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Structural Characterization of Anti-UV Components from *Sasa senanensis* Rehder Extract

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Abstract. *Background: Previous studies have shown antiviral, antibacterial and anti-inflammatory activity of alkaline extract of the leaves of Sasa senanensis Rehder (SE). However, active components have not been identified. We isolated the substances that exhibit anti-UV activity and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity from SE and estimated their putative structures. Materials and Methods: The anti-UV substances (SEE-1 and SEE-2) were isolated from SE by ethanolic extraction, Wakosil chromatography and recycled high-performance liquid chromatography (HPLC) at a yield of 0.22 and 0.18%, respectively. The structural analysis was carried out with ^{1}H-nuclear magnetic resonance (NMR), ^{13}C-NMR and UV absorption. Results: SEE-1 exhibited approximately four-fold higher anti-UV activity and slightly lower DPPH radical-scavenging activity, compared to SE. SEE-1 was identified as p-coumaric acid derivative(s), a lignin precursor. Conclusion: The present study demonstrated for the first time the presence of lignin precursors in SE, which may explain why SE exhibits many of the properties of lignin–carbohydrate complexes.*

The plants in the Sasa genus are distributed only in East Asia and have been used as food and folk medicines. The dried leaves of Sasa plants have been used as herbal tea, functional food and medicines. Alkaline extract of the leaves of *Sasa senanensis* Rehder (SE) (SASA-Health®), which belongs to Group III of over-the-counter drugs (1), is expected to be less hazardous, as compared with Kampo medicines. SE is recognized as being effective in treating fatigue, low appetite, halitosis, body odor and stomatitis, but there is no scientific evidence to demonstrate these phenomena, due to the lack of appropriate biomarkers. SE has shown *in vitro* antiseptic (2), membrane-stabilizing (3), anti-inflammatory (4-6), antibacterial (7, 8), antiviral (7-10), anti-UV (9-12) and radical-scavenging (5, 8-10, 13) activities, and synergistic action with vitamin C (7). SE exhibited several biological properties common to those of lignin–carbohydrate complex (LCC): prominent anti-HIV, anti-UV and synergistic activity with vitamin C (14), as expected from the use of alkaline solution for the extraction of both SE and LCC. On gel filtration fractionation, we successfully concentrated the majority of the anti-UV activity into Fr. I, which was eluted as a single peak, using high-performance liquid chromatography (HPLC) (11). In the present study, we further purified this fraction and identified its putative structure that confers the anti-UV and radical-scavenging activity.

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Key Words: Sasa senanensis Rehder extract, UV protection, DPPH radical, structural analysis.

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) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Sigma Chemical Co. St. Louis, MO, USA; dimethyl sulfoxide (DMSO), Wako Pure Chemical Ind., Ltd., Osaka, Japan; sodium ascorbate, Trolox, *trans-p*-coumaric acid, Tokyo Chemical Industry Co., Ltd., Tokyo, Japan; 1,1-diphenyl-2-picrylhydrazyl (DPPH), Tokyo Chemicals Industry Co., Ltd., Tokyo, Japan.

SE was prepared and supplied by Daiwa Biological Research Institute Co. Ltd., Kawasaki, Kanagawa, Japan. SE (21 ml) was freeze-dried to give a powder (1.1 g).

Isolation of SEE-1 and SEE-2. The purification scheme for anti-UV substances is shown in Figure 1A. SE (2.26 g) was extracted with ethanol. The ethanolic extract (0.15 g) was applied to a Wakosil 40C18 (2.5 i.d.×27 cm) (Wako Pure Chemical Ind., Ltd.) and the fractions that contain SEE-1 and SEE-2 were eluted with H_2O and 20% methanol, respectively. The H_2O extract (0.019 g) was applied to preparative high-performance liquid chromatography (HPLC). The HPLC system used

