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Multiple Biological Complex of Alkaline Extract of the Leaves of *Sasa senanensis* Rehder

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Abstract. *Previous studies have shown anti-inflammatory potential of alkaline extract of the leaves of Sasa senanensis Rehder (SE). The aim of the present study was to clarity the molecular entity of SE, using various fractionation methods. SE inhibited the production of nitric oxide (NO), but not tumour necrosis factor-α by lipopolysaccharide (LPS)-stimulated mouse macrophage-like cells. Lignin carbohydrate complex prepared from SE inhibited the NO production to a comparable extent with SE, whereas chlorophyllin was more active. On successive extraction with organic solvents, nearly 90% of SE components, including chlorophyllin, were recovered from the aqueous layer. Anti-HIV activity of SE was comparable with that of lignin-carbohydrate complex, and much higher than that of chlorophyllin and n-butanol extract fractions. The CYP3A inhibitory activity of SE was significantly lower than that of grapefruit juice and chlorophyllin. Oral administration of SE slightly reduced the number of oral bacteria. When SE was applied to HPLC, nearly 70% of SE components were eluted as a single peak. These data suggest that multiple components of SE may be associated with each other in the native state or after extraction with alkaline solution.*

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Alkaline extract of the leaves of *Sasa senanensis Rehder* or *Sasa albo-marginata* Makino et Shibata (SE) (SASA-Health®), as an over the counter drug, is recognized as being effective in treating fatigue, low appetite, halitosis, body odour and stomatitis. However, there is no scientific evidence that demonstrates these phenomena. SE has been reported to show antiseptic (1), membrane stabilising (2), anti-inflammatory and phagocytic (3), radical scavenging (4), antibacterial, anti-viral and radical scavenging activities (5). It was recently reported that SE inhibited nitric oxide (NO) and prostaglandin E_2 (PGE_2) production by lipopolysaccharide (LPS)-activated mouse macrophage-like cells (RAW264.7) *via* inhibition of inducible NO synthase and cyclooxygenase-2 expression at both protein and mRNA levels (6).

In order to further expand the anti-inflammatory spectrum of SE, we first investigated whether SE inhibits the production of tumour necrosis factor-α (TNF-α), a pro-inflammatory cytokine, by LPS-activated mouse macrophage-like cells (RAW264.7, J774.1). Since chlorophyllin, a modifier of genotoxic effects (7), is present in SE at 0.25-0.26% (w/w) (8), it was also investigated whether chlorophyllin efficiently inhibits NO production by activated macrophages. It was previously reported that SE showed potent anti-HIV activity, comparable to lignin-carbohydrate complex from other species (5). Therefore, the possible anti-HIV activity of lignin-carbohydrate complex prepared from

SE was investigated for the first time. There is no study to date that has investigated the effect of SE on CYP3A activity. It was hypothesized that if SE inhibits CYP3A activity, it may enhance the bio-availability of drugs (especially, CYP3A substrates) that are administered together with SE. Therefore, whether SE actually inhibits CYP3A activity, using grapefruit juice as a positive control (9), was investigated. Based on *in vitro* antiviral and antibacterial activity (5), there is a possibility that oral administration of SE may reduce the number of bacterial in the oral cavity. Therefore this possibility was investigated in this study, using real-time PCR.

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, KS, USA); LPS from *Escherichia coli* (Serotype 0111:B4), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma Chem Co., St. Louis, MO, USA); testosterone (Nacalai Tesque, Inc, Kyoto, Japan); 6β-hydroxytestosterone (Daiichi Pure Chemicals Co Ltd, Tokyo, Japan); corticosterone (internal standard), acetonitrile of HPLC grade, dimethyl sulfoxide (DMSO), all other chemicals of reagent grade (Wako Pure Chem Ind., Osaka, Japan).

SE was prepared and supplied by Daiwa Biological Research Institute Co, Ltd, Kawasaki, Kanagawa, Japan. Lyophilisation and measurement of the dry weight of SE showed that it contained 58.2±0.96 mg solid materials/ml (5).

Preparation of lignin-carbohydrate complex and polysaccharide fractions. SE was centrifuged at 14,400×g for10 min at 15˚C to remove the insoluble materials. When the pH was lowered to 5 by drop-wise addition of acetic acid, fraction (Fr) I was precipitated. Fr I was dissolved in 1.39% $NaHCO_3$ or 1% NaOH, and the insoluble materials were removed. Addition of acetic acid to the supernatant precipitated Frs II and III, respectively. An equal amount of ethanol was added to the supernatant cleared of Fr I in order to precipitate polysaccharide fraction (Fr IV). All fractions were dialysed against water, and lyophilised (Figure 1).

Stepwise extraction of SE with organic solvents. SE (1164 mg) was extracted three times with 1 ml of *n*-hexane, and the *n*-hexane layer was concentrated under vacuum. The aqueous layer was extracted three times with 1 ml of Et_2O. The obtained aqueous layer was further extracted three times with AcOEt, and then *n*-BuOH (Table I).

For large scale fractionation, 254 ml of SE was fractionated into *n*-hexane (26.2 mg), Et_2O (135.2 mg), AcOEt (94.6 mg), *n*-BuOH (511.6 mg) and aqueous fractions (28486.2 mg). *n*-BuOH fraction (500 mg) was applied on a silica gel column and then eluted with $CHCl_3$ (Fr. 1: 3.3 mg), $CHCl_3$:MeOH (49:1) (Fr. 2: 53.6 mg), $CHCl_3$: MeOH (19:1) (Fr. 3: 83.7 mg), $CHCl_3$:MeOH (9:1) (Fr. 4: 14.3 mg), $CHCl_3$:MeOH (9:1) (Fr. 5: 45.4 mg), $CHCl_3$:MeOH (4:1) (Fr. 6: 58.9 mg), and MeOH (Fr. 7: 145.0 mg).

