

Comparative Study of Biological Activity of Three Commercial Products of *Sasa senanensis* Rehder Leaf Extract

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Abstract. Background: We have previously reported that alkaline extract of *Sasa senanensis* leaves (SE) has several biological activities characteristic of lignin-carbohydrate complex (LCC). In the present study, we compared the biological activity of three commercially available products of SE (products A, B and C). Materials and Methods: Cell viability of mock-infected, HIV-infected, UV-irradiated cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. Radical intensity 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: Product A is a pure SE that contains Fe(II)-chlorophyllin, whereas products B and C contain Cu(II)-chlorophyllin and less LCC. Product C is supplemented with ginseng and pine (*Pinus densiflora*) leaf extracts. Product A exhibited 5-fold higher anti-HIV, 4-fold higher anti-UV, 5-fold higher hydroxyl radical-scavenging, and 3-fold lower CYP3A4 inhibitory activities as compared to those of product B, and 5-fold higher, 1.5-fold higher, comparable, and 7-fold lower activities, respectively, as compared to those of product C. Conclusion: The present study demonstrates for the first time the superiority of product A over products B and C, suggesting the beneficial role of LCC and Fe(II)-chlorophyllin.

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Key Words: *Sasa senanensis* Rehder, leaf extract, anti-HIV, anti-UV, radical scavenging, CYP3A4 inhibition, Fe(II), lignin.

Alkaline extract of the leaves of *Sasa senanensis* Rehder (SE) or *Sasa albo-marginata* Makino et Shibata (SASA-Health[®]) (Daiwa Biological Research Institute Co., Ltd., Kanagawa, Japan) has been recognized as over the counter (OTC) drug effective in treating fatigue, low appetite, halitosis, body odour and stomatitis. These beneficial clinical effects of SE have been partially supported by the following *in vitro* activities: e.g., antiseptic (1), membrane-stabilising (2), anti-inflammatory (3-5), antibacterial (6, 7), anti-viral (6, 7), anti-UV (8) and radical-scavenging (4, 7, 9) activities, and synergistic action with vitamin C (6). Among these activities, anti-HIV and anti-UV activity, and synergism with vitamin C are characteristic of lignin-carbohydrate complex (LCC) (10). We recently found that nearly 70% of SE components were eluted as a single peak on high-performance liquid chromatography (HPLC), suggesting that multiple components of SE may be associated with each other in the native state or after extraction with alkaline solution (7).

The OTC drugs are classified into three groups (I, II and III) in Japan. The safety of OTC drugs increases in the order of I<II<III (11). SE, which belongs to Group III, is expected to be less hazardous, as compared with Kampo Medicines, which belong to Group II. In Japan, three commercial products of SE (products A, B and C) are available in drug stores. Product A is a 100% pure extract of *Sasa senanensis* Rehder containing Fe(II)-chlorophyllin, in which Mg(II) in the centre is replaced by Fe(II) by adding FeCl₂. Products B and C contain Cu(II)-chlorophyllin and lesser amount of LCC. Product C is supplemented with ginseng extract (1.8 mg/ml) and pine (*Pinus densiflora*) leaf extract (3 mg/ml), with paraben as a preservative. Here, we compared the biological activity of products A, B and C for the first time.

To our knowledge there has been no study that has investigated the effect of SE on cytochrome P-450 (CYP)3A activity. We recently reported that the CYP3A-inhibitory activity of SE (product A) was significantly lower than the one observed in grapefruit juice and chlorophyllin (7). We also compared the three products for their CYP3A4-inhibitory activity, using grape juice as a positive control (12).

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), diethylenetriaminepentaacetic 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 sulfate (79 kDa; Ajinomoto Co. Inc., Tokyo, Japan).

Product A (SE) was prepared and supplied by Daiwa Biological Research Institute Co. Ltd., Kawasaki, Kanagawa, Japan. Product B was purchased from Sunchlon Co. Ltd., Ueda-shi, Nagano, Japan. Product C was purchased from Wakanyaku Medical Institute, Ltd., Shinjuku-ku, Tokyo, Japan. One millilitre of products A, B and C was freeze dried to produce powder (66.1, 77.6 and 28.5 mg, respectively).

Assay for anti-HIV activity. Human T-cell leukemia virus I (HTLV-I)-bearing CD4 positive human T cell line, MT-4 cells, were 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 product samples and the relative viable cell number was determined by 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 up to the 50% compared to the one 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: $SI = CC_{50}/EC_{50}$ (13).

