

Biological Activity of SE-10, Granulated Powder of *Sasa senanensis* Rehder Leaf Extract

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Abstract. *Background:* We have previously reported that alkaline extract of *Sasa senanensis* leaves (SE) showed potent anti-HIV, anti-UV and radical scavenging activity. *In the present study,* we investigated the biological activities of SE-10, a granulated powder of SE supplemented with lactose, lactitol, trehalose and tea extract. *Materials and Methods:* Cell viability of mock-infected, HIV-infected, and UV-irradiated cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method. Scavenging activity of superoxide anion and hydroxyl radicals was determined by electron-spin resonance spectroscopy. Cytochrome P-450 (CYP)3A4 activity was measured by β -hydroxylation of testosterone in human recombinant CYP3A4. *Results:* SE-10 had slightly higher anti-HIV and anti-UV activities, but slightly lower radical-scavenging and CYP3A4-inhibitory activities, as compared with SE. *Conclusion:* The present study demonstrates that the biological activities of SE were well preserved during the manufacturing process of SE-10.

Over the counter drugs are classified into three groups (I, II and III), based on their safety (1). Alkaline extract of the

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leaves of *Sasa senanensis* Rehder (SE) or *Sasa albo-marginata* Makino et Shibata (SASA-Health[®]) (Daiwa Biological Research Institute Co., Ltd., Kanagawa, Japan), which belong to Group III, are expected to be less hazardous compared with Kampo Medicines, which belong to Group II.

SE has been recognized as being effective in treating fatigue, poor appetite, halitosis, body odour and stomatitis. SE exhibited *in vitro* antiseptic (2), membrane-stabilising (3), anti-inflammatory (4-6), antibacterial (7, 8), antiviral (7, 8), anti-UV (9) and radical-scavenging (5, 7, 10) activities, and synergistic action with vitamin C (7). Some of these activities (anti-HIV, anti-UV, and radical-scavenging activity, and synergism with vitamin C) are characteristic of the lignin-carbohydrate complex (LCC) (11). In accordance with this, SE exhibited higher anti-HIV, anti-UV and radical scavenging activity than its related products (product B and product C) (12), which were prepared by different extraction methods. Furthermore, SE inhibited cytochrome P-450 (CYP)3A activity to a lesser extent than grapefruit juice [generally accepted as a CYP3A inhibitor (13)], chlorophyllin, products B and C (8, 12), suggesting that the adverse effect of drugs concomitantly administered with SE may be milder than that with product B or C. We also found that product C, supplemented with ginseng and pine (*Pinus densiflora*) leaf extracts, had significantly lower anti-HIV and anti-UV activity as compared with SE (12), suggesting that SE without supplementation has sufficient biological activity.

SE-10 is a granulated powder of SE supplemented with lactose, lactitol, trehalose and tea extract, and is sold as dried and packaged powder in drug stores. We investigated here whether SE-10 has comparable biological activity to that of SE.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle's medium (DMEM): Gibco BRL, Grand Island, NY, USA; fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), hypoxanthine (HX), xanthine oxidase (XOD), diethylenetriaminepenta-acetic acid (DETAPAC), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO): Dojin, Kumamoto, Japan; RPMI-1640 medium, azidothymidine (AZT), 2',3'-dideoxycytidine (ddC): Sigma Chemical Co. St. Louis, MO, USA; dimethyl sulfoxide (DMSO), dextran sulfate (5 kDa): Wako Pure Chemical Ind., Ltd., Osaka, Japan; sodium ascorbate: Tokyo Chemical Industry Co., Ltd., Tokyo, Japan; curdlan sulphate: 79 kDa: Ajinomoto Co. Inc., Tokyo, Japan.

SE-10 and SE were provided by Daiwa Biological Research Institute Co. Ltd., Kawasaki, Kanagawa, Japan. One millilitre of SE was freeze-dried to produce the powder (66.1 mg). One package (1.5 g powder) of SE-10 contained 661 mg dried SE powder, 0.9 g lactate, 0.2 g lactitol, 0.15 g trehalose and 6 mg tea extract.