HPLC analysis of crude SE extract. SE (5 μl) was applied to an HPLC column: Develosil RPAQUEOUS (C-30) and eluted, and analysed for 70 min, using the following conditions: Mobile phase: A (0.1 % TFA in H_2O), B (CH_3CN), gradient (0-10 min: B10%, 10-50 min: B10%-B50%, 50-70 min: B50%); flow rate: 1.0 ml/min; detection: UV 210-400 nm; column temperature: 40˚C.

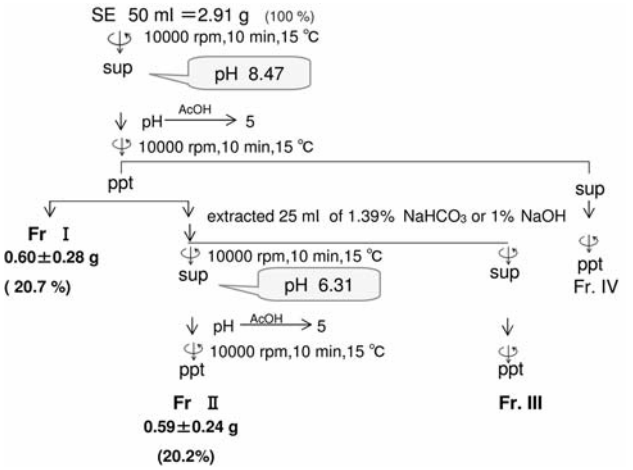
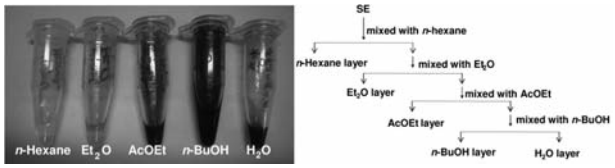


Figure 1. *Fractionation of SE. Yield of fractions (Frs) I and II represents the mean±SD from three independent experiments.*

Table I. *Distribution of chlorophyllin and components that inhibited the NO production by macrophages into fractions separated by successive organic solvent extractions.*

	Yield mg(%)	Volume ml	Conc. mg/ml	CC_{50} mg/ml	IC_{50} mg/ml	SI CC_{50}/IC_{50}
Initial	1164 (100)	20.0	58.2	13.6184	1.597	8.53
n-Hexane	7.4 (0.6)	0.4	18.5	0.1715	0.0387	4.43
Et_2O	6.2 (0.5)	0.4	15.5	0.1253	0.0245	5.00
AcOEt	13.2 (1.1)	0.4	33.0	0.2478	0.03193	7.76
n-BuOH	67.6 (5.8)	0.8	84.5	1.2288	0.1068	11.51
H_2O	943 (81.0)	20.0	47.2	22.8598	2.12	10.78
Total	1037.4 (89.1)					



Cell culture. RAW264.7 cells (10), J774.1 cells (11), human oral normal cells (gingival fibroblast HGF, pulp cell HPC, periodontal ligament fibroblast HPLF) (5) and human oral squamous cell carcinoma cell lines (HSC-2, HSC-3, HSC-4) (Riken Cell Bank, Ibaraki) (5) were cultured in DMEM supplemented with 10% heat-inactivated FBS under a humidified 5% CO_2 atmosphere. Human promyelocytic leukemia cell line HL-60 and human T-cell leukemia virus I (HTLV-I)-bearing CD4 positive human T cell line, MT-4 were cultured in suspension in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, as described previously (5).

