in vivo *22*: 471-476 (2008)

Antiviral, Antibacterial and Vitamin C-synergized Radical-scavenging Activity of *Sasa senanensis* Rehder Extract

HIROSHI SAKAGAMI[1,2], SHIGERU AMANO[3], HIROTAKA KIKUCHI[4], YUKIO NAKAMURA[4], REINA KUROSHITA[5], SHIGERU WATANABE[5], KAZUE SATOH[6], HIDEO HASEGAWA[7], AKIKO NOMURA[7], TAISEI KANAMOTO[8], SHIGEMI TERAKUBO[8], HIDEKI NAKASHIMA[8], SUMIKO TANIGUCHI[9] and TAKAAKI OIZUMI[9]

[1]*Meikai Pharmaco-Medical Laboratory (MPL),* [2]*Divisions of Pharmacology,* [3]*Microbiology,* [4]*Endodontics and* [5]*Pediatric Dentistry, Meikai University School of Dentistry, Sakado, Saitama;* [6]*Department of Anatomy, School of Medicine, Showa University, Tokyo;* [7]*The Japanese Association of Clinical Research on Supplements (JACRS), Saitama;* [8]*Department of Microbiology, St. Marianna University School of Medicine, Kanagawa;* [9]*Daiwa Biological Research Institute Co., Ltd., Kawasaki, Kanagawa, Japan*

Abstract. *Sasa senanensis Rehder extract (SE) showed slightly higher cytotoxicity against human squamous cell carcinoma cell lines and human glioblastoma cell lines, as compared with human oral normal cells (gingival fibroblast, pulp cell, periodontal ligament fibroblast), and was more cytotoxic to human myelogenous and T-cell leukemia cell lines. SE showed a bacteriostatic effect on Fusobacterium nucleatum and Prevotella intermedia, but almost completely eliminated hydrogen sulfide (H_2S) and methyl mercaptan (CH_3SH) produced by these bacteria. SE protected human T-cell leukemia MT-4 cells from the cytopathic effect of human immunodeficiency virus (HIV) infection, and its anti-HIV activity was much higher than that of tannins and flavonoids, comparable with that of natural and synthetic lignins. SE also protected the MDCK cells from the cytopathic effect of influenza virus infection. SE synergistically enhanced the superoxide anion and hydroxyl radical-scavenging activity of vitamin C. The present study suggests the functionality of SE as a complementary alternative medicine.*

Sasa senanensis Rehder extract (SE) has been reported to show anti-septic (1), membrane stabilizing (2), anti-inflammatory (3) and radical scavenging activities (4). Chemical analysis of SE suggests the presence of lignin as a major ingredient. We have also observed that SE contains a huge quantity of acid-precipitable substances (Furou *et al*., unpublished data). Lignin carbohydrate has displayed several unique biological activities such as anti-human immunodeficiency virus (HIV) activity and synergistic actions with vitamin C (5). In order to explore novel functions of SE, its possible anti-HIV, anti-influenza virus, antibacterial and radical scavenging activities were investigated.

Correspondence to: Hiroshi Sakagami, Division of Pharmacology, Department of Diagnostic and Therapeutic Sciences, Meikai University School of Dentistry, Sakado, Saitama 350-0283, Japan Tel: +81 49 279 2758, Fax: +81 49 285 5171, e-mail: sakagami@dent.meikai.ac.jp

Key Words: SE, lignin, radical-scavenging activity, anti-HIV activity, antibacterial activity, vitamin C.

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) (JRH Bioscience, Lenexa, KS, USA); RPMI1640, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), hypoxanthine (HX), xanthine oxidase (XOD) (Sigma Chemical Ind., St. Louis, MO, USA); diethylenetriaminepentaacetic acid (DETAPAC), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) (Dojin, Kumamoto, Japan). SE was prepared and supplied by Daiwa Biological Research Institute Co., Ltd., Kawasaki, Kanagawa, Japan. Lyophilization and measurement of the dry weight of SE showed that it contained 58.2±0.96 mg solid materials/mL.