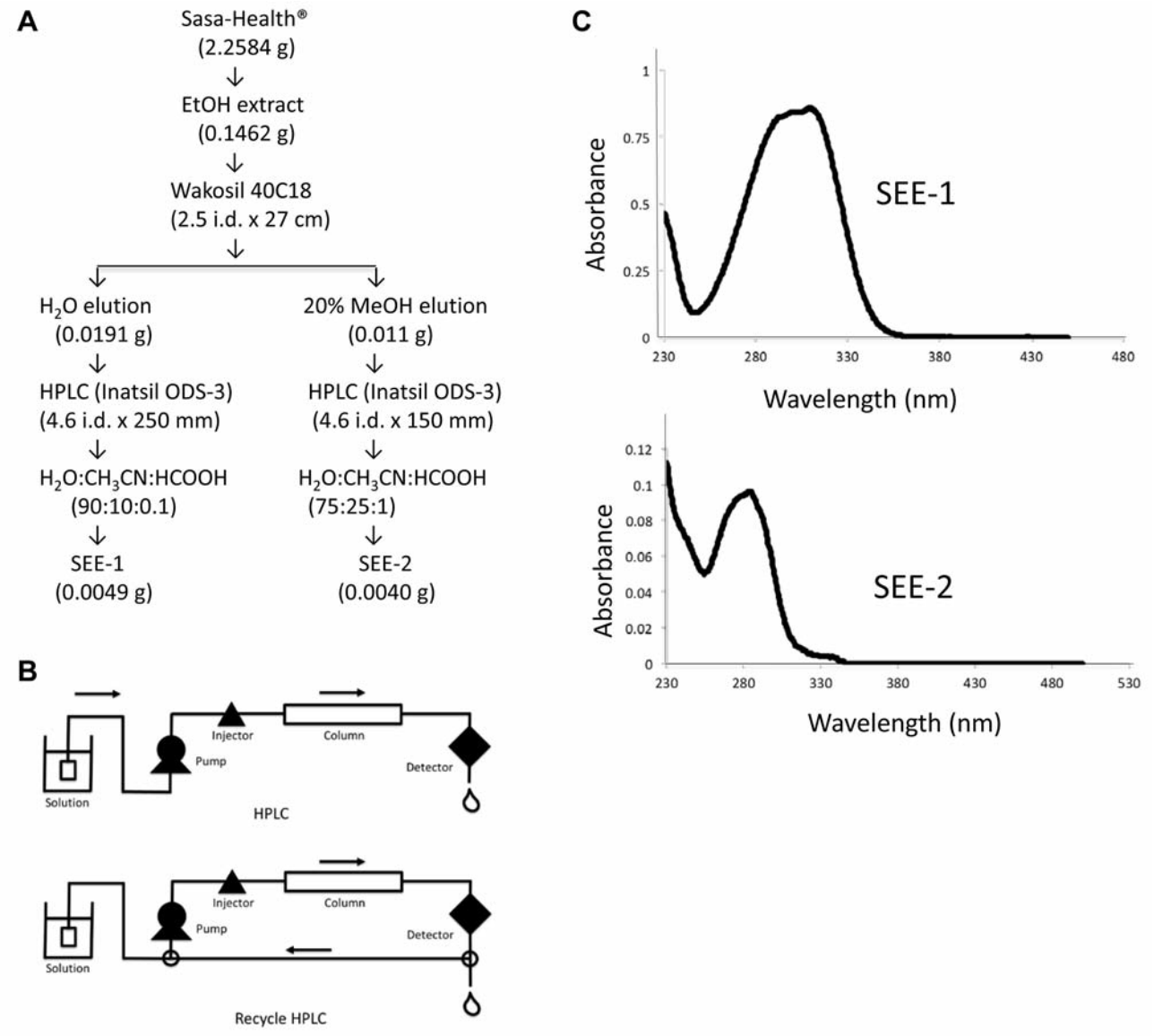


Figure 1. *Isolation of SEE-1 from Sasa-Health®. A: Procedure for purification of SEE-1 and SEE-2, using recycle HPLC (B). C: UV absorption spectra of SEE-1 and SEE-2.*