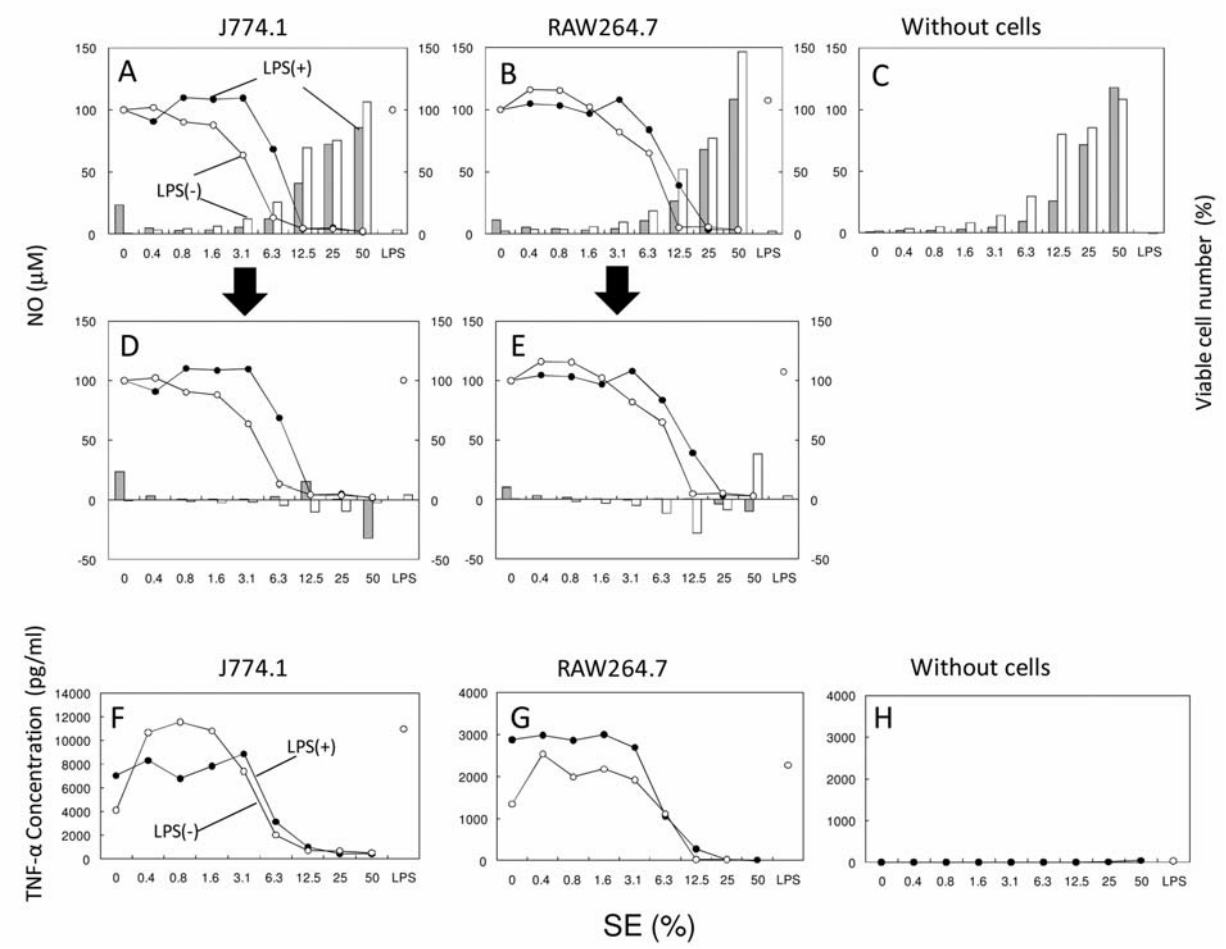


Figure 2. *Effect of SE on NO (A) and TNF-α (B) production by mouse macrophage-like cells (J774.1, RAW264.7). J774.1 (A, D, F) or RAW264.7 cells (B, E, G) were incubated for 24 hours with the indicated concentrations of SE in the absence (open symbol) or presence (closed symbols) of 100 ng/ml LPS, and the viable cell number (circle), NO (A-E) or TNF-α (F-H) (bar) was determined. Since SE has color that interferes the NO determination, the data of without cell (C) was subtracted from A and B to yield D and E. Since SE itself did not affect the TNF-α determination (H), data of F and G were used without subtraction of data of H. Each value represents mean of duplicate determinations.*

Determination of viable cell number. RAW264.7 or J774.1 were inoculated at 1.5×10^6/ml (100 μl) in a 96-microwell plate (Becton Dickinson, Labware, NJ, USA) and incubated for 1-2 hours. Near-adherent cells were replaced with fresh culture medium and treated for 24 hours with the indicated concentrations of SE in phenol red-free DMEM supplemented with 10% FBS in the presence or absence of LPS (0.1 μg/ml). Viable cell number was then determined by the MTT method. In brief, cells were incubated for 30 minutes with 0.2 mg/ml of MTT. After removal of the medium, the cells were lysed with DMSO, and the absorbance at 540 nm was recorded using microplate reader (12, 13).

Near-confluent human normal (HGF, HPC, HPLF) and tumour cells (HSC-2, HSC-3, HSC-4) were treated for 48 hours with various concentrations of test samples, and the relative viable cell number of adherent cells was then determined by the MTT method. 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 tumour-specificity index (TS) was measured by the following equation: $TS=[CC_{50}(HGF)+CC_{50}(HPC)+CC_{50}(HPLF)]/[CC_{50}(HSC\text{-}2)+CC_{50}(HSC\text{-}3)+CC_{50}(HSC\text{-}4)+CC_{50}(HL\text{-}60)]\times(4/3)$.

Assay for NO and TNF-α production. RAW264.7 or J774.1 cells were inoculated at 1.5×10^6/ml (100 μl) in a 96-microwell plate and incubated for 1-2 hours. Near confluent cells were treated for 24 hours without or with the indicated concentrations of SE (0.9-50%) in phenol red-free DMEM supplemented with 10% FBS in the presence or absence of LPS (0.1 μg/ml). The NO and TNF-α released into the culture medium was quantified as follows.

NO was determined by Griess reagent, using the standard curve of NO_2^-. To eliminate the interaction between sample and Griess reagent, the NO concentration was also measured in the culture medium without the cells, and this value was subtracted from that obtained with the cells. Then the concentration that inhibited the LPS-stimulated NO production by 50% (50% inhibitory concentration: IC_{50}) was determined from the dose-response curve (14). The efficacy of inhi-

Table II. *Anti-HIV activity and anti-inflammatory activity of SE components*.