Assay for anti-HIV activity. Human T-cell leukemia virus I (HTLV-I)-bearing CD4-positive human T-cell line, MT-4, was cultured in RPMI-1640 medium supplemented with 10% FBS and infected with HIV-1_{IIIB} at a multiplicity of infection of 0.01. HIV- and mock-infected (control) MT-4 cells (3×10^4 cells/96-microwell) were incubated for five days with different concentrations of SE-10 or SE and the relative viable cell number was determined by the MTT assay. The concentration that reduced the viable cell number of the uninfected cells by 50% (CC_{50}) and the concentration that increased the viable cell number of the HIV-infected cells to the 50% that of control (mock-infected, untreated) cells (EC_{50}) were determined from the dose-response curve with mock-infected and HIV-infected cells, respectively. The anti-HIV activity was evaluated by the selectivity index (SI), which was calculated using the following equation: $SI = CC_{50}/EC_{50}$ (14).

Assay of anti-UV activity. Human oral squamous cell carcinoma HSC-2 cells (provided by Professor Nagumo, Showa University) were inoculated into 96-microwell plates (3×10^3 cells/well, 0.1 ml/well) and were incubated for 48 h to allow cell attachment. The culture supernatant was replaced with 100 μ l phosphate-buffered saline without calcium and magnesium [PBS(-)] that contained different concentrations of SE-10 or SE, placed at 21 cm distance from a UV lamp (wavelength: 253.7 nm) and were exposed to UV irradiation (6 J/m²/min) for 1 min. The cells were then incubated for a further 48 hours in DMEM containing 10% FBS to determine the relative viable cell number by the MTT assay. From the dose-response curve, the CC_{50} and the concentration that increased the viability of UV-irradiated cells up to 50% that of control (unirradiated, untreated) cells (EC_{50}) were determined. The SI was determined using the following equation: $SI = CC_{50}/EC_{50}$ (15, 16).

Radical-scavenging activity. The free radical intensity was determined at 25°C, using electron-spin resonance (ESR) spectroscopy (JEOL JES REIX, X-band, 100 kHz modulation frequency; JEOL Ltd., Tokyo, Japan) (17). The instrument settings were: centre field, 335.5 \pm 5.0 mT; microwave power, 16 mW; modulation amplitude, 0.1 mT; gain, 630; time constant, 0.03 s and scanning time, 2 min.

For the determination of the superoxide anion (in the form of DMPO-OOH), produced by the HX-XOD reaction (total volume: 200 μ l) [2 mM HX in 0.1 M phosphate buffer (PB) (pH 7.4) 50 μ l, 1 mM DETAPAC 10 μ l, 10% DMPO 30 μ l, test sample (in PB) 40 μ l, PB 40 μ l, XOD (0.5 U/ml in PB) 30 μ l], the time constant was changed to 0.03 s (17).

For the determination of the hydroxyl radical (in the form of DMPO-OH), produced by the Fenton reaction (200 μ l) [1 mM FeSO₄ (containing 0.2 mM DETAPAC) 50 μ l, 0.1 M PB (pH 7.4) 50 μ l, 92 mM DMPO 20 μ l, test sample (in H₂O) 50 μ l, 1 mM H₂O₂, 30 μ l], the gain was changed to 160 (17).

The concentration that reduced the radical intensity of DMPO-OOH and DMPO-OH by 50% (IC_{50}) was determined by the dose-response curve.

Measurement of CYP3A4 activity. CYP3A4 activity was measured by β -hydroxylation of testosterone in human recombinant CYP3A4 (Cypex Ltd., Dundee, UK). The reaction mixture, containing 200 mM potassium phosphate buffer (pH 7.4), NADPH regenerating system (1.3 mM NADPH, 1.3 mM glucose-6-phosphate, 0.2 U/ml glucose-6-phosphate dehydrogenase, and 3.3 mM MgCl₂) along with 0, 0.01, 0.1, 0.5, 1.0 and 2% of SE-10 or SE or vehicle (water) and the human recombinant CYP3A4 (16.5 pmol/ml), was preincubated at 37°C for 2 min. The reaction was started by the addition of 300 μ M testosterone substrates. The final volume of the reaction mixture was 250 μ l with a final DMSO concentration of 2%. The reaction was stopped by the addition of 500 μ l ethyl acetate after 15 min. After centrifugation (15,000 \times g, 5 min), 400 μ l of supernatant were collected, dried, and resuspended in 200 μ l of methanol. Analyses of the metabolites were performed by high-performance liquid chromatography (HPLC) (JASCO PU2089, AS2057, UV2075 ChromNAV) equipped with a TSK gel ODS-120A, 4.6 mm ID \times 25 cm, 5 μ m column (TOSOH, Tokyo, Japan). The mobile phase consisted of 70% methanol and 30% water. The metabolites were separated using an isocratic method at a flow rate of 1.0 ml/min. Quantification of the metabolites was performed by comparing the HPLC peak area at 254 nm to that of 11 α -progesterone, the internal standard. The retention times for 6 β -hydroxytestosterone and 11 α -progesterone were approximately 5.0 and 6.7 min, respectively. The concentration that inhibited the CYP3A4 activity by 50% (IC_{50}) was determined from the dose-response curve.