was composed of a JASCO PU-980 pump and a JASCO UV-970 UV/VIS detector (JASCO Corporation, Tokyo, Japan). The separation column was Inertsil ODS-3 (4.6 i.d.×250 mm; Tosoh Co., Tokyo, Japan). SEE-1 (0.0049 g) was eluted with H_2O:acetonitrile:formic acid (90:10:0.1). The 20% methanolic extract (0.013 g) was applied to HPLC (Inertsil ODS-3) (4.6 i.d.×150 mm) and SEE-2 (0.0040 g) was eluted with H_2O:acetonitrile:formic acid (75:25:1).

HPLC analysis. The HPLC system was composed of 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 280 nm and the sample was injected manually. Solvent A (5% acetonitrile containing 0.1% acetic acid) and solvent B (acetonitrile) were used as the mobile phase, with a flow rate of 1.0 ml/min and gradient of B 0% (0-10 min), 10% (10-15 min), 25% (15-30 min), 40% (30-40 min), 100% (40-50 min) and holding at 100% (to 60 min).

Isolation of luteolin glycosides. Luteolin 6-C-glucoside, luteolin 6-*C*-arabinoside and luteolin 7-*O*-glucoside were isolated from *Sasa senanensis* Rehder as described previously (12).

Spectral analysis. UV spectrum was measured on a UV-visible recording spectrophotometer UV-240 (Shimazu, Kyoto, Japan). NMR spectra were obtained on an Avance system (Bruker Biospin, Yokohama, Japan) at 400 MHz for ^{1}H and 100 MHz for ^{13}C, respectively (Agilent-400 MR-vnmrs 400 Spectrometer, Agilent Technologies, Inc., Santa Clara, CA, USA).

