells were cultured for a further 48 h. The viable cell number was determined by the MTT method, and expressed as a percentage of control unirradiated and untreated cells. Each value represents the mean±SD of triplicate assays.*

Table I. *Anti-UV activity of lignin-carbohydrate complex (LCC) and related compounds.*

	CC_{50} (mg/ml)	EC_{50} (mg/ml)	SI(CC_{50}/EC_{50})
Exp. 1: LCC Fr. VI from			
Pine cone of *Pinus elliottii* var. Elliottii.	>5	0.14	>38.1
Pine cone of *Pinus parviflora* Sieb. et Zucc.	>5	0.14	>37.0
Seed shells of *Pinus parviflora* Sieb. et Zucc.	4.5	0.18	25.6
Pine cone of *Pinus taeda* L.	4.5	0.19	24.8
Pine cone of *Pinus densiflora* Sieb. et Zucc.	>5	0.51	>9.8
Pine cone of *Pinus thunbergii* Parl.	1.1	0.14	7.6
Mean			>23.8±13.0
Exp. 2: LCC Fr. VII from			
Seed shells of *Pinus parviflora* Sieb. et Zucc.	>5	0.24	>21.1
Pine cone of *Pinus elliottii* E.	>5	0.41	>12.3
Pine cone of *Pinus taeda* L.	>5	0.49	>10.3
Pine cone of *Pinus parviflora* Sieb. et Zucc.	>5	0.64	>7.8
Pine cone of *Pinus thunbergii* Parl.	>5	0.72	>7.1
Mean			>11.7±5.6
Exp. 3: Chemically modified glucans and lignin:			
DMAE-laminarin	>5	–	–
DMAE-pullulan P-5	>5	–	–
DMAE-pullulan P-25	>5	–	–
DMAE-pullulan P-100	>5	–	–
DMAE-pullulan P-400	>5	–	–
DMAE-dextran	>5	–	–
Paramylon sulfate (SR=0.07%) (MW 123 kD)	>5	–	–
Paramylon sulfate (SR=4.0%) (MW 132 kD)	>5	–	–
Paramylon sulfate (SR=7.2%) (MW 139 kD)	>5	–	–
P-201	>4	>0.49	>8.2
P-252	>4	>0.56	>7.1
Sodium ascorbate (mM) (positive control)	>5	0.056	89.3
Exp. 4: Anti -oxidants			
Sodium ascorbate (mM)	19.9	0.3	64.2
Vanillin	33.2	0.5	63.8
Gallic acid	1.9	0.4	5.4
EGCG	>4	0.15	>2.6
Ar-trumeron	–	–	–
Turmeric	–	–	–
Exp. 5: Combination of LCC and vitamin C			
Alkali-lignin 0 mg/ml +sodium ascorbate (mM)	>16	0.3	>61.5
Alkali-lignin 0.1mg/ml +sodium ascorbate (mM)	>16	0.2	>94.1
Alkali-lignin 0.3 mg/ml +sodium ascorbate (mM)	–	–	–
Alkali-lignin 1 mg/ml +sodium ascorbate (mM)	–	–	–

induced a plateau level of anti-UV activity that could not be accurately determined by the scale over EC_{50} (<0.06) and CC_{50} values (>16 mg/ml) of alkali-lignin (Figure 4C and D).

Discussion

The present study demonstrated that LCC Fr. VI from various sources of plants (SI>23.8±13.0, n=6) and that from commercially available LCC (alkali-lignin) (SI>61.5) had comparable or slightly higher anti-UV activity as compared with SE (SI=9.6-42) (10) and LEM (SI=13.9) (9). Furthermore, LCC enhanced the anti-UV activity of vitamin C (Figure 4), as did SE (see Figure 2 in ref. 10) and LEM (see Figure 6 in ref. 9). These data suggest that the anti-UV activity of SE and LEM may be due to LCC present in these extracts. Taken together with our previous data, it was demonstrated, for the first time, that the anti-UV activity of LCCs (SI>23.8) was higher than that of gallic acid