Cell culture. Human oral normal cells (gingival fibroblast HGF, pulp cell HPC, periodontal ligament fibroblast HPLF) were prepared from periodontal tissues, according to the guideline of the Intramural Board of Ethics Committee (No. 0206) after obtaining the informed consent from the patients. Since these normal cells have a limited lifespan due to their *in vitro* senescence, the cells at the 8-12 population doubling levels were used for the present study. These normal cells, human oral squamous cell carcinoma cell lines (HSC-2, HSC-3, HSC-4, Ca9-22, NA) (kindly supplied by Prof. Nagumo, Showa University) and human glioblastoma cell lines (T98G, U87MG) (kindly supplied by Dr. Iida, Showa University) were cultured in DMEM medium

supplemented with 10% heat-inactivated FBS in a humidified 5% CO_2 atmosphere. Human myelogenous leukemic cell lines HL-60, ML-1 and KG-1 (supplied by Prof. Takeda, Science University of Tokyo) and human T-cell leukemia virus I (HTLV-I)-bearing CD4 positive human T-cell line, MT-4 (supplied by Prof. Yamamoto, Tokyo Medical Dental University) were cultured in suspension in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, as described previously (6). Madin-Darby canine kidney (MDCK) cells (Dainippon Sumitomo Pharma Company Ltd, Osaka, Japan) were cultured in Eagle's minimum essential medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 10% FBS (Hyclone Ltd., UT, USA).

Assay for cytotoxic activity. Near-confluent cells were treated for 48 h with various concentrations of test samples, and the relative viable cell number of adherent cells was then determined by the MTT method, as described previously (6). The viable cell number of HL-60 cells was determined by hemocytometer after staining with trypan blue. The 50% cytotoxic concentration (CC_{50}) was determined from the dose-response curve. The tumor-specificity index (TS) was measured by the following equation: TS= [CC_{50} (HGF) + CC_{50} (HPC) + CC_{50} (HPLF)] / [CC_{50} (HSC-2) + CC_{50} (HSC-3) + CC_{50} (HSC-4) + CC_{50} (Ca9-22) + CC_{50} (NA) + CC_{50} (T98G) + CC_{50} (U87MG) + CC_{50} (HL-60) + CC_{50} (ML-1) + CC50 (KG-1) + CC_{50} (MT-4)] x (11/3) (Table I).

Antimicrobial activity and volatile sulfur compounds (VSC) determination. Fusobacterium nucleatum ATCC 31647 (Summit Pharmaceuticals International Corporation, Tokyo, Japan) and *Prevotella intermedia* ATCC 25611 (Riken Bioresource Center, Saitama, Japan) were grown anaerobically (10% CO2, 10% H_2, 80% N_2) in Gifu Anaerobic Medium (GAM) broth (Nissui Medical Co., Tokyo, Japan) supplemented with hemin (5 μg/mL) and menadione (1 μg/mL). The 50% minimal inhibitory concentrations (MIC_{50}) of SE were determined by the broth dilution method in a modification of the National Committee for Clinical Laboratory Standards (NCCLS) (7). The formation of hydrogen sulfide (H_2S), methyl mercaptan (CH_3SH) and dimethyl sulfide ($(CH_3)_2S$) were determined by gas chromato-graphy. Before each test, a working solution of SE (5%=2.9 mg/mL, in supplemented GAM) was prepared from the stock solution (100%=58.2 mg/mL). A serial two-fold dilution of the working solution with GAM broth was carried out in a 15 mL capped centrifugation tube (Sumitomo, Japan) (total volume of 3 mL), and then an equal amount of bacterial suspension (2×10^6 CFU/mL) was added (total volume=6 mL). The tubes were incubated in an anaerobic box with an atmosphere generation system [Anaerobic Box (ANX-1), Hirasawa Works Inc., Tokyo, Japan)] at 37˚C for 24 h. Firstly, the gas (5 mL) in the tube was collected by syringe, and the H_2S, CH_3SH and $(CH_3)_2S$ were immediately quantified by gas chromatography (Shimazu EC14B, Kyoto, Japan), with carrier gas (N_2, 150 kPa), detector (hydrogen 75 kPa, air 75 kPa, 120˚C), and column oven (60˚C). Secondly, the turbidity (absorbance at 620 nm) of the bacterial suspension was measured using a microplate reader (Multiskan Biochromatic, Labsystem, Osaka, Japan). Non-inoculated supplemented GAM broth with added inhibitor, incubated under the same conditions, was used as a blank.