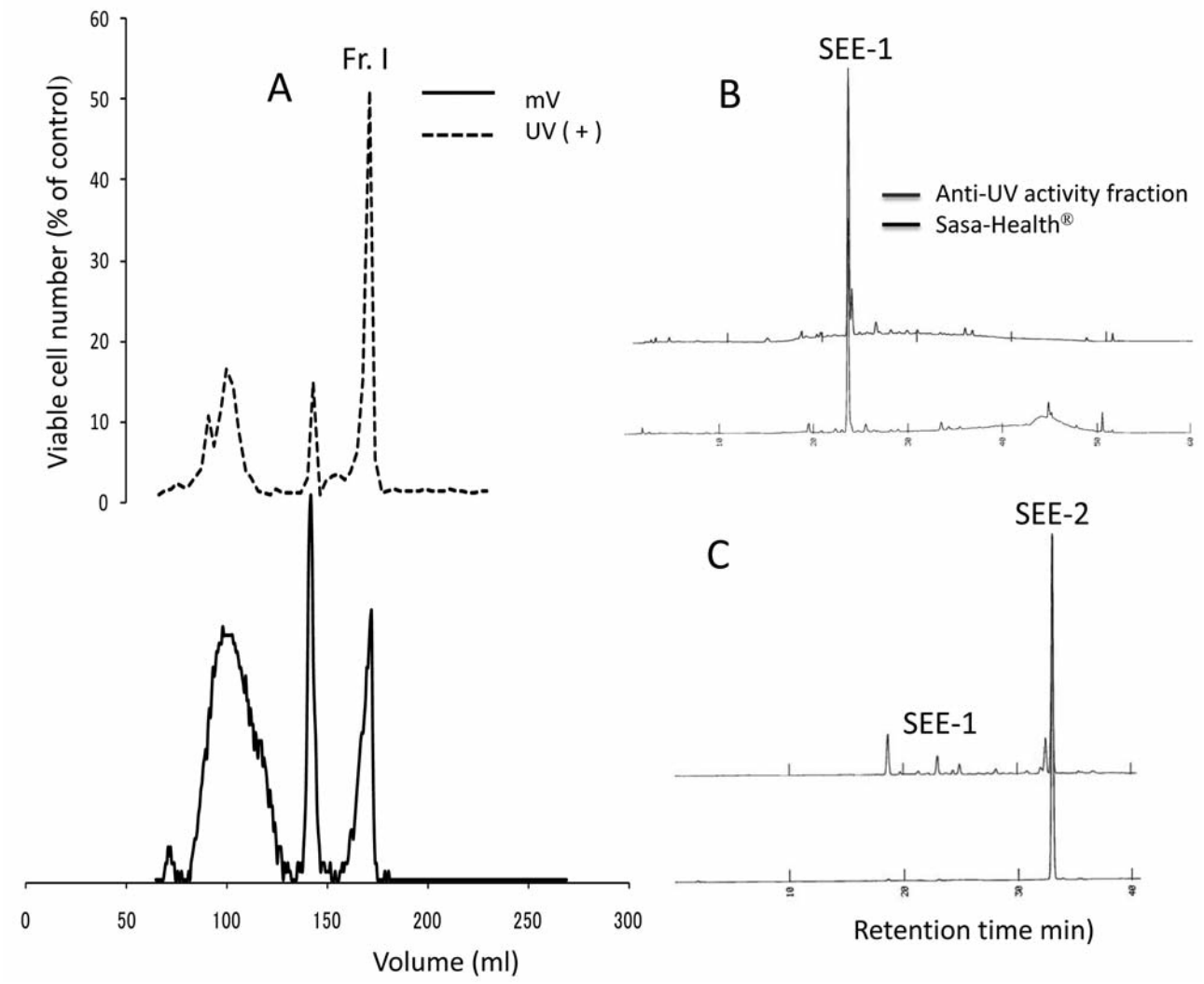
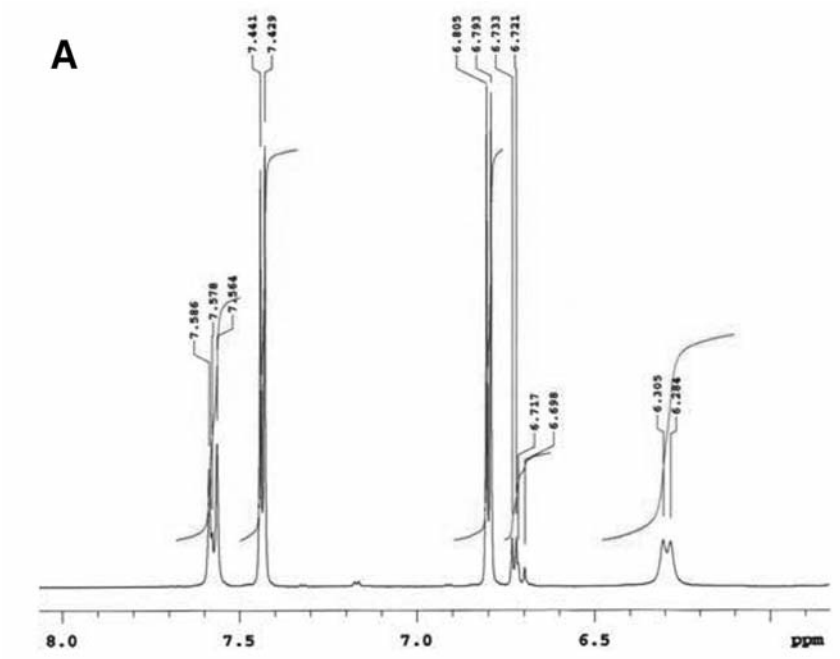
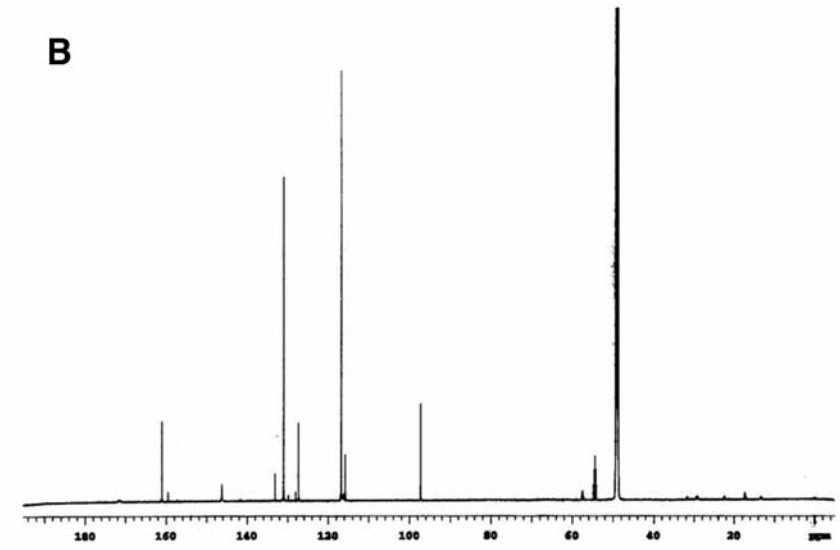