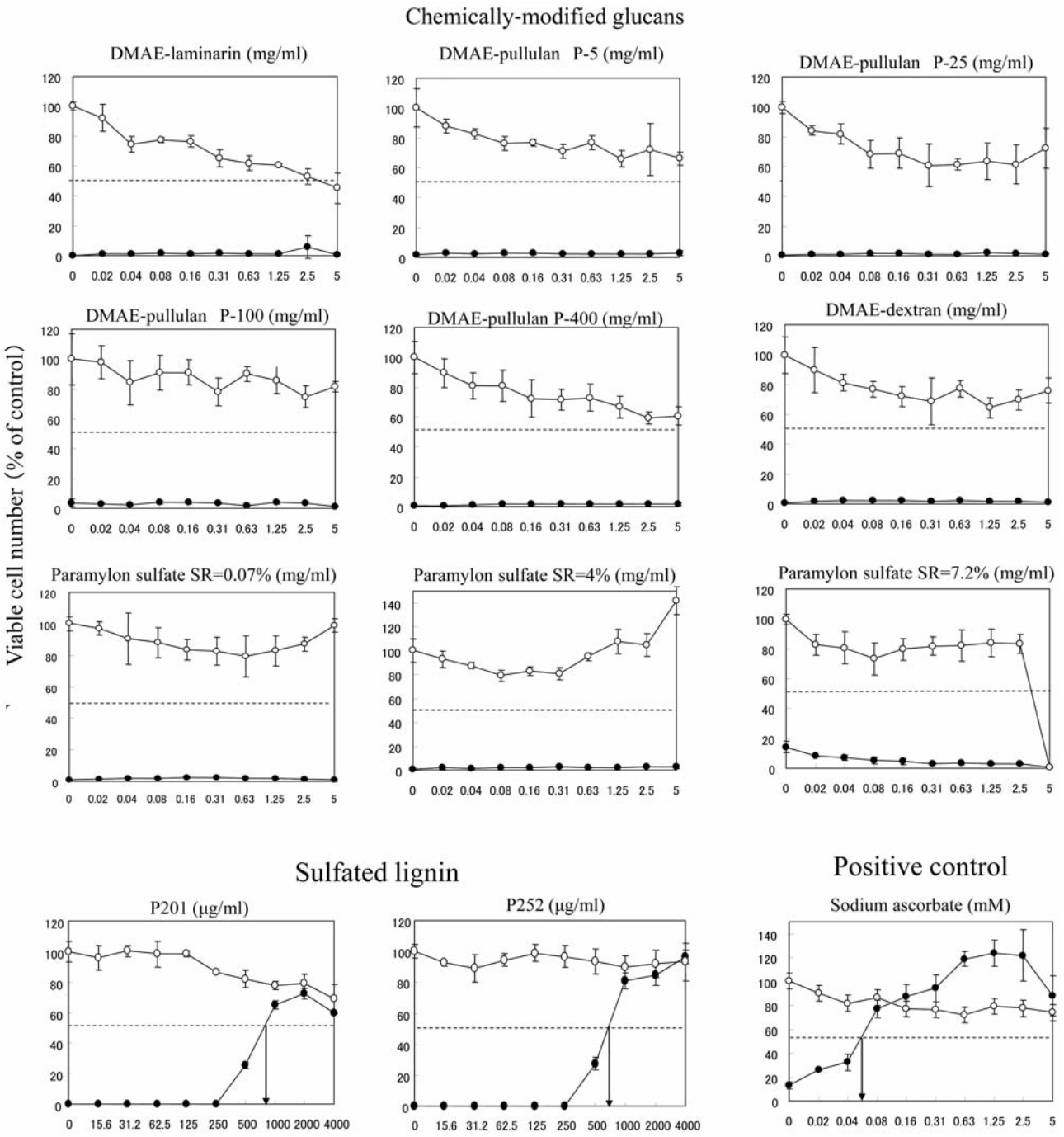

Figure 2. *Effect of chemically–modified glucans on UV-induced cytotoxocity. Near-confluent HSC-2 cells were replenished with PBS(–) in the absence (control) or presence of various concentrations of chemically–modified glucans or sodium ascorbate (used as positive control). The cells were then exposed to UV irradiation for 1 min, and after 48 h the viable cell number was determined in fresh culture medium, by MTT the method. Each value represents the mean±SD of triplicate assays as a percentage to that of the control.*