	Anti-HIV activity			Anti-inflammatory activity		
	CC_{50} (μg/ml)	EC_{50} (μg/ml)	SI	CC_{50} (μg/ml)	IC_{50} (μg/ml)	SI
SE	638±12	18±3.1	36	4132±1164	396±87	10.3
Lignin Fr I (acid precipitation)	591	16	37	>5000	455±51	>11.3
Lignin Fr II (acid precipitation×2)	566	9.8	58	>5000	889±92	>5.8
Lignin Fr III (acid precitation×2)	735	12	62			
Polysaccharide Fr IV	>1000	>1000	≤1	>5000	>5000	≤1.0
Lignin Fr I (acid precipitation)	321±36	10±1.4	32			
Lignin Fr II (acid precipitation×2)	246±3.3	9.3±0.8	27			
n-BuOH extract Fr 0	91	>800	<1			
n-BuOH extract Fr 1	325	>400	<1			
n-BuOH extract Fr 2	71	>800	<1			
n-BuOH extract Fr 3	86	>800	<1			
n-BuOH extract Fr 4	180	>800	<1			
n-BuOH extract Fr 5	126	>800	<1			
n-BuOH extract Fr 6	397	>800	<1			
n-BuOH extract Fr 7	429	59	7			
Chlorophyllin (μg/ml)	97	18	5.4	36.4±1.7	1.5±0.22	25.1
Chlorophyll (μg/ml)				41.0±9.8	11.7±1.3	3.5

Lignin-carbohydrate complexes (Frs I, II, III) and polysaccharide (Fr IV) were prepared as described in Figure 1. *n*-BuOH fractions were separated as described in the Materials and Methods. Anti-inflammatory activity was assayed by the ability to inhibit the NO production by LPS-stimulated RAW264.7 cells, as described in Figure 2.

Table III. Tumour-specificity of *n*-BuOH extracts of SE.

	CC_{50} (μg/ml)							
	Human tumor cell lines				Human normal oral cells			
Fr	HSC-2	HSC-3	HSC-4	HL-60	HGF	HPC	HPLF	TS
0	201±41	340±3	184±34	77±8	331±7	332±8	338±3	1.7
1	384±36	>400	346±22	178±7	>400	>400	>400	><1.2
2	64±13	122±1	75±4	35±2	143±11	105±21	152±1	1.8
3	89±1	151±7	80±2	37±1	176±24	205±30	208±4	2.2
4	288±2	>400	243±14	70±8	>400	>400	>400	>1.6
5	258±6	>400	317±3	86±1	>400	>400	>400	>1.5
6	327±2	>400	303±3	141±19	>400	>400	>400	>1.4
7	>400	>400	>400	309±5	>400	>400	>400	>1.1

Each value represents the mean±SD of triplicate determinations.

bition of NO production was evaluated by the selectivity index (SI), which was calculated using the following equation: $SI=CC_{50}/IC_{50}$.

The TNF released into culture medium was determined by ELISA (Quantikine ELISA kit, R&D systems, Minneapolis, MN, USA).

Assay for anti-/HIV-I_{IVB} HIV activity. MT-4 cells were infected with HIV_{-1IIIB} 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 (15). 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 cytochrome P-450 3A (CYP3A) activity. CYP3A activity was assayed by the ability to oxidise 6β-position of testosterone, using rat liver microsome fraction (16). Testosterone (0.2 mM) was

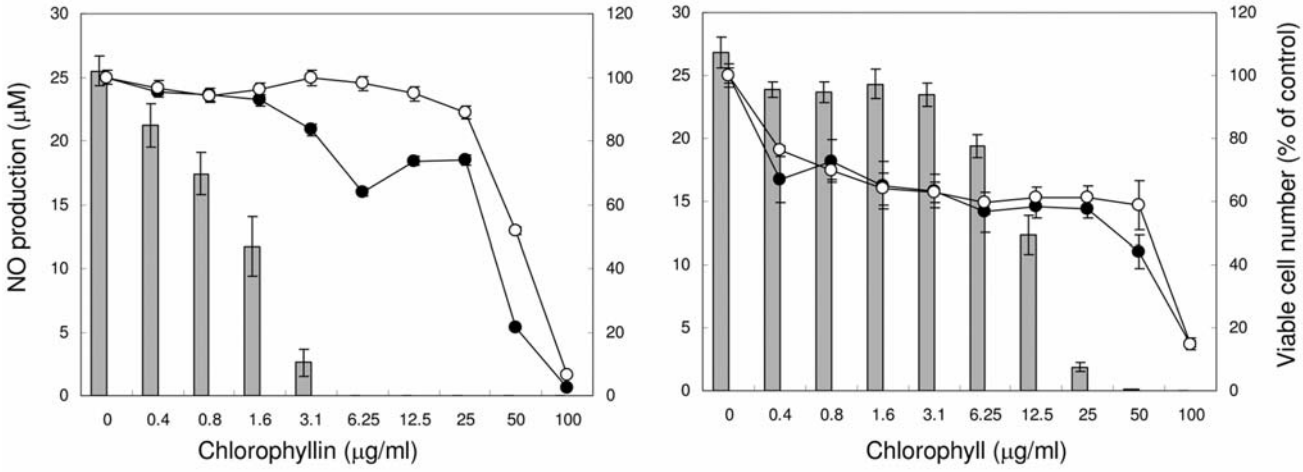


Figure 3. *Chlorophyllin inhibited NO production by activated macrophages more potently than did chlorophyll. RAW264.7 cells were incubated for 24 hours with the indicated concentrations of chlorophyllin (A) or chlorophyll (B) in the absence (no color) or presence (colored) of 100 ng/ml LPS, and viable cell number (circle) and NO in the culture medium (bar) were determined. Each value represents the mean±SD of triplicate determinations.*