Assay of anti-UV activity. HSC-2 cells (provided by Prof. Nagumo, Showa University) were inoculated into 96-microwell plates (3×10^3 cells/well, 0.1 ml/well) and incubated for 48 hours 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 products, placed at 21 cm distance from a UV lamp (wavelength: 253.7 nm) and 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% compared to that of control (unirradiated, untreated) cells (EC_{50}) were determined. The SI was

determined using the following equation: $SI = CC_{50}/EC_{50}$ (14, 15).

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) (16). The instrument settings were: centre field, 335.5 ± 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, 0.5 mM DETAPAC 20 μ l, 8% DMPO 30 μ l, sample (in PB) 40 μ l, PB 30 μ l, XOD (0.5 U/ml in PB) 30 μ l], the time constant was changed to 0.03 s (16).

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, sample (in H₂O) 50 μ l, 1 mM H₂O₂, 30 μ l], the gain was changed to 160 (16).

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, U.K.). 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 the product samples or vehicle (DMSO) 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 was collected, dried, and resuspended in 200 μ l of methanol. Analyses of the metabolites were performed by HPLC (JASCO PU2089, AS2057, UV2075, ChromNAV) equipped with 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 the one 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.

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

Results

Anti-HIV activity. Products A, B and C all effectively dose-dependently reduced the cytopathic effect of HIV infection (closed symbols in Figure 1), although their anti-HIV activity

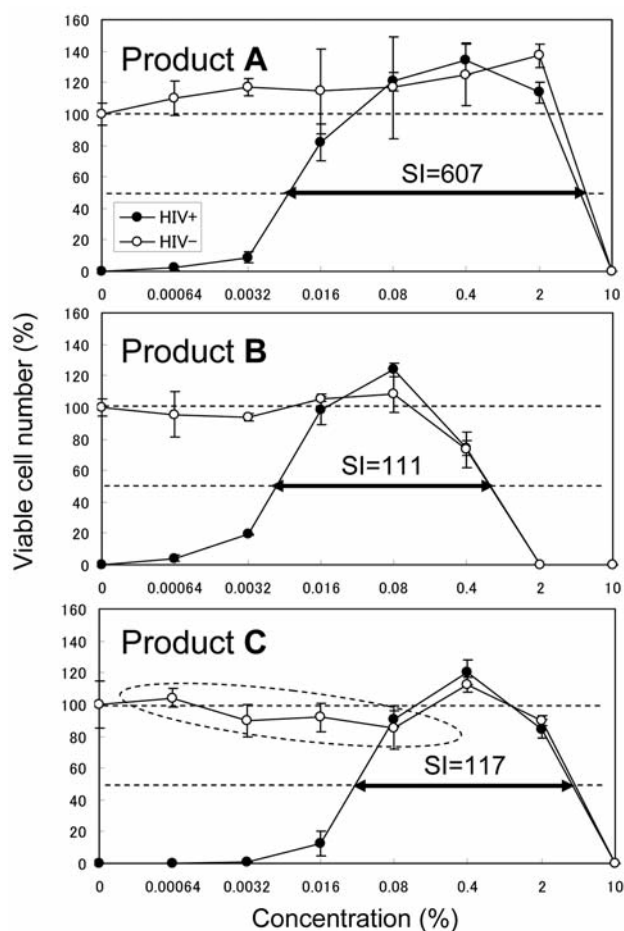


Figure 1. Anti-HIV activity of three commercial products of *Sasa senanensis* Rehder extract. HIV-1_{III_B}-infected (HIV+) and mock-infected (HIV-) MT-4 cells were incubated for 5 days with the indicated concentrations of products A (upper panel), B (center panel) and C (lower panel) and the viable cell number was determined by the MTT assay and expressed as a percentage that of the control. Data represent the mean±standard deviation from triplicate assays. It should be noted that product C exhibited a weak cytostatic effect at lower concentrations (indicated by dotted circle).

was much lower than the one of the positive controls [dextran sulfate (SI=1378), curdlan sulfate (SI=5606), azidothymidine (SI=17746), 2',3'-dideoxycytidine (SI=5123)] (Table I). The potency of anti-HIV activity was in the order of product A (SI=607) > product C (SI=117) > product B (SI=111).