Determination of optimal exposure time. Normal human oral cells, gingival fibroblast (HGF), pulp cells (HPC) and periodontal ligament fibroblast (HPLF) (16), and HSC-2 cells were incubated for the indicated times without or with SE-10 or SE. The medium was replaced with fresh medium, and cells were further incubated for 48 hours to determine the viable cell number by MTT method.

Statistical treatment. Experimental values are expressed as the mean \pm standard deviation (SD). Statistical analysis was performed by using the Student's *t*-test. A *p*-value <0.05 was considered to be significant.

Results

Components of SE-10 and SE. Components of SE-10 and SE are listed in Table I. SE-10 contained higher amounts of glucose and galactose (derived from lactose), and caffeine (derived from tea extract), but other components were comparable, when corrected for the fold dilution.

Table I. Composition of SE-10 and SE.

	SE-10		SE	
	(mg/100 g)	(mg/SE 100 g)*	(mg/100 ml)	(mg/100 g)**
Water	500		93800	-
Protein	10400	23600	1500	22700
Lipid	1200	2730	200	3030
Ash content	5400	12300	900	13600
Sugar	66100	150000	1200	18200
Glucose	26600	60000	90	1360
Arabinose	2500	5700	380	5700
Xylose	7600	17300	1060	16000
Galactose	25600	58000	180	2700
Dietary fibre	14400	32700	2100	31800
Water-soluble	8900	20000	1400	21200
Water-insoluble	5500	12500	700	10600
Tannin	3930	8930	N.D.	
Arginine	100	280	19	290
Lysine	410	930	59	890
Histidine	160	360	23	350
Phenylalanine	580	1320	86	1300
Tyrosine	370	840	63	950
Leucine	910	2070	135	2040
Isoleucine	360	820	53	800
Methionine	230	520	32	480
Valine	640	1450	95	1440
Alanine	730	1660	105	1590
Glycine	710	1600	99	1500
Proline	570	1295	84	1270
Glutamic acid	1290	2932	186	2800
Serine	160	364	21	320
Threonine	110	250	13	200
Aspartic acid	1090	2480	159	2400
Tryptophan	210	480	28	420
Folic acid	0.058	0.13	0.008	0.12
Lutein	2.3	5.2	0.3	4.5
Caffeine	27	61	U.D.	
Sodium	2390	5432	395	5980
Iron	13.1	30	1.02	15
Calcium	15.7	36	1.0	15
Potassium	50.7	115	4.9	74
Magnesium	5.5	13	0.5	8
Zinc	0.63	1.4	0.08	1.2
Vitamin A (retinol equivalent)	0.022	0.05	0.003	0.05
α -Carotene	0.042	0.1	U.D.	
β -Carotene	0.241	0.5	0.032	0.5
Vitamin K ₁	0.040	0.09	0.006	0.09
α -Tocopherol	0.4	0.9	U.D.	
γ -Tocopherol	0.2	0.5	U.D.	

*Corrected for SE content (44%); **corrected, assuming that 1 ml contains 66.1 mg SE. N.D., Not determined. U.D., undetectable. The summation of SE-10 and SE was 170-180 g, and therefore these values should be utilized to assess the relative amounts.