Figure 2. *Anti-UV activity of gel permeation chromatography (GPC) fractions. (A) Fractionation of alkaline extract of the leaves of Sasa senanensis Rehder (SE, Sasa-Health®) by gel filtration on Wakosil 40C18. The eluate was collected every 10 min and the elution curve was made by plotting the eluate absorbance at 254 nm (monitored by mV, indicated by solid line). To determine anti-UV protective activity of each fraction HSC-2 cells were exposed to 1-min UV irradiation in PBS(–) with 70% of each fraction. The viable cell number was determined the by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (11) (indicated by dotted line). B and C: Fractionation of SEE-1 and SEE-2 by HPLC (Inertsil ODS-3). The detection scales are the same (B, C).*

Assay for UV protection. The human oral squamous cell carcinoma HSC-2 cells were inoculated at 3×10^3 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 48, the media of the near-confluent cells attached to 96-microwell plates were replaced with PBS(–) containing different concentrations of test substances or fractions. PBS(–), instead of DMEM plus 10% FBS, was used as the medium during UV irradiation, since the latter contained radical scavenger(s) or UV-absorbing substance(s) such as phenol red and proteins. The cells were then placed at 20.5 cm distance from a UV lamp (wavelength: 253.7 nm) and were 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 before determination of the relative viable cell number by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. In brief, treated cells were incubated for another 4 h in fresh culture medium containing 0.2 mg/ml MTT. The cells were then lysed with 0.1 ml of DMSO and the absorbance of the cell lysate was determined at 540 nm using a microplate reader (Biochromatic Labsystem, Helsinki, Finland). From the dose–response curve, the 50% cytotoxic concentration (CC_{50}) and the concentration that increased the viability of UV-irradiated cells to 50% of the control (EC_{50}) were determined. The selectivity index (SI) was determined by the following equation: $SI=CC_{50}/EC_{50}$ (11).