(component of tannin) (SI=5.4), EGCG (major component of green tea) (SI>2.6) (Table I), tea extracts (green tea, black tea, Jasmine tea, oolong tea, barley tea and Kohki tea) (SI=1.0-3.4) (9), luteolin 6-C-β (SI=7.9), luteolin7-*O*-β-D-glucoside (SI=6.0), luteolin 6-*C*-α-L-arabinoside (SI=6.2), tricin (SI=2.7) (18), chlorophyll (SI=0.2), chrolophyllin (SI=0.5) (10), and hot water extract of Kampo medicines (SI=1-2) (19). This finding further adds one valuable

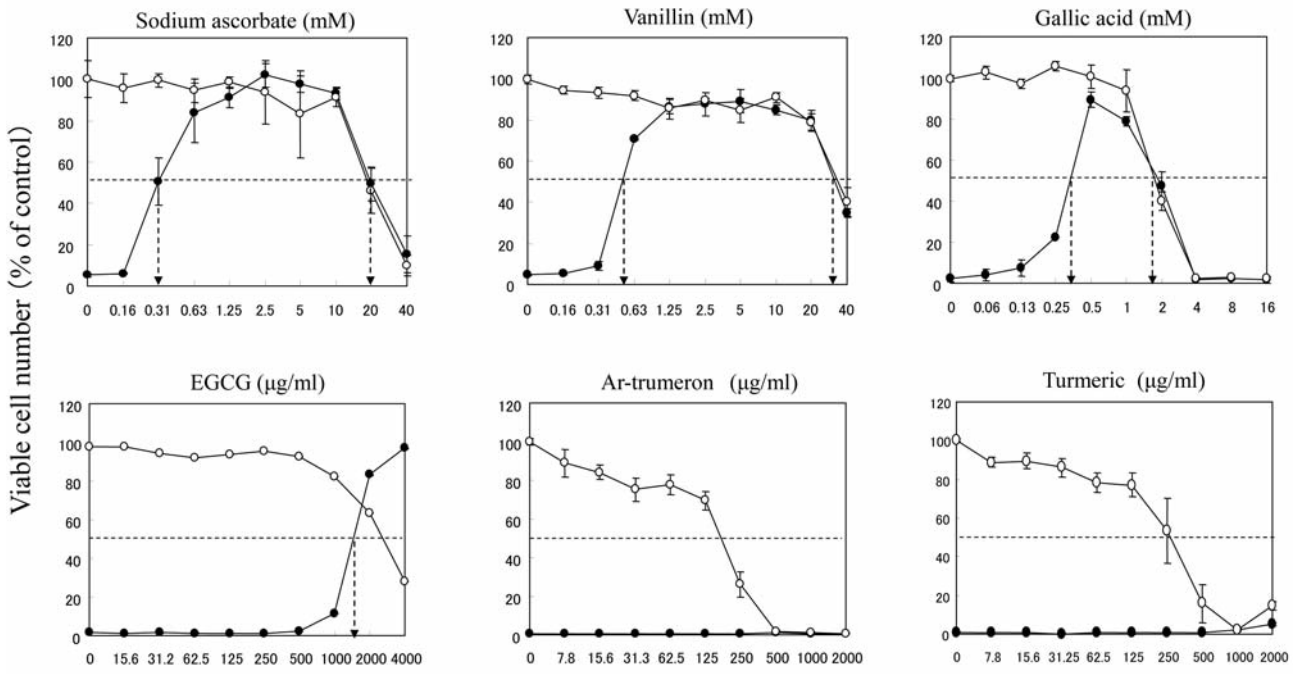

Figure 3. *Effect of anti-oxidants on UV-induced cytotoxocity. Near-confluent HSC-2 cells were replenished with PBS(–) in the absence (control) or presence of anti-oxidants. The cells were then exposed to UV irradiation for 1 min, and after 48 h the viable cell number was determined in fresh culture medium, as described in Figure 1. Each value represents the mean±SD of triplicate assays.*