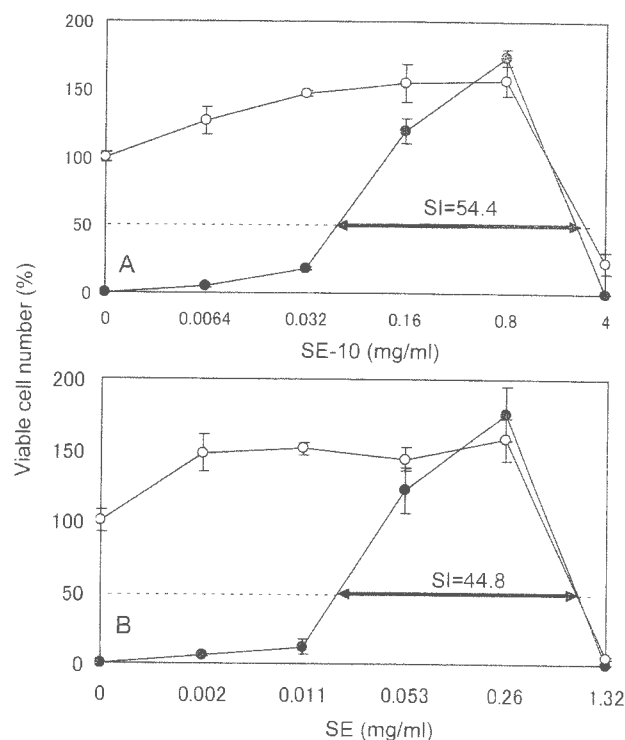


Figure 1. Anti-HIV activity of SE products. HIV-1_{IIIIB}-infected (HIV+) and mock-infected (HIV-) MT-4 cells were incubated for 5 days with the indicated concentrations of SE-10 (A) or SE (B), and the viable cell number was determined by the MTT assay and expressed as a percentage that of the control. Data represent the mean \pm standard deviation from triplicate assays. SI: selectivity index. It should be noted that the sample concentrations in the horizontal axis are plotted logarithmically by a factor of (1/5)ⁿ to achieve the evaluation of anti-HIV activity in wide ranges of concentrations.

Anti-HIV activity. When MT-4 cells were infected with HIV-1_{IIIIB} at a multiplicity of infection of 0.01, essentially all cells died (closed symbols in Figure 1). Both SE-10 and SE reduced the cytopathic effect caused by the HIV infection (Figure 1). Their SIs were at the same level, 54.4 and 44.8, respectively, and 10- to 100-fold lower than that of positive controls (dextran sulfate SI=160, curdlan sulfate SI=781, azidothymidine SI=6931, 2',3'-dideoxycytidine SI=905) (Table II).

Anti-UV activity. We recently reported that UV irradiation induced non-apoptotic cell death without induction of internucleosomal DNA fragmentation in HSC-2 cells (15). UV irradiation (6 J/m²/min, 1 minute) resulted in the significant loss of viable cells after 48 hours' incubation (closed symbols in Figure 2). Addition of SE-10 and of SE during the UV irradiation protected the cells from UV-induced cell injury when optimal concentrations of SE-10 (2-63 mg/ml) and SE (1-17 mg/ml) were selected; higher concentrations were cytotoxic (Figure 2). From the CC₅₀ and

Table II. Anti-HIV activity of SE-10 and SE. Data for SE-10 and SE were derived from the dose-response curve shown in Figure 1

	CC ₅₀	EC ₅₀	SI
SE-10 (mg/ml)	2.88	0.0529	54.4
SE (mg/ml)	0.825	0.0184	44.8
Positive controls			
Dextran sulfate (µg/ml)	125	0.78	160
Curdlan sulfate (µg/ml)	453	0.58	781
AZT (µM)	201	0.029	6931
ddC (µM)	1991	2.20	905

CC₅₀: 50% Cytotoxic concentration; EC₅₀: 50% effective concentration; SI: selectivity index=CC₅₀/EC₅₀; AZT: azidothymidine; 2',3'-dideoxycytidine.

Table III. Anti-UV activity of SE-10 and SE. Each value was determined from the dose-response curve. The data of experiment (Exp.) I for SE-10, SE and sodium ascorbate were derived from Figure 2.

	CC ₅₀	EC ₅₀	SI
SE-10 (mg/ml) (Exp. I)	159	1.28	124
SE-10 (mg/ml) (Exp. II)	135	0.86	157
SE-10 (mg/ml) (Exp. III)	137	1.30	105
Mean			129±26
SE (mg/ml) (Exp. I)	25.8	0.60	43.0
SE (mg/ml) (Exp. II)	24.0	0.65	36.9
SE (mg/ml) (Exp. III)	24.2	0.68	35.6
Mean			38.5±4.0
Positive controls:			
Sodium ascorbate (mM) (Exp. I)	>32	0.37	>86.5
Sodium ascorbate (mM) (Exp. II)	>32	0.38	>84.2
Sodium ascorbate (mM) (Exp. III)	>32	0.32	>99.1
Mean			>89.9±8.0

CC₅₀: 50% Cytotoxic concentration; EC₅₀: 50% effective concentration; SI: selectivity index=CC₅₀/EC₅₀.