C

	^{13}C	^{1}H
1	127.4	
2,6	131.0	7.44 (d, *J*=8.6Hz)
3,5	116.8	6.80 (d, *J*=8.6Hz)
4	161.0	
7	146.2	7.58 (d, *J*=15.5Hz)
8	115.8	6.29 (br.d, *J*=14.7Hz)
9	159.5	

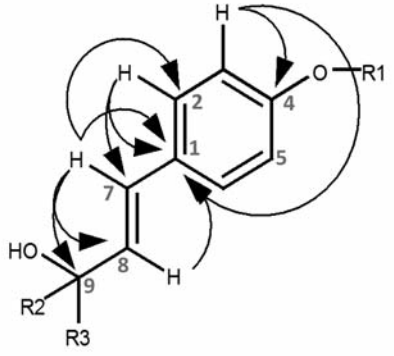


Figure 3. *Structural determination of SEE-1. A: ^{1}H-NMR spectrum; B: ^{13}C-NMR spectrum; C: data of A and B; D: putative structure of SEE-1.*

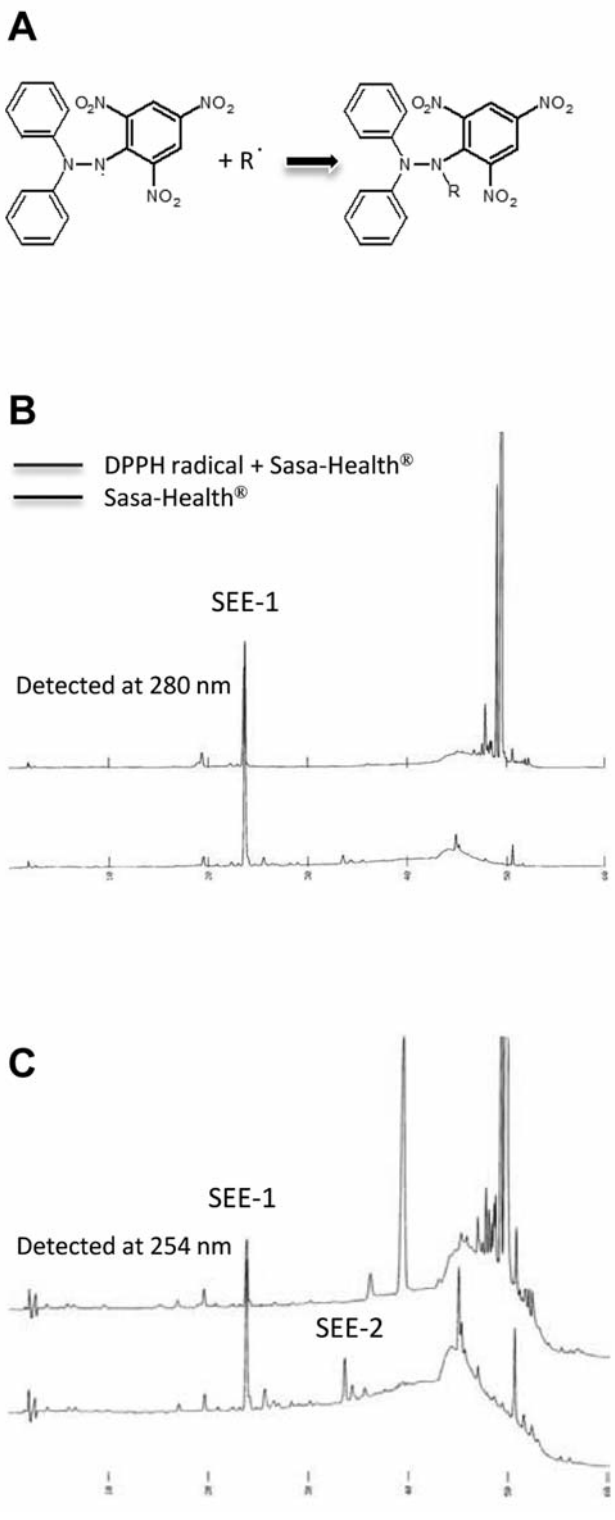


Figure 4. *1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity of Sasa-Health® by HPLC analysis. A: Reaction scheme of DPPH radical and anti-UV substance (indicated by •R). B and C: Elution profile of Sasa-Health® alone (lower) and with DPPH radical (upper) on HPLC (Inertsil ODS-3) fraction, detected at 280 nm for SEE-1 (B) and 254 nm for SEE-2 (C).*