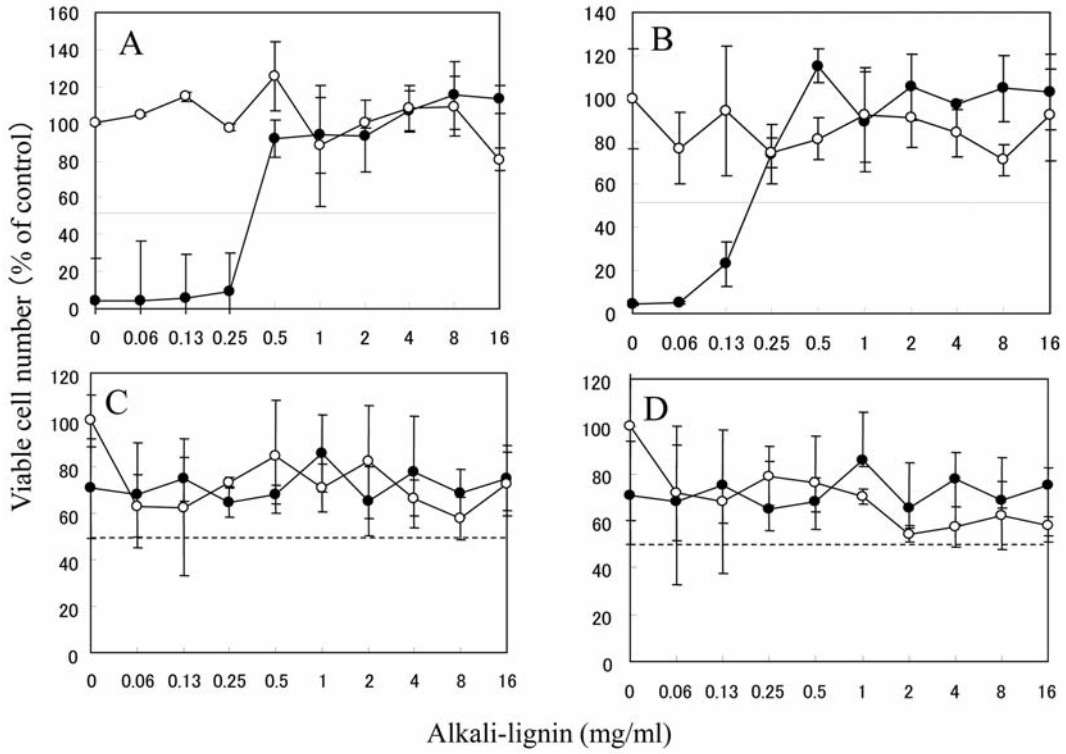

Figure 4. *Effect of combination of alkali-lignin and sodium ascorbate on UV-induced cytotoxocity. Near-confluent HSC-2 cells were exposed to UV irradiation for 1 min in PBS(–) without (control) or with the indicated concentrations of alkali-lignin without (A) or with 100 (B), 300 (C) or 1000 (D) μg/ml sodium ascorbate (VC). The cells were cultured in fresh medium for 48 h and then the viable cell number (expressed as a percentage that of the control unirradiated and untreated cells) was determined, as described in Figure 1. Each value represents the mean±SD of triplicate assays.*

biological activity to LCCs that has prominent anti-HIV activity. High anti-UV activity of LCC may suggest their anti carcinogenic activity against ROS, including hydroxyl radical produced by UV irradiation. Anti-aging activity of LCC using 8-oxoguanine assay is under way.

We found that the anti-UV activity of LCC Fr. VII (SI>11.7±5.6) was one half that of LCC Fr. VI (SI>23.8±13.0). This can be explained by the fact that Fr. VII is composed mostly of polysaccharides with minor lignin content (13). We also found that the anti-UV activity of sulfated lignin (SI=7.1-8.1) was approximately one third that of LCC Fr. VI. This suggests that the introduction of a sulfate group to LCC does not enhance its anti-UV activity, in contract to prominent anti-HIV activity of sulfated polysaccharide (17).

In conclusion, the present study demonstrated that LCL shows much higher anti-HIV and anti-UV activity as compared with lower molecular weight polyphenols, such as tannins and flavonoids. This strongly suggests that the use of an alkaline solution (that extracts LCC) rather than hot water (that extracts Kampo medicine) may be a better choice to prepare anti-HIV/UV activity in higher yield.

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