Assay for HIV activity. MT-4 cells were infected with HIV-1_{IIIB} at a multiplicity of infection (m.o.i.) of 0.01. HIV- or mock-infected (control) MT-4 cells were incubated for 5 days with various concentrations of test samples, and the relative viable cell number was determined by MTT assay. The 50% cytotoxic concentration (CC_{50}) and 50% effective concentration (EC_{50}) were determined from the dose-response curve with mock-infected or HIV-infected cells, respectively (8). All the data represent the mean values of triplicate measurements. The anti-HIV activity was evaluated by selectivity index (SI), which was calculated by the following equation: SI=CC_{50}/EC_{50}

Assay for anti-influenza A (H1N1) virus activity. MDCK cells were infected with influenza A (H1N1) virus at 100 $TCID_{50}$ (50% tissue culture infectious dose). The virus- or mock-infected (control) MDCK cells were incubated for 5 days with a serial two-fold dilution of test sample starting at 0.63%. During the incubation, at 3 days after infection, the maintenance medium in each well was exchanged for fresh medium which contained the same concentration of test sample as before the exchange. The relative viable cell number was determined by MTT assay. The CC_{50} and EC_{50} were determined from the dose-response curve with mock-infected or virus-infected cells, respectively. The antiviral activity was evaluated using an SI, which was calculated as above.

Table I. *Cytotoxic activity of SE against human normal and tumor cells.*

		CC_{50}	
		% (v/v)	mg/mL
Human normal cells			
Gingival fibroblast (HGF)		6.96	4.05
Pulp cell (HPC)		7.54	4.38
Periodontal ligament fibroblast (HPLF)		6.19	3.60
	(mean)	6.90	4.01
Human oral squamous cell carcinoma cell lines			
HSC-2		8.49	4.94
HSC-3		5.99	3.49
HSC-4		5.69	3.31
Ca9-22		4.20	2.44
NA		6.71	3.91
	(mean)	6.22	3.62
Human glioblastoma cell lines			
T98G		6.92	4.03
U87MG		3.94	2.29
	(mean)	5.43	3.16
Human myelogenous leukemia cell lines			
HL-60		1.14	0.66
ML-1		0.39	0.23
KG-1		2.00	1.16
	(mean)	1.18	0.68
Human T-cell leukemia cell line			
MT-4		1.41	0.82
TS value		1.62	