Table I. *Anti-UV activity of alkaline extract of the leaves of Sasa senanensis Rehder (SE, Sasa-Health®) and SEE-1.*

	EC_{50} (μg/ml)	CC_{50} (μg/ml)	SI
SE (Sasa-Health®)	513.3±28.9	7780±1612	15.2±2.3
SEE-1	137	>500	>3.6
Luteolin 6-*C*-glucoside	129.0±9.1	>800	>6.2
Luteolin 6-*C*-arabinoside	156.3±7.9	>800	>5.1
Luteolin 7-*O*-glucoside	157.4±10.5	>800	>5.1
Chlorophyllin	349.2	653.1	1.9

SEE-1: One of the anti-UV substances isolated from SE; EC_{50}: the concentration that increased the viability of UV-irradiated cells to 50% of the control; CC_{50}: 50% cytotoxic concentration; SI, selectivity index (CC_{50}/EC_{50}). Data of luteolin glycosides and chlorophyllin are from (12).

Table II. *1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging of alkaline extract of the leaves of Sasa senanensis Rehder (SE, Sasa-Health®) and SEE-1.*

	IC_{50} (μg/ml)	IC_{50} (μM)
SE (Sasa-Health®)	148.64±8.75	
SEE-1	243.6±10.75	
Luteolin 6-*C*-glucoside	12.91±0.43	28.82±0.96
Luteolin 6-*C*-arabinoside	10.87±0.90	26.02±2.15
Luteolin 7-*O*-glucoside	10.10±1.17	22.55±2.62
trans-p-Coumaric acid	3221.02±38.28	19621.20±233.21
Trolox	6.34±0.12	25.33±0.46
Sodium ascorbate	7.69±0.30	38.81±1.50

IC_{50}: Concentration that reduced the intensity of DPPH radical by 50%.

Measurement of DPPH-scavenging activity. DPPH (final concentration: 6.8 mg/ml) and SE (final concentration: 2.6 mg/ml) were mixed and stood for 30 min at room temperature. SE alone, and the mixture were subjected to HPLC separation: using a Inertsil ODS-3 column (4.6 i.d. ×150 mm), mobile phase: A: H_2O:CH_3CN:HCOOH=95:5:0.1, B: CH_3CN, with gradient of 0-10 min 0% B, 15-30 min 25% B, 30-40 min 40% B, 40-50 min 100% B, 50-60 min 100% B, at a flow rate of 1.0 ml/min. Substances that scavenge DPPH radical can be detected where the absorbance at 280 and 254 nm is reduced by mixing with SE.

Statistical analysis. Experimental values are expressed as the mean±standard deviation (SD) of triplicate assays.

Results

Purification of anti-UV substance. When SE was separated on gel filtration based on molecular weight, anti-UV activity was distributed into three peaks (Figure 2A) (cited from ref.

11). From these, the highest anti-UV activity was eluted in the last peak with the smallest molecular weight (elution volume: 155-177 ml) (indicated by Fr. I) (Figure 2A)(11). When Fr. I was applied to HPLC (Inatsil ODS-3), a major single peak (SEE-1) having sharp anti-UV activity eluted at a retention time of 23.5 min (Figure 2B). The yield of SEE-1 from the starting material (SE) was 0.22%.

SEE-1 was subject to structural analysis with UV absorption (Figure 1C), ^{1}H-NMR and ^{13}C-NMR spectra analyses (Figure 3A-C). ^{1}H-NMR spectra (Figure 3A) demonstrated the signals of 1,4-substituted benzene (δ7.44, 6.80) and *trans*-olefin (δ7.58, 6.29). The cross peaks were found between the H-7 and C-2/-7 in HMBC spectrum. These data suggest the binding of olefin to the benzene ring. The cross peak was also found between H-7 and C-9. ^{13}C-NMR spectra (Figure 3B) demonstrated that the signal at C-9 was sifted to up-field than the carbonyl group of both *cis* and *trans* caffeic acid. These data suggest that the putative structure of SEE-1 may be that of *trans-p*-coumaric acid derivatives attached to unidentified substances at R1, R2 and R3 positions (Figure 3D).