EC₅₀ values, the SI values were calculated (Table III). Repeated experiments demonstrated that SE-10 exhibited 3-fold higher anti-UV activity (SI=129) than SE (SI=38.5), comparable with that of sodium ascorbate (positive control) (SI>89.9) (Table III).

Radical-scavenging activity. Both SE-10 and SE efficiently scavenged the superoxide anion (detected as DMPO-OOH), generated by HX and XOD reaction (Figure 3). From the dose-response curve, the IC₅₀ of SE-10 was calculated to be 0.225 mg/ml. Since SE-10 contains 44% SE in weight, the IC₅₀ value was corrected to 0.099 mg SE equivalent/ml, a value approximately 13% higher than that of SE (IC₅₀=0.0873 mg/ml).

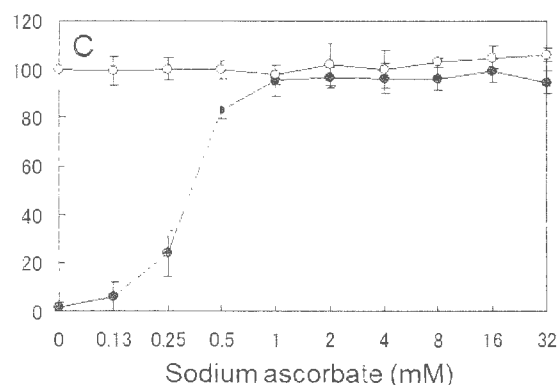
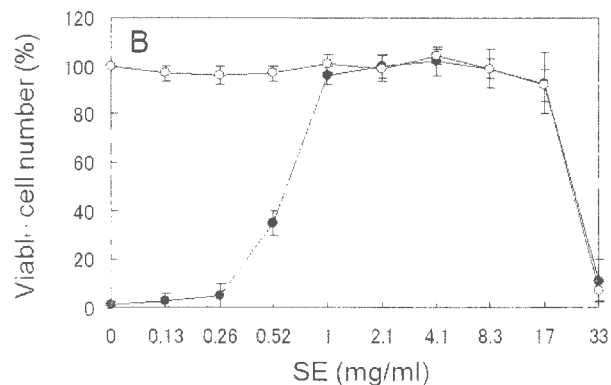
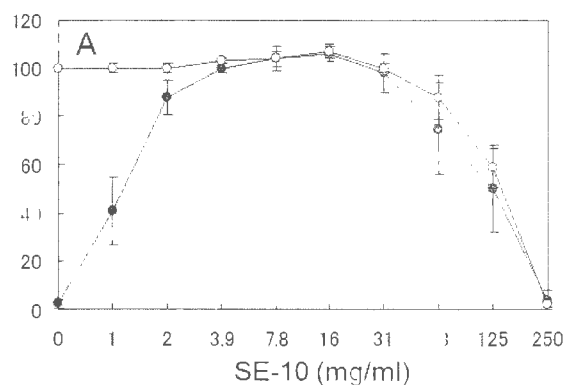


Figure 2. Anti-UV activity of SE products and sodium ascorbate. HSC-2 cells were exposed (UV+) or not (UV-) to UV irradiation (6 J/m²/min) for 1 min in PBS(-) containing the indicated concentrations of SE-10 (A), SE (B) or sodium ascorbate (C), and then cultured for 48 hours. Viable cell number was then determined by MTT assay and expressed as a percentage that of the control (non-irradiated and incubated without SE product). Each value represents the mean±S.D. of triplicate assays. CC₅₀, EC₅₀ and SI values are listed in Table III (Exp. I). Another two independent experiments performed in triplicates produced essentially similar results (Exps. II and III in Table III). It should be noted that the sample concentrations are plotted logarithmically by a factor of (1/2)ⁿ.