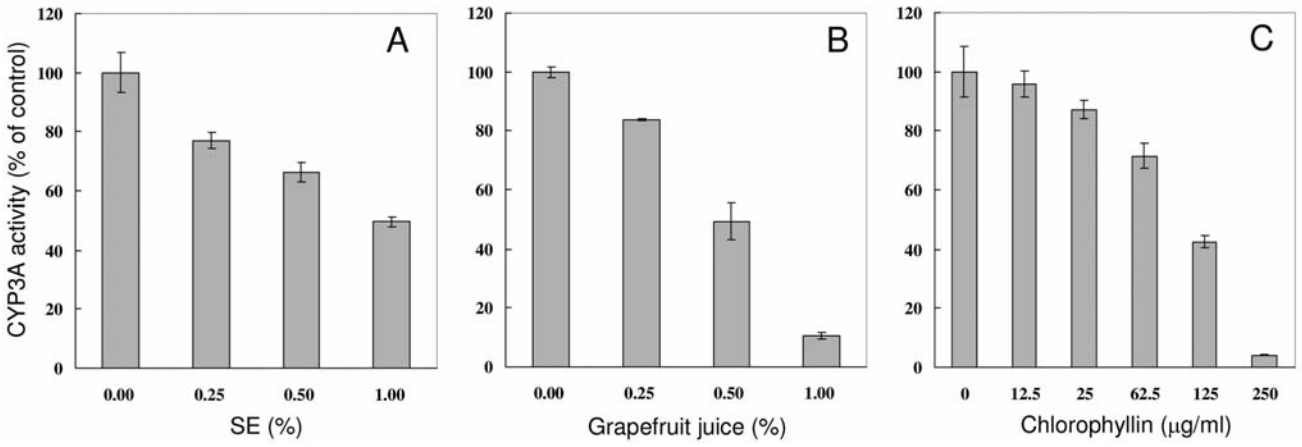


Figure 4. *Inhibition of CYP3A activity by SE, grapefruit juice and chlorophyllin. CYP3A activity was measured by hydroxylase activity of 6β-position of testosterone. Each value represents the mean±SD of triplicate determinations.*

incubated for 5 min at 37˚C in 0.1 M sodium phosphate buffer (pH 7.4) containing 1 mM NADPH and rat microsome. Ten-volume of corticosterone-acetonitrile solution (2 μg/ml) was then added to the reaction mixture as an internal marker. After 20 s mixing, the mixture was centrifuged at 16,000 ×*g* for 5 min at 4˚C, and 6β-hydroxytestosterone in the obtained supernatant was determined using an LC/ESI/MS system (Shimadzu LCMS-2010EV). Reverse phase Capcell Pak MGII-ODS column (2.0 mm×15 cm; particle size: 5 μm) (Shiseido Co, Ltd, Tokyo, Japan) was used as solid phase. H_2O/acetonitrile was used as mobile phase (flow rate: 0.2 ml/min). The concentration of acetonitrile in mobile phase was 10-100% (0-8 min) and 100% (8-20 min). For mass spectrometry, anion mode (interface voltage: 4.5 kV, detector voltage: 1.5 kV, heat block temperature: 200˚C, CDL temperature: 250˚C) was used to detect the molecular ion (m/z: 303.2 and 345.2) of 6β-hydroxytestosterone and corticosterone. Nitrogen (flow rate: 1.5 l/min) was used as nebuliser gas. 6β-Hydroxytestosterone standard solution (1-60 μM) was used to determine the standard curve.

Determination of oral bacterial DNA. Three healthy male volunteers (A, B, C) had taken 10 ml of 10% (v/v) SE (diluted 10-fold with water) three times a day, within 30 min after each meal, retaining it for approximately 1 min in the oral cavity and then swallowing. Bacteria from saliva and tongue coat were collected just before the lunch at day 1, 3, 7, 14, 21, 28 after the start of SE administration. As a control, bacteria collection was performed 5 times (once a day for 5 days) before SE administration. Bacteria were collected from the periodontal pocket before SE administration, and 7 days after SE administration. The number of bacteria was then measured by real-time PCR, according to the guidelines of the intramural Ethics Committee