Anti-UV activity. SEE-1 (EC_{50}=137 μg/ml) exhibited approximately three to four-fold higher anti-UV activity than SE (EC_{50}=513 μg/ml) and chlorophyllin (EC_{50}=349 μg/ml), which was however comparable with that of luteolin glycosides (EC_{50}=129-157 μg/ml) (Table I). On the basis of the SI, SEE-1 (SI>3.6) has higher anti-UV activity than chlorophyllin (SI=1.9).

Anti-DPPH radical scavenging activity. DPPH radical-scavenging activity overlapped with the elution peak of SEE-1 (retention time=23.5 min) (Figure 4B). SEE-1 exhibited slightly lower DPPH radical-scavenging activity (IC_{50}=244 μg/ml) as compared with SE (IC_{50}=149 μg/ml), but was much lower than that of luteolin glycosides (IC_{50}=10-13 μg/ml) and of popular antioxidants, Trolox (IC_{50}=6.3 μg/ml) and sodium ascorbate (IC_{50}=7.7 μg/ml). It was unexpected that *trans-p*-coumaric exhibited the least DPPH radical-scavenging activity (IC_{50}=3221 μg/ml). This further supports the theory that SEE-1 has structure similar to *trans-p*-coumaric acid, but attached to unidentified substances.

Discussion

The present study determined for the first time the low molecular anti-UV substance of SE as *p*-coumaric acid derivative(s) attached to unidentified substances. The alkaline extraction, which is an indispensable process for the preparation of SE, as well as LCC, produces many degradation products of LCC, including *trans-p*-coumaric and its polymers. Gradual degradation of lignin structure with time under alkaline solution makes the complete structural determination of SEE-1 difficult.

The present study demonstrated for the first time the presence of lignin precursors in SE. This finding explains why SE exhibited many properties of LCC. Three major products of bamboo leaf extract are available in drug stores in Japan. Sasa Health® (SE) is a pure alkaline extract of the leaves of *Sasa senanensis* Rehder containing Fe (II)-chlorophyllin, in which Mg (II) is replaced by Fe (II) by adding $FeCl_2$. Sunchlon® is an alkaline extract of *Sasa* Makino et Shibata that contains Cu (II)-chlorophyllin, but approximately 80% of the LCC has been removed as a precipitate during the manufacturing process (15). Shojusen® is a hot water extract of the leaves of *Sasa krilensis* Makino et Sibata, supplemented with ethanolic extract of the leaves of *Pinus densiflora* Sieb et Zucc., ethanolic extract of the roots of *Panax ginseng* C.A. Meyer and paraben as a preservative (16). We found that Sasa Health®, which contains higher amounts of LCC than the other two products exhibited the greatest anti-HIV and anti-UV activity (9). We recently reported that a total of 35 Kampo medicine and constitutive plant extracts, prepared by hot water extraction, had disappointingly lower anti-HIV and anti-UV activity (17), as compared with purified LCC from various plant species (18). These data confirm that alkaline extraction can achieve a higher yield of LCC, as compared with hot water extraction. SE as well as LCC, having prominent antiviral potency, may be applicable in the treatment of virally-induced diseases (19-21).

We also isolated an anti-UV substance (SEE-2) (at the yield of 0.18%) from SE (Figure 1A), with similar chromatographic techniques (retention time=22.8 min) (Figure 2C). SEE-2 has also DPPH radical scavenging activity (Figure 4C), but different UV absorption properties from SEE-1 (Figure 1C), suggesting that SEE-2 may have different backbone structure from SEE-1. Further analyses are necessary to further study SEE-2.

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