SE-10 and SE also efficiently scavenged the hydroxyl radical (detected as DMPO-OH), generated by the Fenton reaction (Figure 4). From the dose-response curve, the IC₅₀ value was calculated to be 0.736 mg/ml (0.324 mg SE

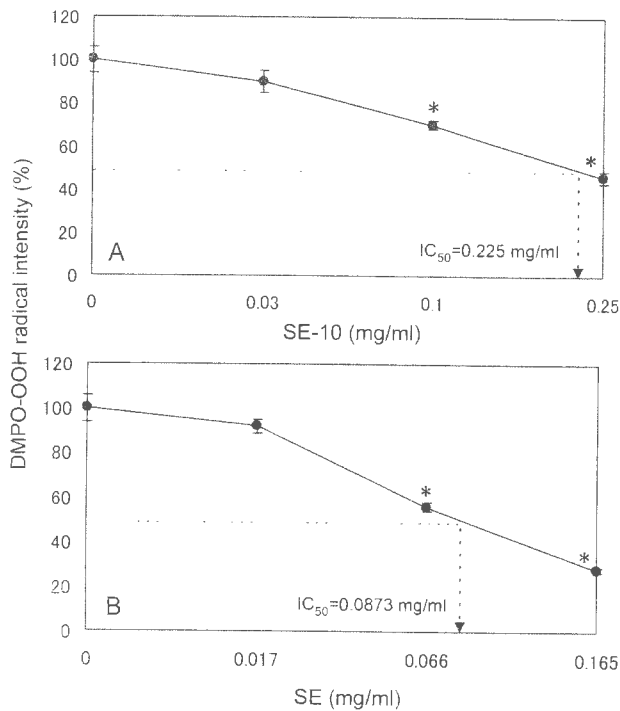


Figure 3. Superoxide anion-scavenging activity of SE-10 (A) and SE (B). The radical intensity of superoxide anion radical (measured as DMPO-OOH) produced by the hypoxanthine-xanthine oxidase reaction was measured by ESR spectroscopy. Each value represents the mean \pm S.D. of triplicate assays. * p <0.01 relative to the control (0%). It should be noted that the sample concentrations are plotted arbitrarily to achieve more gradual decrease in the activity.

equivalent/ml), a value approximately 38% higher than that of SE (IC₅₀=0.235 mg/ml).

CYP3A4-inhibitory activity. SE-10 and SE dose-dependently inhibited the β -hydroxylation of testosterone, generally used for the assay of CYP3A4 activity (Figure 5). SE-10 (IC₅₀=0.516 μ g SE equivalent/ml) had an approximately 16% lower CYP3A4-inhibitory activity (IC₅₀=0.445 μ g/ml).

Determination of optimum exposure time. When HGF, HPC, and HPLF cells were exposed to SE-10, the viability of the cells depended both on the exposure time (6-35 minutes) and concentration (0.4-100 mg/ml) (Figure 6A-D). Exposure to SE-10 at 50 mg/ml (22 mg SE equivalent/ml) for 35 minutes did not induce apparent cytotoxicity in any cells (Figure 6A-D). Exposure to 100 mg/ml of SE-10 (44 mg SE equivalent/ml) for 6 minutes also did not induce apparent cytotoxicity (Figure 6A-D). Similarly, exposure to SE (0.13-16.5 mg/ml) was not cytotoxic towards any cell line (Figure 6E-H).

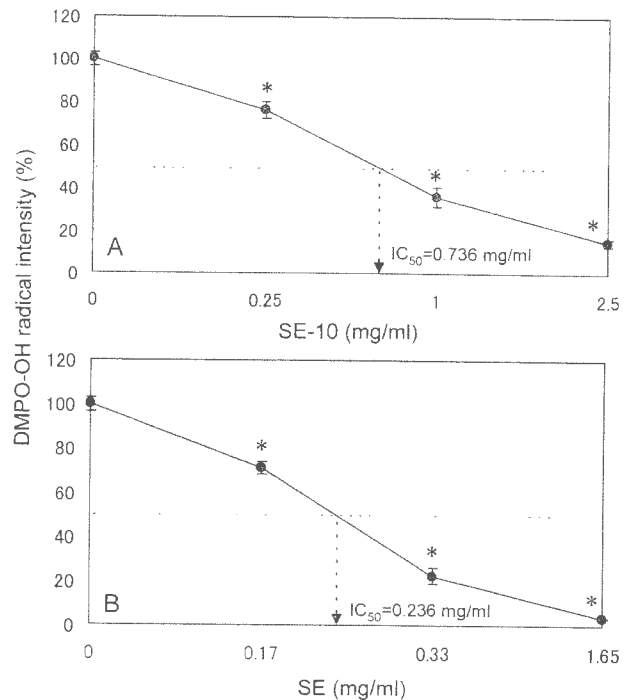


Figure 4. Hydroxyl radical-scavenging activity of SE-10 (A) and SE (B). The radical intensity of hydroxyl radical (measured as DMPO-OH) produced by the Fenton reaction was measured by ESR spectroscopy. Each value represents the mean \pm S.D. of triplicate assays. * p <0.01 relative to the control (0%). It should be noted that the sample concentrations are plotted arbitrarily to achieve more gradual decrease in the activity.