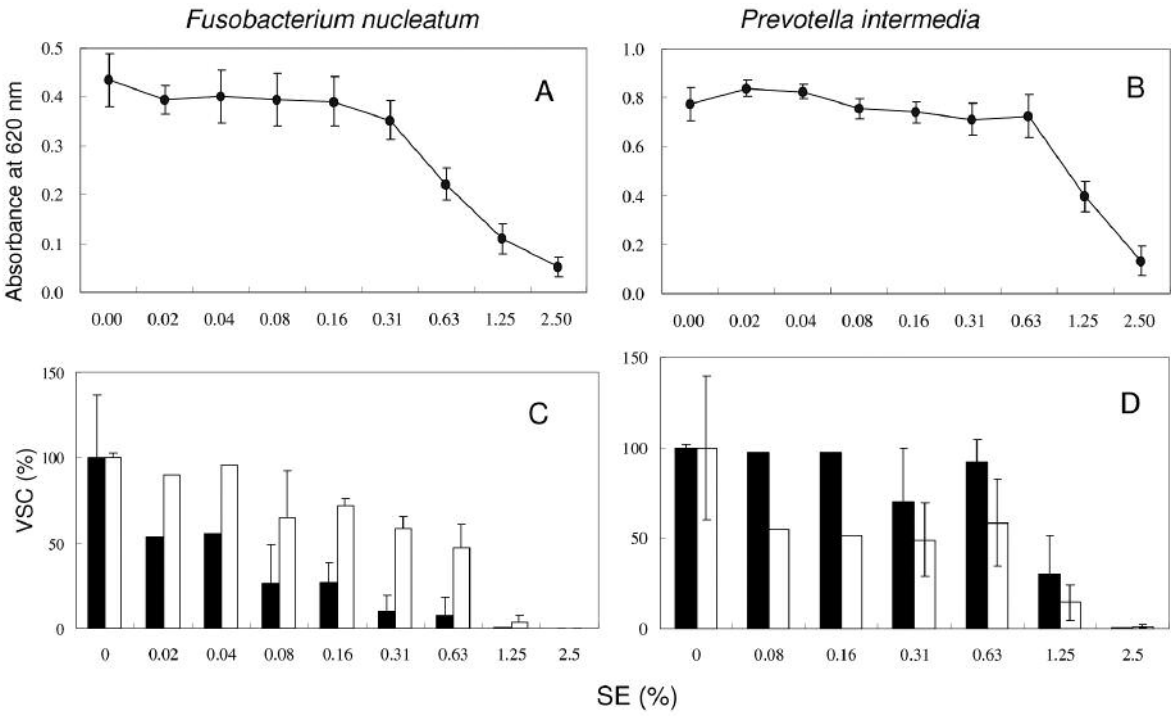

Figure 1. *Antibacterial activity of SE. Fusobacterium nucleatum (A, C) and Prevotella intermedia (B, D) were cultured anaerobically for 24 hours at 37˚C with the indicated concentrations of SE in capped 15-cm centrifugation tubes. The VSC released into the culture medium (black bar, H_2S; white bar, CH_3SH) was quantified by gas chromatography (C, D). Bacterial growth was measured by recording the absorbance at 620 nm, using a microplate reader (A, B). (A, B) Bacteriostatic activity of SE. Each point represents mean±S.D. from triplicate assays. (C, D) Effect on VSC. Data with bars represent mean±S.D. from triplicate assays, while those without bars are the value from a single assay.*

Radical scavenging activity. The radical intensity was determined at 25˚C in 0.1 M Tris-HCl (pH 7.4), 0.1 M Na_2CO_3/$NaHCO_3$ (pH 10.0) or 0.1 M KOH (pH 13.0), using electron-spin resonance (ESR) spectroscopy (JEOL JES REIX, X-band, 100 kHz modulation frequency) (9). The instrument settings were: center field, 335.5±5.0 mT; microwave power, 16 mW; modulation amplitude, 0.1 mT: gain, 630, time constant, 0.03 sec; scanning time, 2 min.

For determination of the superoxide anion (in the form of DMPO-OOH), produced by the HX-XOD reaction (total volume: 200 μL) (2 mM HX in 0.1 M phosphate buffer [pH 7.4] [PB] 50 μL, 0.5 mM DETAPAC 20 μL, 8% DMPO 30 μL, sample 40 μL, H_2O 30 μL, XOD [0.5 U/mL in PB] 30 μL), the same instrument settings described above were used (9).

For the determination of the hydroxyl radical (in the form of DMPO-OH), produced by the Fenton reaction (200 μL) (1 mM $FeSO_4$ [containing 0.2 mM DETAPAC] 50 μL, 0.1 M phosphate buffer [pH 7.4] 50 μL, 92 mM DMPO 20 μL, sample 50 μL, 1 mM H_2O_2, 30 μL), the gain was changed to 400 (9).

Results

Cytotoxic activity. SE showed slightly higher cytotoxicity against the human squamous cell carcinoma cell lines (HSC-2, HSC-3, HSC-4, Ca9-22, NA) (mean CC_{50}=6.22%, 3.62 mg/mL) and the human glioblastoma cell lines (T98G, U-87MG) (mean CC_{50}=5.43%, 3.16 mg/mL), as compared with the human oral normal cells (HGF, HPC, HPLF) (mean CC_{50}=6.90%, 4.01 mg/mL), and was more cytotoxic to the human myelogenous leukemic cell lines (HL-60, ML-1, KG-1) (CC_{50}=1.18%, 0.68 mg/mL) and the human T-cell leukemia cell line (MT-4) (CC_{50}=1.41%, 0.82 mg/mL), with an approximate tumor specificity index of 1.62 (Table I).

Antibacterial activity. SE showed a bacteriostatic, but not a bactericidal effect on *Fusobacterium nucleatum* and *Prevotella intermedia* (Figure 1A, 1B). The MIC_{50} for the *F. nucleatum* and *P. intermedia* was calculated to be 0.63 and 1.25%, respectively, and at the highest concentration (2.5%), 12.0 and 17.2% of the bacteria remained viable, respectively.

Gas chromatography demonstrated that these bacteria produced H_2S and CH_3SH, but not $(CH_3)_2S$. SE more efficiently reduced the production of H_2S in *F. nucleatum*, with a 50% inhibitory concentration (IC_{50}) of 0.04% (Figure 1C). On the other hand, SE more efficiently reduced the production of CH_3SH in *P. intermedia*, with an IC_{50} of 0.16% (Figure 1D). A higher concentration of SE (2.5%) completely eliminated both H_2S and CH_3SH.