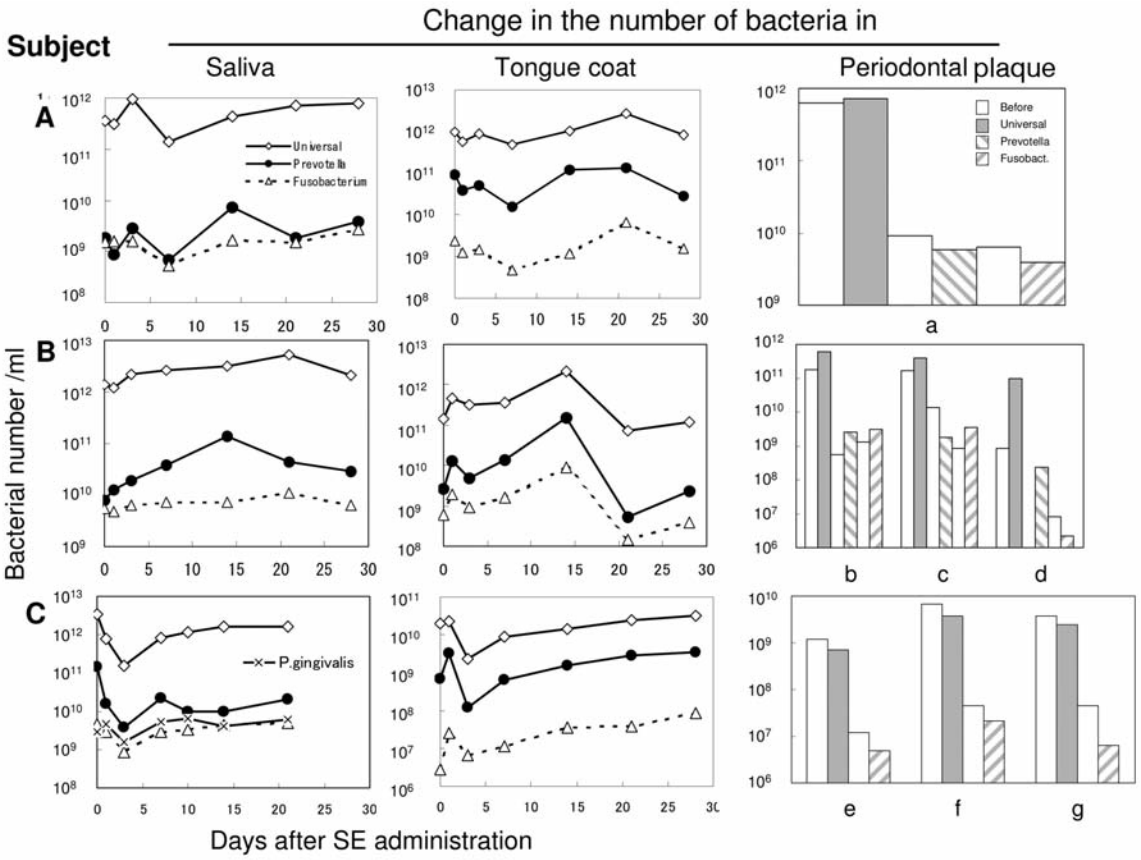


Figure 5. *Changes in the number of bacteria in saliva, tongue coat and periodontal pocket after administration of SE to three healthy males A (53 years old), B (57 years old) and C (33 years old). (a-g) mean the location where sample was collected: (a) the upper left permanent second molar, (b) the lower right permanent first premolar, (c) the lower left permanent first premolar, (d) the upper left permanent first molar, (e) the lower left permanent third molar, (f) the upper left permanent second molar, (g) the lower left permanent first molar. White bars, number of bacteria before SE administration; gray bars, number of bacteria 7 days after SE administration.*

(approved as No A0901), after obtaining the informed consents from three healthy volunteers (A, B, C). Bacteria in saliva (1 ml), the tongue coat (collected by scraping with loop) and the periodontal pocket [corrected by sterile paper point (Johnson & Johnson, New Brunswick, NJ, USA)] were washed three times with 1 ml PBS(–).

DNA was extracted from these three samples using Ultra Clean Microbial DNA Isolation Kit (MO BIO Laboratories, Inc, Carlsbad, CA, USA). After adjusting the DNA concentration to 2 ng/5 μl, real-time PCR was performed with LightCycler^R 480 (Roche Molecular Biochemicals, Mannheim, Germany), using specific primers (5'-3') (40 μM): Total bacteria (Universal) (forward: TCCTACGGGA GGCACAGT; reverse: GGACTACCAGGGTATCTAATCCTGTT; Taqman probe: CGTATTACCGCGGCTGCTGGCAC), *Fusobacterium* (*F. nucleatum, F . periodonticum, F. alocis, F. simiae*) (forward: AAGCGCGTCTAGGTGGTTATGT; Reverse: TGTAGTTCCGCTT ACCTCTCCAG; Taqman probe: CAACGCAATACAGAGTTGA GCCCTGCATT), *Prevotella* (forward: CCAGCCAAGTAGCGT GCA; reverse: TGGACCTTCCGTATTACCGC; Taqman probe: AATAAGGACCGGCTAATTCCGTGCCAG), *Porphyromonas gingivalis* (forward: TCGGTAAGTCAGCGGTGAAAC; reverse: GCAAG CTGCCTTCGCAAT; Taqman probe: CTCAACGTTCAGCCTGCC GTTGAAA). Concentration of Taqman fluorescent probe was 11 μM (Universal, *Fusobacterium*), 13 μM (*Prevotella*) and 14 μM (*P. gingivalis*).

Reaction mixture (20 μl) contains DNA solution (5 μl), Light Cycler^R 480 Probe Master (10 μl), Taqman probe (0.2 μl), forward primer (0.2 μl), reverse primer (0.2 μl), dH_2O (4.4 μl). Amplification program: Initial denaturation (95˚C, 5 min), amplification reaction (95˚C, 10 min), annealing elongation reaction (45 cycles of 60˚C, 30 s and 72˚C, 1 s), heating up to 95˚C and then cooled down to 50˚C. Standard curve of DNA of each bacterial was produced, using 10-10^6-fold diluted sample [*P. intermedia* (ATCC 25611), *P. gingivalis* (ATCC 33277), *F. nucleatum* (ATCC 31647)].

Results

Effect of SE on NO and TNF-α production by mouse macrophage-like cells. Colorimetric determination of NO by the Griess method is considerably affected by the colour of SE. It was therefore essential to subtract the absorbance value derived from SE (Figure 2C). When this value was