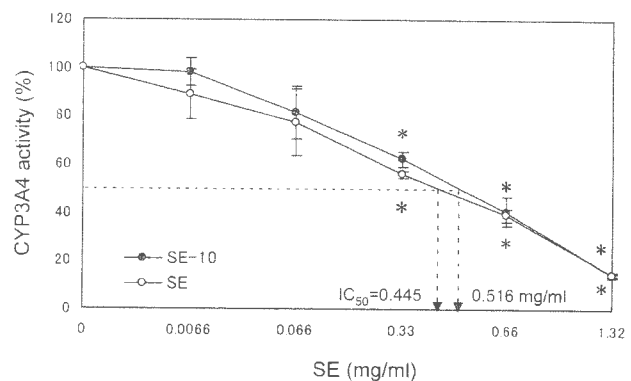


Figure 5. Cytochrome P450 enzyme (CYP)3A4 inhibitory activity of SE products. Each value represents the mean \pm S.D. of triplicate assays. * p <0.01 relative to the control (0%). It should be noted that the sample concentrations are plotted arbitrarily to achieve more gradual decrease in the activity.

Discussion

The present study demonstrated that both SE-10 (SI=54.4) and SE (SI=44.8) exhibited much higher anti-HIV activity, as compared with hundreds of tannin-related compounds