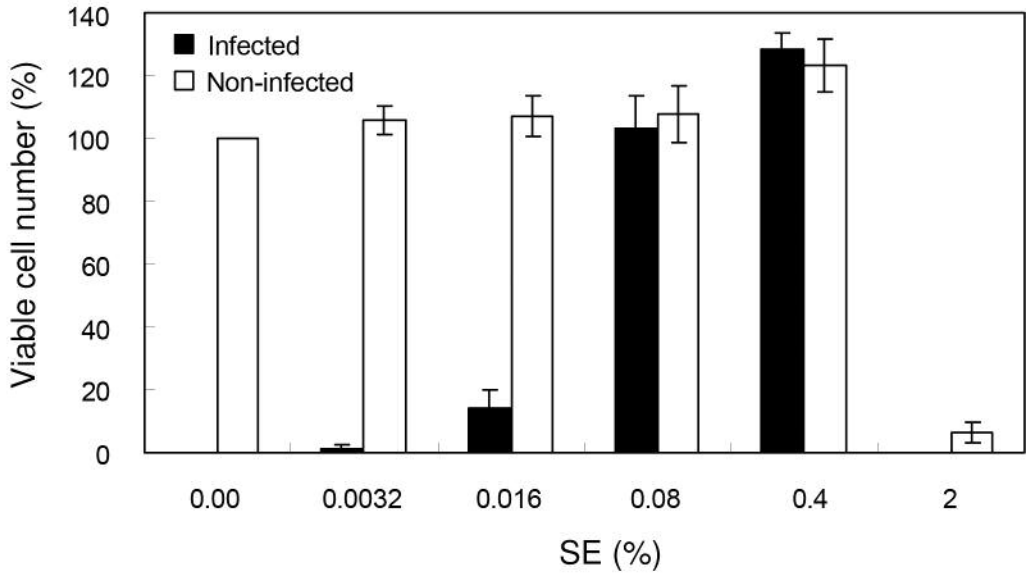

Figure 2. *Anti-HIV activity of SE. HIV-infected or mock-infected MT-4 cells were incubated for 5 days with the indicated concentrations of SE, and the viable cell number was determined by MTT method. Each value represents mean±S.D. from three independent experiments.*

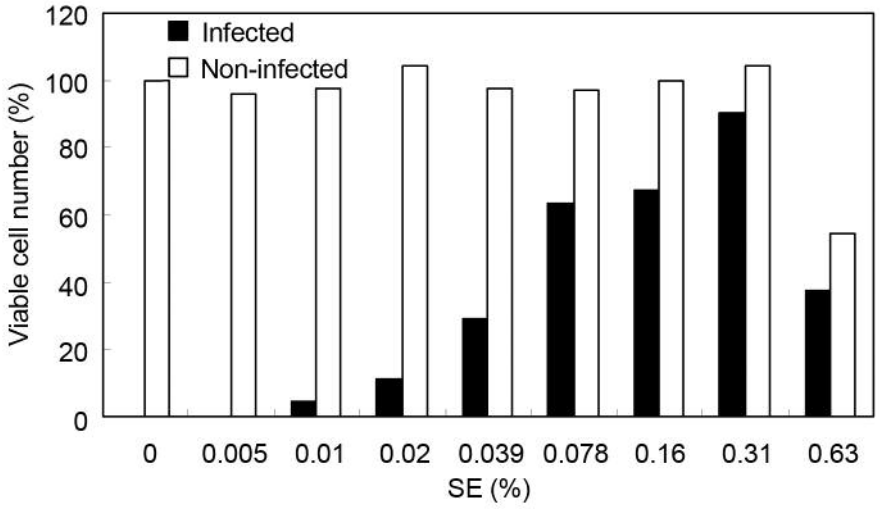

Figure 3. *Anti-influenza virus activity of SE. Influenza virus-infected or mock-infected MDCK cells were incubated for 3 days with the indicated concentrations of SE, and the viable cell number was determined by MTT method. Each value represents the mean from triplicate assays.*

Antiviral activity. SE protected the MT-4 cells from the cytopathic effect of HIV infection (CC_{50}=1.10±0.02%, EC_{50}=0.033±0.006%, SI=36±6) (Figure 2). SE also protected the MDCK cells from the cytopathic effect of influenza virus infection (CC_{50}=0.67%, EC_{50}=0.060%, SI=11) (Figure 3).