Anti-UV activity. We recently reported that UV irradiation induced non-apoptotic cell death in HSC-2 cells, without induction of caspase-3 activation and internucleosomal DNA fragmentation (14). Products A, B and C all dose-dependently reduced UV-induced cytotoxicity (Figure 2). The potency of anti-UV activity was in the order of product A (SI=18.4) > product C (SI=12.6) > product B (SI=3.7) (Exp. I, Table II).

Table I. Anti-HIV activity of three commercial products of *Sasa senanensis* Rehder extract. Each value was determined from the dose-response curve in Figure 1.

Test material	CC ₅₀	EC ₅₀	SI
Product A (%)	5.465	0.009	607
Product B (%)	0.666	0.006	111
Product C (%)	4.083	0.035	117
DS (μg/ml)	137.78	0.10	1378
CRDS (μg/ml)	504.56	0.09	5606
AZT (μM)	230.70	0.013	17746
ddC (μM)	2766.19	0.54	5123

CC₅₀: 50% Cytotoxic concentration; EC₅₀: 50% effective concentration; SI: selectivity index; DS: dextran sulfate, CRDS: curdlan sulfate, AZT: azidothymidine, ddC: 2',3'-dideoxycytidine.

Table II. Anti-UV activity of three commercial products of *Sasa senanensis* Rehder extract. Each value was determined from the dose-response curve with triplicate assay in experiments I and II (Figure 2).

	CC ₅₀ (mg/ml)	EC ₅₀ (mg/ml)	SI
Exp. 1			
Product A	12.4	0.674	18.4
Product B	0.735	0.199	3.7
Product C	6.18	0.490	12.6
Exp. 2			
Product A	12.0	0.568	21.1
Product B	1.83	0.357	5.1
Product C	>5.70	0.459	>12.4
Sodium ascorbate (mM)	9.3	0.28	33.2

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

Repeated experiment gave a similar order of potency of these products [product A (SI=21.1) > product C (SI=>12.4) > product B (SI=5.1)] (Exp. II, Table II). The anti-UV activity of product A was approximately 59% of that of vitamin C (SI=33.2), as positive control (Table II).

Radical-scavenging activity. Products A, B and C dose-dependently reduced the intensity of superoxide anion (O₂⁻) (detected as DMPO-OOH) generated by HX and XOD reaction (Figure 3A). The potency of O₂⁻ scavenging activity of the three products was comparable: product A (IC₅₀=0.46 mg/ml), product B (IC₅₀=0.52 mg/ml) and product C (IC₅₀=0.54 mg/ml) (Table III).

Products A, B and C dose-dependently reduced the intensity of hydroxyl radical (•OH) (detected as DMPO-OH) generated by the Fenton reaction (Figure 3B). The potency