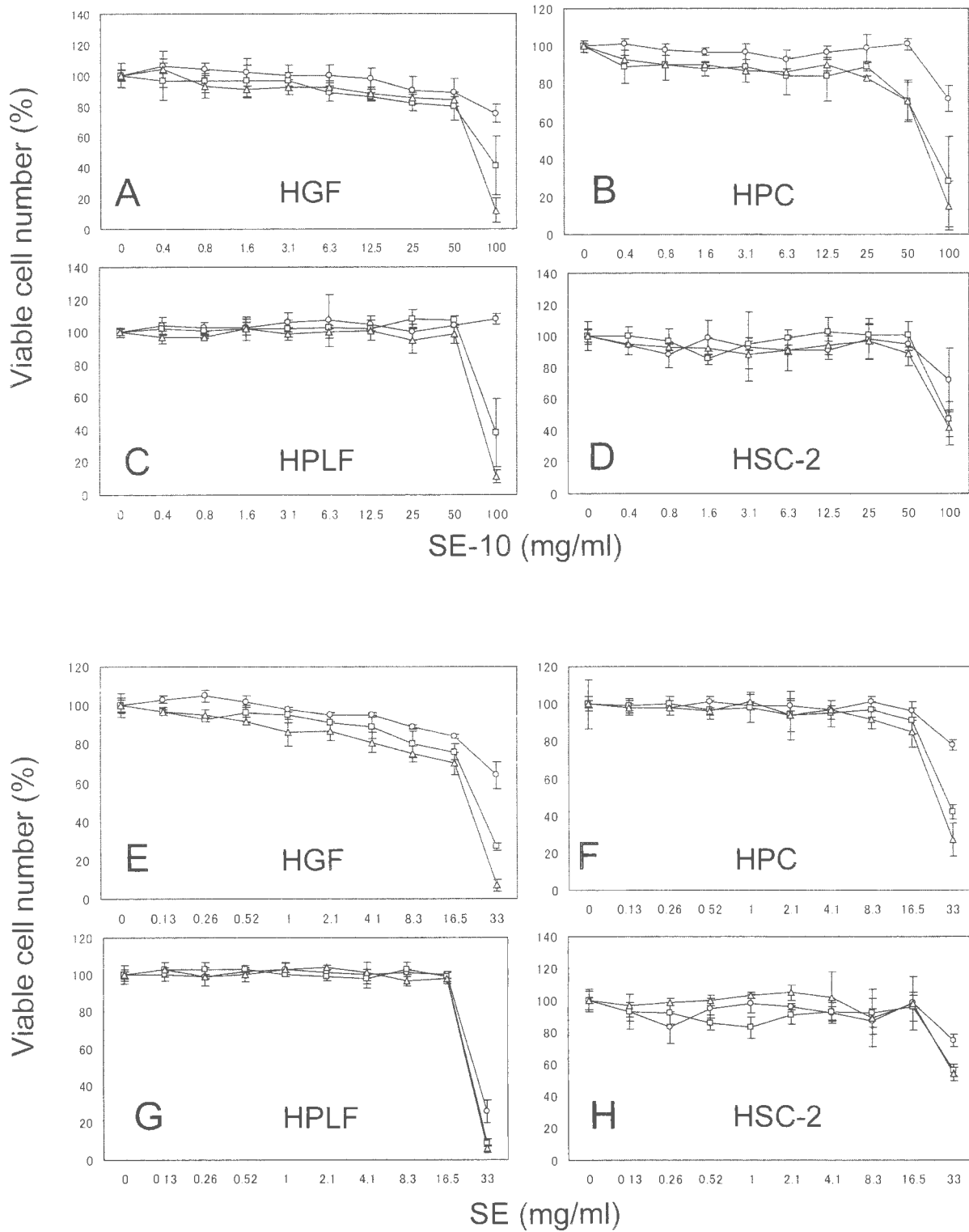


Figure 6. Determination of optimum exposure time to SE products. Near confluent human gingival fibroblast (HGF, A, E), human pulp cell (HPC, B, F), human periodontal ligament fibroblast (HPLF, C, G) and human oral squamous cell carcinoma (HSC-2, D, H) cells were incubated for 6 (○), 20 (□) or 35 (Δ) minutes in DMEM without (control) or with the indicated concentrations of SE-10 (A-D) or SE (E-H). The medium was then replaced with fresh medium that did not contain SE-10 nor SE, and were further incubated for 48 hours to determine the viable cell number by MTT method. Each value represents the mean±S.D. of triplicate assays. It should be noted that the sample concentrations are plotted logarithmically by a factor of (1/2)ⁿ.

(SI=1-14) (11), flavonoids (SI=1) (18), luteolin glycosides (SI=2-7) and even Tricin (SI=24) (19) that showed potent anti-human cytomegalovirus activity (20), possibly due to the presence of LCC. It was unexpected that the anti-HIV activity of SE-10 was slightly higher than that of SE (Figure 1). This may be due to the presence of oligomeric tannins, the anti-HIV activity of which has been reported to increase with polymerization (14).

We have previously reported that the anti-UV activity of SE was higher than that of (-)-epigallocatechin gallate (EGCG) (major green tea component) (SI=7.7), gallic acid (structural unit of tannin) (SI=17.1), chlorophyll a (SI<0.2), chlorophyllin (SI=0.5) (9) and tea extracts (green tea, black tea, jasminated, oolong tea, barley tea, Kohki tea) (SI<3.4) (21). Surprisingly, the present study demonstrated that the anti-UV activity of SE-10 was 3.4-fold higher than that of SE, and comparable with that of sodium ascorbate (Figure 2, Table III). The higher anti-UV activity of SE-10 may be due to the presence of tannins or trehalose having anti-stress activity (22-24). As yet, we have been unable to achieve the identification of tannin species in SE-10, possibly due to the association of polyphenolic substances with water-soluble higher molecular weight materials (8). The potent anti-UV activity of SE products may be derived from their radical-scavenging activity (Figures 3 and 4). These data demonstrate that beneficial biological activities of SE are well preserved during the manufacturing process of SE-10.

We also found that SE-10 had a slightly lower CYP3A4-inhibitory activity than SE (Figure 5). Combined with our recent report (12), CYP3A4 inhibitory activity increases in the following order (from lower to higher): SE-10<SE< products B and C. SE-10 and SE seem likely to be safer drugs as compared with products B and C, since the latter are expected to enhance the side-effects of CYP3A4-metabolizable drugs.

Considering the potent anti-HIV, antibacterial and anti-inflammatory activities of SE and SE-10, these products may be applicable for the treatment of virally-induced diseases, such as stomatitis and oral lichen planus. The effects of these agents on the viability and function of oral cells are under way.

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