Synergistic radical-scavenging activity with vitamin C. ESR spectroscopy showed that SE (50%=29.1 mg/mL) did not produce any detectable ESR signal at pH 7.4 (radical intensity (RI)<0.089) and pH 10.0 (RI<0.11). At pH 13.0, a weak broad peak, similar to that of typical lignin (10), appeared (RI=0.14) (Figure 4).

When the superoxide (generated by the HX-XOD reaction) was mixed with DMPO, four radical peaks derived from the spin adduct (DMPO-OOH) were detected by ESR spectroscopy (data not shown). SE synergistically enhanced the superoxide anion-scavenging activity of vitamin C (Table II).

When the hydroxyl radical (generated by the Fenton reaction) was mixed with DMPO, four radical peaks derived

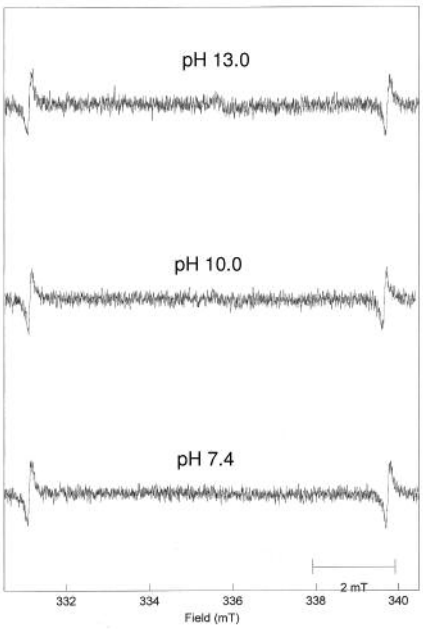

Figure 4. *Radical production by SE. SE (100%) was mixed with equal volume of 0.2 M buffer solution at the indicated pH, and the radical intensity was then measured by ESR.*

from the spin adduct (DMPO-OH) appeared (data not shown). SE synergistically enhanced the hydroxyl radical-scavenging activity of vitamin C (Table III).

Discussion

The present study demonstrated some novel biological activities of SE. Although SE did not show high tumor-specific cytotoxicity, it was highly cytotoxic to three human myelogenous leukemic cell lines (HL-60, ML-1, KG-1) and one T-cell leukemic cell line (MT-4). The type of cell death induced by SE remains to be investigated.

SE showed a bacteriostatic, but not a bactericidal effect on *F. nucleatum* and *P. intermedia*, which are involved in periodontal diseases. Although the inhibition of bacterial growth was incomplete, SE almost completely eliminated H_2S and CH_3SH from the bacterial cultures. This suggests that SE may metabolize or capture these volatile compounds, in addition to its antibacterial activity. SE seems thus to be applicable in dentistry as a mouth wash.

SE showed potent anti-HIV activity (SI=36). It should be noted that the anti-HIV activity of SE is much higher than that of tannins (8) and flavonoids (11, 12), and comparable with that of natural lignin (13) and dehydrogenation polymers of phenylpropenoids (so-called "synthetic lignin") (14). We have recently found that mulberry juice containing insoluble fiber showed weak, but

Table II. *Synergistic superoxide scavenging-activity of SE and vitamin C.*

	DMPO-OOH radical intensity (% of control)	
	0.5%	0.25% + 5 μM vitamin C
SE	48.3	47.1<60.4 [(48.3+72.4)/2]
10 μM Vitamin C	72.4	

Table III. *Synergistic hydroxyl radical-scavenging activity of SE and vitamin C.*

	DMPO-OH radical intensity (% of control)	
	1%	0.5% + 5 μM vitamin C
SE	65.4	75.5<87.3 [(65.4+109.1)/2]
10 μM Vitamin C	109.1	

significant anti-HIV activity (SI=3.7), whereas blueberry juice without fiber had less anti-HIV activity (SI<1.0) (8). The anti-HIV activity of the mulberry juice was concentrated in the lignin fractions IV and VI (SI=7.2 and 7.0, respectively), prepared by acid-precipitation (pH 5.0) and ethanol (83%) precipitation of the insoluble fiber, respectively (15). This suggests that the insoluble fibers present in other fruits and vegetables may be an excellent source of anti-HIV substances.

SE was found to enhance the superoxide and hydroxyl radical scavenging activity of vitamin C. This suggests its applicability as an enhancer of the action of antioxidants.