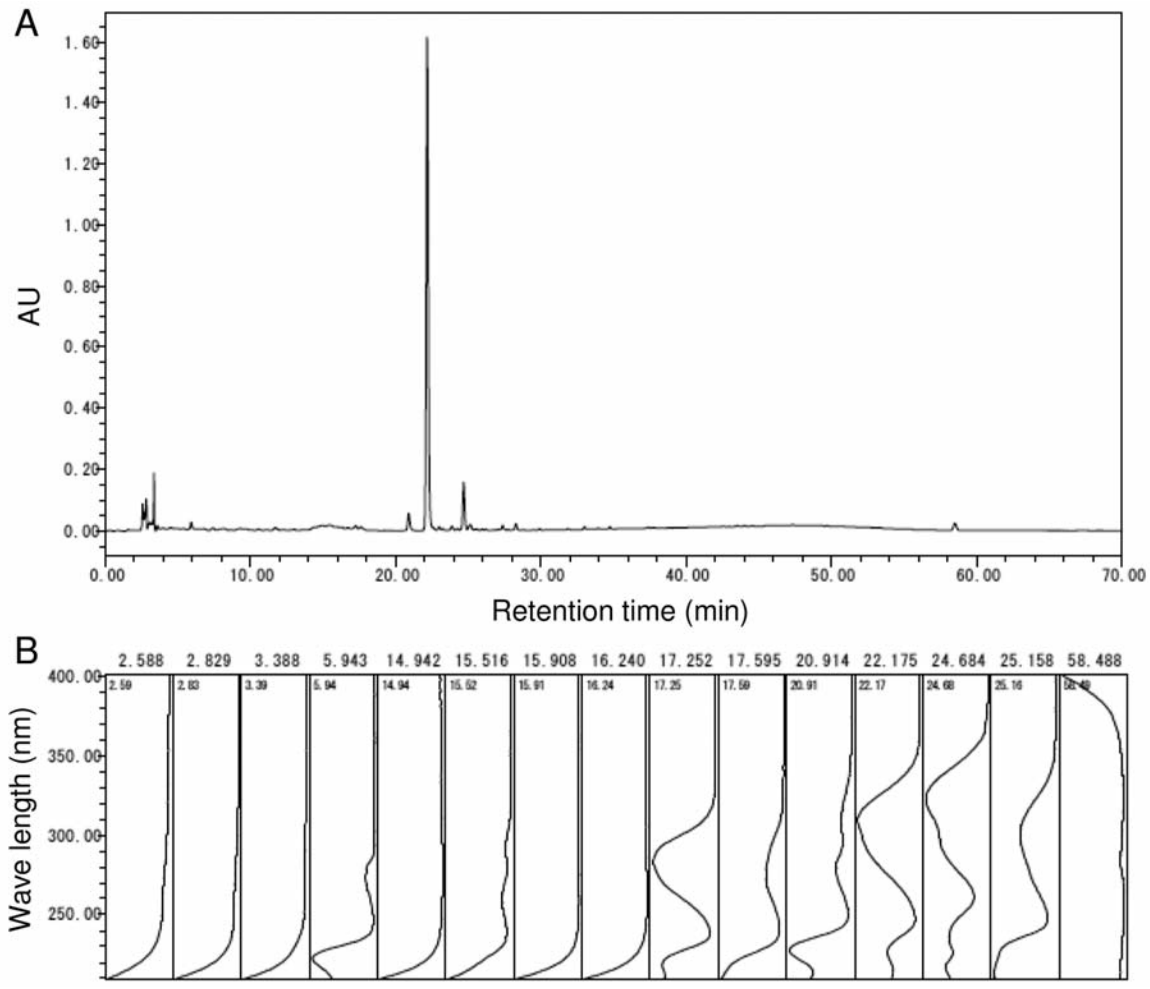


Figure 6. *HPLC analysis of crude SE extract. A: Autoscale chromatogram; B: spectral index*

subtracted from each point (Figure 2A B), it was found that SE did not induce NO production, but rather inhibited the NO production induced by LPS (100 ng/ml) in both J774.1 and RAW264.7 cells (Figure 2D, E) (SI=10.3) (Table II). Similar results were obtained when SE was autoclaved before being added to the cells (data not shown).

On the contrary, it was found that SE itself did not interfere with the TNF-α determination (Figure 2H), indicating that it is not necessary to subtract the value of SE alone. SE slightly enhanced the TNF-α production at lower concentrations, whereas it inhibited the TNF-α production at higher concentrations, due to its cytotoxicity (Figure 2F, G). Similar results were obtained when autoclaved SE was used (data not shown).

Effect of SE components on NO production. Commercially available chlorophyllin inhibited NO production by LPS-activated RAW264.7 cells (SI=25.1), whereas chlorophyll was much less active (SI=3.5) (Figure 3, Table II). When SE was extracted with various organic solvents, 90.8% (corrected for the recovery) of SE components were recovered from the aqueous layer, followed by *n*-BuOH (6.5%), AcOEt (1.2%), Et_2O (0.6%) and *n*-hexane layers (0.7%) (Table I). It was unexpected that chlorophyllin was distributed into these fractions. The majority of the activity that inhibited NO production by LPS-activated RAW264.7 cells was recovered from the water (SI=10.78) and *n*-BuOH layers (SI=11.51) (Table I). SE has approximately 20% (w/w) lignin-carbohydrate complex (Figure 1). Lignin carbohydrate complexes (Frs. I-III) inhibited the NO production comparably (SI≥5.8->11.3), whereas polysaccharide fraction (Fr. IV) was inactive (SI≥<1.0) (Table II). These data suggest that chlorophyllin in SE may be associated with hydrophilic substances.

Anti-HIV activity of lignin-carbohydrate complex in SE. SE showed potent anti-HIV activity (SI=36), comparable with

lignin carbohydrate complexes prepared without (Fr. I) (SI=32-37) or with the repeated cycle of acid precipitation and solubilisation (Frs. II, III) (SI=27-62) (Table II). On the contrary, polysaccharide fraction (Fr IV) was inactive (SI≥1). *n*-BuOH fraction (no. 0) and its seven subfractions (No 1-7), separated by silica gel column chromatography, showed essentially no anti-HIV activity, except for the most hydrophilic fraction no. 7 (SI=7) (Table II). *n*-BuOH fractions showed no tumour-specific cytotoxicity (Table III).