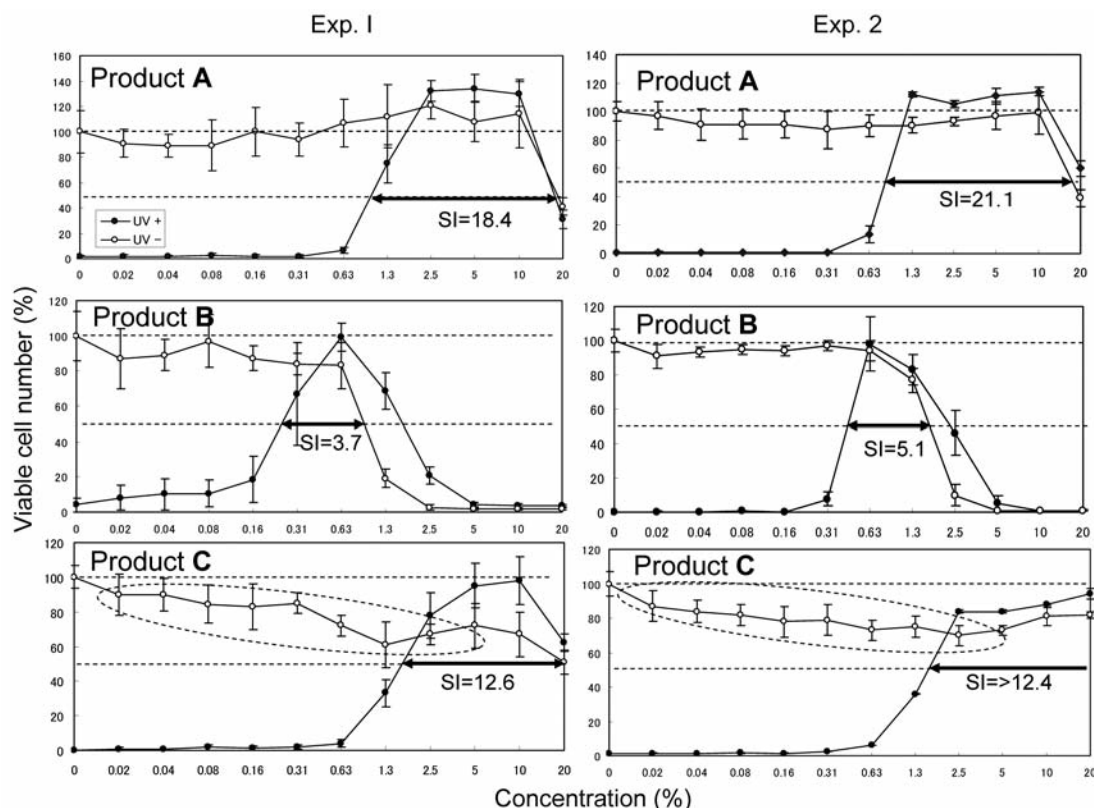


Figure 2. Anti-UV activity of three commercial products of *Sasa senanensis* Rehder extract. HSC-2 cells were exposed (UV+) or not (UV-) to UV irradiation (6 J/m²/min) for 1 min in PBS(-) containing the indicated concentrations of products A (upper panel), B (center panel), and C (lower panel), and then were 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). Experiments were performed twice (Exps. I and II), and each value represents the mean \pm S.D. of triplicate assays. It should be noted that product C exhibited a weak cytostatic effect at lower concentrations (indicated by dotted area). SI: selectivity index.

of products A and C was comparable with each other (IC₅₀=2.1 and 1.9 mg/ml, respectively), but 4-fold higher than that of product B (IC₅₀=8.0 mg/ml) (Table III).

CYP3A4-inhibitory activity. Products A, B and C dose-dependently inhibited the β -hydroxylation of testosterone, generally used for the assay of CYP3A4 activity (Figure 4 and Table IV). Product C exhibited the highest CYP3A4-inhibitory activity (IC₅₀=58 μ g/ml), followed by product B (IC₅₀=124 μ g/ml) and then product A (IC₅₀=403 μ g/ml). Product B inhibited the CYP3A4 to an extent similar to that attained by Cu(II)-chlorophyllin (7); product A inhibited CYP3A4 to lower extent than that achieved by grapefruit juice (7).

Discussion

The present study demonstrated for the first time that all three SE products exhibited potent anti-HIV, anti-UV and radical-scavenging activity. All these data are summarized in Table V. Product A was found to have 4 to 5-fold higher anti-HIV, anti-UV and \cdot OH radical-scavenging activity as

compared with product B. This suggests the beneficial role of LCC and Fe(II)-chlorophyllin. Product C was found to have much higher biological activity than product B, possibly due to the supplementation of ginseng and pine (*Pinus densiflora*) leaf extracts, but the potency of product C was still less than that of product A.

The present study also demonstrated that the CYP3A4 inhibitory activity of product C was nearly 7-fold higher compared to the one of product A. This suggests that product C may better enhance the bioavailability of drugs (especially, CYP3A substrates) that are administered together with it, as compared with product A, and thus it is important to perform therapeutic drug monitoring of product C to minimize the possible side-effects of an accompanying drug that is metabolized by CYP3A. It should be noted that lower concentrations of product C (0.02-2.5%) exhibited slight, but reproducible cytostatic activity (indicated by dotted circles in Figures 1 and 2). Further experiments are required to identify the cytostatic principle (whether it is the exogenous plant extracts or preservative). All data suggest the superiority of product A over products B and C.