In conclusion, SE has some lignin-like activities, such as anti-HIV activity, anti-influenza virus activity, synergistic action with vitamin C and acid-precipitability. The biological actions of purified lignin from SE and comparison with those of SE remains to be investigated.

Acknowledgements

This study was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan (Sakagami, No.19592156).

References

1 Chuyen NV, Kurata T and Kato H: Anti-septic activity of *Sasa senanensis Rehder* extract. J Antibac Antifung Agents *11*: 69-75, 1983 (in Japanese).

2 Ohizumi T, Kodama K, Tsuji M and Okuchi K: The effect of *Sasa senanensis Rehder* extract and crude herb medicine extract on the membrane. Showa Med J *49*: 315-321, 1989.

3 Ohizumi T, Shirasaki K, Tabata T, Nakayama S, Okazaki M and Sakamoto K: Pharmacological studies of *Sasa senanensis Rehder* extract on anti-inflammatory effect and phagocytic activity. Showa Med J *48*: 595-600, 1988 (in Japanese).

4 Komatsu M and Hiramatsu M: Free radical scavenging activity of Sasa Senanensis Rehder extract. KISO TO RINSHO *31*: 3321-3324, 1997 (in Japanese).

5 Sakagami H, Hashimoto K, Suzuki S, Ogiwara T, Satoh K, Ito H, Hatano T, Yoshida T and Fujisawa S: Molecular requirement of lignin for expression of unique biological activity. Phytochemistry *66*: 2107-2119, 2005.

6 Sekine T, Takahashi J, Nishishiro M, Arai A, Wakabayashi H, Kurihara T, Kobayashi M, Hashimoto K, Kikuchi H, Katayama T, Kanda Y, Kunii S, Motohashi N and Sakagami H: Tumor-specificity and type of cell death induced by trihaloacetylazulenes in human tumor cell lines. Anticancer Res *27*: 133-144, 2007.

7 NCCLS. Methods for dilution antimicrobial susceptibility test for bacteria that grow aerobically; approved standard M7-A6, 6th ed. National Committee for Clinical Laboratory Standards, Wayne, Pa., 2003

8 Nakashima H, Murakami T, Yamamoto N, Sakagami H, Tanuma S, Hatano T, Yoshida T and Okuda T: Inhibition of human immunodeficiency viral replication by tannins and related compounds. Antiviral Res *18*: 91-103, 1992.

9 Sakagami H, Asano K, Satoh K, Takahashi K, Terakubo S, Shoji Y, Nakashima H and Nakamura W: Anti-stress activity of mulberry juice in mice. In Vivo *20*: 499-504, 2006.

10 Satoh K, Sakagami H and Nakamura K: Enhancement of radical intensity and cytotoxic activity of ascorbate by PSK and lignins. Anticancer Res *16*: 2981-2986, 1996.

11 Fukai T, Sakagami H, Toguchi M, Takayama F, Iwakura I, Atsumi T, Ueha T, Nakashima H and Nomura T: Cytotoxic activity of low molecular weight polyphenols against human oral tumor cell lines. Anticancer Res *20*: 2525-2536, 2000.

12 Shirataki Y, Motohashi N, Tani S, Sakagami H, Satoh K, Nakashima H, Mahapatra SK, Ganguly K, Dastidar SG and Chakrabarty AN: *In vitro* biological activity of prenylflavanone. Anticancer Res *21*: 275-280, 2001.

13 Sakagami H, Satoh K, Ida Y, Koyama N, Premanathan M, Arakaki R, Nakashima H, Hatano T, Okuda T and Yoshida T: Induction of apoptosis and anti-HIV activity by tannin- and lignin-related substances. *In*: Plant Polyphenols 2. Chemistry, Biology, Pharmacology, Ecology. GG Gross, RW Hemingway and T Yoshida (eds.). Prenum Press, pp. 595-611, 1999.

14 Nakashima H, Murakami T, Yamamoto N, Naoe T, Kawazoe Y, Konno K and Sakagami H: Lignified materials as medicinal resources. V. Anti-HIV (human immunodeficiency virus) activity of some synthetic lignins. Chem Pharm Bull *40*: 2102-2105, 1992.

15 Satoh K, Ida Y, Ishihara M and Sakagami H: Interaction between sodium ascorbate and polyphenols. Anticancer Res *19*: 4177-4186, 1999.

Received January 2, 2008
Revised March 11, 2008
Accepted March 27, 2008