Effect of SE and cholorophyllin on CYP3A activity. SE, grapefruit juice and chlorophyllin dose-dependently inhibited the CYP3A activity (hydroxylation activity of 6β-position of testosterone), with 50% inhibitory concentration (IC_{50}) being 0.99% (576 μg/ml), 0.49% and 109 μg/ml, respectively (Figure 4). This indicates that CYP3A inhibitory activity of SE is less than half of that of grapefruit juice and less than 1/5 of that of chlorophyllin.

Investigationf SE in healthy volunteers. Effect of oral administration of SE on oral bacterial number was next investigated (Figure 5). In the saliva, *Porphyromonas gingivalis* was detected in volunteer C. Number of all bacteria was decreased at day 7 after SE administration, and thereafter increased up to day 28 in volunteer A. There was no apparent change in the number of bacteria in volunteer B. Number of bacteria declined until day 7, and gradually returned to the initial level thereafter.

In the tongue coat, *Porphyromonas gingivalis* was undetectable in all 3 volunteer. No apparent changes in the number of bacteria were found in volunteer A. The number of bacteria declined at day 21 in volunteer B. The number of bacteria declined at day 3, and gradually returned to the initial level thereafter in volunteer C.

In the periodontal pocket, the number of *Prevotella* and *Fusobacterium* declined, but total number of anaerobic bacteria did not change in volunteer A. The effect of SE was not apparent in volunteer B. The number of total bacteria and *Fusobacterium* declined in volunteer C.

Discussion

The present study demonstrated that SE did not inhibit the production of TNF-α by LPS-activated macrophages, while it inhibited their production of NO. This suggests the different regulation between LPS-stimulation of TNF-α production and that of NO production in macrophages.

It was found that the majority of chlorophyllin and the activity that inhibited the NO production by macrophages was recovered from the water layer. This suggests the possibility that chlorophyllin in SE may be associated with hydrophilic substances, especially lignin-carbohydrate complex in the native state or after extraction with alkaline solution, since the preparative method of SE is the same with that of lignin-carbohydrate complex (17). This was supported in this study by the observation that lignin-carbohydrate complex of SE has greenish color (absorption peak=655 nm), characteristic to chlorophyllin (absorption peak=629 nm), expected to contain 1.7-2.6% chlorophyllin in the molecule, and that 68.5% of SE eluted as a single peak at the retention time of 22.175 min in HPLC (Figure 6). Upon binding to chlorophyllin, lignin-carbohydrate complex may obtain the activity of inhibiting the NO production by activated macrophages. It was also found that among seven *n*-BuOH fractions, only no.7, that has the higher polarity, showed some, but much lower anti-HIV activity (SI=7) than lignin-carbohydrate complex (SI=27-62) (Table II). This suggests that major anti-HIV activity of SE is exerted by lignin-carbohydrate complex.

SE contains other bioactive phenolic compounds. 4',5,7-trihydroxy-3,5'-dimethoxylflavone (Tricin) from SE potently inhibited the cytopathic effect of cytomegalovirus (18). Apigenin 6-C-β-D-xylopyranosyl-8-C-β-D-glucopyranoside (19), kurilensin A and B (20) in related *Sasa* plants scavenged the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. Tricin and (–)-syringaresinol inhibited P-glycoprotein, thus enhancing the cytotoxic activity of daunomycin against human breast cancer cells (21). It is not clear to which extent these compounds are responsible for total biological activity of SE.

The current preliminary experiment has shown that oral administration of SE reduced the number of total bacteria, *Fusobacterium*, and *Prevotellain* in volunteers A and C, but not in subject B at day 7. This suggests that SE may exert some anti-bacterial activity at early stage after SE administration, although further clinical studies with more numbers of subjects are necessary to confirm this possibility.

Stomatitis is generated in oral mucosa by various stimuli, and aggravated by immunosuppression, and viral and bacterial infections. Oral submucous fibrosis (OSF) is always associated with a subepithelial inflammatory reaction followed by fibroelastic changes of the lamina propria, accompanied by epithelial atrophy (22). Currently, intralesional steroids, hyaluronidase, micronutrients, minerals, carbon dioxide laser, pentoxifylline, lycopene, immunised milk, interferon gamma, turmeric, hyalase, chymotrypsin and collagenaseare are the main treatment modalities (23). Mucositis (sore mouth) induced by chemoradiotherapy *via* toxic free radicals is one of the serious side effects of cancer therapy for oral cancer (24). Recently, rebamipid (novel anti-ulcer drug) (25), recombinant human epidermal growth factor (26) are used for the treatment of mucositis. However, natural therapies that have antiviral and antibacterial activity have not been applied to stomatitis. SE is recognised as general drug that is effective in treating stomatitis by the Ministry of Health, Labour and Welfare, Japan, and its application as a preventiative and therapeutic agent for stomatitis is therefore promising.

The present study also revealed that SE inhibited CYP3A activity to some extent. It is therefore important to monitor the side effect of accompanying drug that is metabolised by CYP3A.

SE showed broad and potent anti-viral activity and synergism with vitamin C (5). Since viral infection is one of the major risk factors of oral cavity cancer (27), anti-viral action of SE may reduce the incidence of virus-induced carcinogenesis. These unique properties of SE suggest its functionality as an alternative medicine.

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