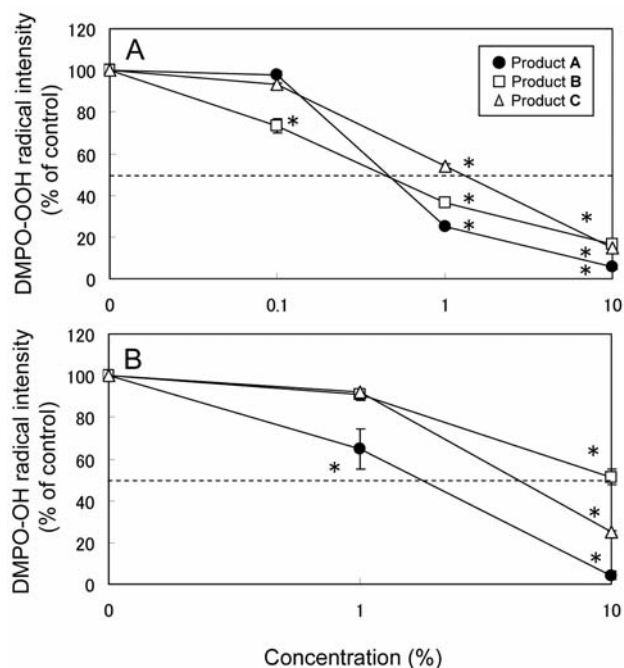


Figure 3. Effect of products A, B and C on the radical intensity of superoxide anion radical, measured as DMPO-OOH produced by hypoxanthine-xanthine oxidase reaction (A) and hydroxyl radical, measured as DMPO-OH produced by Fenton reaction (B). Each value represents the mean \pm S.D. of triplicate assays. * p <0.05 relative to the control (0%).

Table III. Radical scavenging activity of three commercial products of *Sasa senanensis* Rehder extract. Each value was calculated from the data of Figure 3.

	O ₂ ⁻ -Scavenging activity (IC ₅₀)	•OH-Scavenging activity (IC ₅₀)
Product A	0.69% (0.46 mg/ml)	3.2% (2.1 mg/ml)
Product B	0.67% (0.52 mg/ml)	10.3% (8.0 mg/ml)
Product C	1.9% (0.54 mg/ml)	6.6% (1.9 mg/ml)

IC₅₀: The concentration that reduced the radical intensity by 50%.

Supplementation of ginseng and pine (*Pinus densiflora*) leaf extracts cannot compensate for the loss of LCC (unpublished data), indicating that the pure SE is sufficient alone for expressing its full biological activity, and the effects of any manipulation (such as removal of LCC) and supplementation with other plant extracts should be carefully considered.

Product A had much higher anti-HIV activity (SI=639) as compared with tricin (SI=27), a potent anti-human cytomegalovirus agent (17), and luteolin glycosides (18) (SI=2~7) from *Sasa senanensis* Rehder. The potent anti-viral

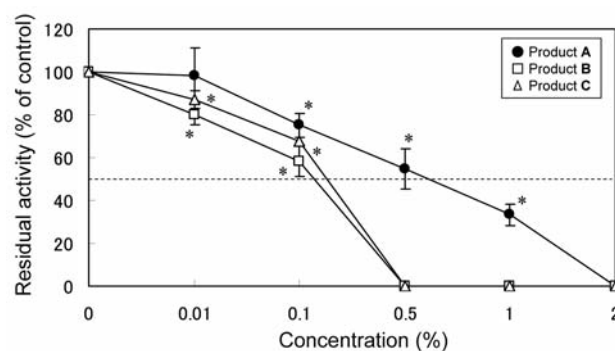


Figure 4. CYP3A4 inhibitory activity of products A, B and C. Each value represents the mean \pm S.D. of triplicate assays. * p <0.05 relative to the control (0%).

Table IV. CYP3A4 inhibitory activity of three commercial products of *Sasa senanensis* Rehder extract. Each value was calculated from the dose-response curve in Figure 4.

	CYP3A4 inhibition (IC ₅₀)
Product A	0.61% (403 μ g/ml)
Product B	0.16% (124 μ g/ml)
Product C	0.20% (58 μ g/ml)
Reference substances [see (7)]	
Product A	0.99% (576 μ g/ml)
Cu (II)-chlorophyllin	109 μ g/ml
Grapefruit juice	0.49%

IC₅₀: The concentration that inhibits the CYP3A4 activity by 50%.

Table V. Summary of biological activities of three SE products

	Relative biological activity (% of product A)		
	Product A	Product B	Product C
Anti-HIV activity	100	18	19
Anti-UV activity			
(Exp. 1)	100	22	72
(Exp. 2)	100	24	>57
O ₂ ⁻ -Scavenging activity	100	88	85
•OH-Scavenging activity	100	26	111
CYP3A4-inhibitory activity	100	325	695

activity together with the antioxidant and the anti-inflammatory activity of product A suggest its possible applicability to the prevention of or therapy for virally mediated diseases.

Although OTC drugs, categorized as Group III, are the least hazardous, it may be preferable to test their safety before marketing, using *in vitro* biological assay systems such as the ones that are described in this study.

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