Chapter 8

Functional Evaluation of Sasa Makino et Shibata Leaf Extract as Group III OTC Drug

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1. Introduction

Over the counter (OTC) drugs in Japan are classified into three groups (I, II and III), based on the safety [1]. Group I drugs have the highest risk of exerting the adverse effects on our health. The intensity of such side effects declines in the order of Group I, II and III. Only Group III drugs with the least side effects can be purchased through the internet.

	Safety	
Group I	+	
Group II	++	Kampo medicine, herb extracts
Group III	+++	herb extracts, SE

Table 1. Classification of OTC drugs in Japan, based on the safety.

Kampo Medicines, classified as Group II, are usually available as hot water extracts of more than two different plant species. Recently, the presentation of the detailed compositional analysis by HPLC has become mandatory for the publication of the biological activity of Kampo Medicines. However, we often experience the loss of biological activity of Kampo medicines during the purification steps, thus making it difficult to assign the active principles. Herb extracts are classified into Group II and Group III. Three major products of bam-

boo leaf extract (products A, B, C) are classified into Group III (Table 2), and other drugs are classified into Group I.

Three major products of bamboo leaf extract		
Product A (=SE)	Fe (II)-chlorophyllin	Pure *Sasa senanensis* Rehder extract
Product B	Cu (II)-chlorophyllin	LCC was removed
Product C	Cu (II)-chlorophyllin	Supplemented with ginseng and pine (*Pinus densiflora*) leaf extracts.

Table 2. Three major products of Bamboo leaf extract available in the drug store in Japan.

Two bamboos, "Take" and "Sasa" (Japanese names) belong to grasses, but are not strictly distinguished each other botanically. There are 70 genera of bamboos in the world and 14 genera (approximately 600 species) in Japan. Sasa culms are 1-2 m high, 5-8 mm in diameter, robust, ramose at lower portions. Leaf-blades are oblong-lanceolate, 20-25 cm long and 4-5 cm broad (Figure 1A, B). They are distributed into Saghalien, the Kuriles, Hokkaido, Honshu, Shikoku and Kyushu in Japan. Product A (Sasa Health®, referred to as "SE") (Figure 1C) is a pure alkaline extract of the leaves of *Sasa senanensis* Rehder (dry weight: 58.8 mg/ml [2-4]) that contains Fe (II)-chlorophyllin, in which Mg (II) is replaced by Fe (II) by adding $FeCl_2$. SE-10 (Figure 1D) is a granulated powder of SE supplemented with lactose, lactitol, trehalose and tea extract, and sold as dried and packaged powder in drug stores.

Products B (Sunchlon®, referred to as "BLE") is an alkaline extract of Sasa Makino et Shibata (dry weight 77.6 mg/ml [4]) that contains Cu (II)-chlorophyllin, but approximately 80% of lignin-carbohydrate complex (LCC) has been removed as precipitate [5].

Product C (Shojusen®, referred to as "KS) is a hot water extract of the leaves of *Sasa krilensis Makino* et Sibata (27.0 mg/ml), supplemented with ethanol extract of the leaves of *Pinus densiflora* Sieb et Zucc. (1.2 mg/ml), ethanol extract of the roots of *Panax ginseng* C.A. Meyer (0.92 mg/ml) and paraben as a preservative [6] (Table 2).

These bamboo leaf products is recognized as being effective in treating various malaises including fatigue, low appetite, halitosis, body odor and stomatitis [7-10]. However, there is no scientific evidence that demonstrates their efficacy due to the lack of appropriate biomarkers, although their *in vitro* antiseptic [11], membrane stabilizing [12], anti-inflammatory [13-16], phagocytic [17], radical scavenging [2, 4, 18, 19], anti-oxidant [20-23], antibacterial [2, 9], anti-viral [2, 4, 18, 19, 24] and antitumor activities [2, 25, 26] have been reported. SE showed several common biological properties with LCCs, that is, the prominent anti-HIV, anti-UV and synergistic activity with vitamin C [27, 28].

Lignins are major class of natural products present in the natural kingdom, and are formed through phenolic oxidative coupling processes in the plant [29]. Lignins are formed by the dehydrogenative polymerization of three monolignols: *p*-coumaryl, *p*-coniferyl and sinapyl alcohols [29]. These monolignols were produced from $_L$-phenylalanine by general phenyl-

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Figure 1. The primeval forest of *Sasa senanensis* Rehder (A), its leaves (B), SE (C) and SE-10 (D).

propanoid pathway [30]. Some polysaccharides in the cell walls of lignified plants are linked to lignin to form lignin-carbohydrate complexes (LCCs). Considering that both of SE and LCC are prepared by extraction with alkaline solution, it is not surprising that they display common biological activities with each other. Furthermore, we have recently identified the anti-UV substances of SE as *p*-coumaric acid derivative(s), one of lignin precursors [31]. Alkaline extraction step that is necessary for the preparation of SE provides higher amounts of LCC as compared with hot-water extracted Kampo medicines. One or two-order higher anti-HIV activity of both SE and LCC over tannins and flavonoids suggest their possible applicability towards virally-induced diseases.

However, there is a possibility that the components from SE and other plants are associated with each other, thus modify their biological activities. Also, SE components may inhibit the activity of CYP3A4, the most abundant drug-metabolizing enzyme, so as to increase the bioavailability of co-administered drugs (especially, CYP3A4 substrates). Lastly, the clinical evidences that demonstrate how the treatment of SE products improves the patient's conditions are limited.

Based on these circumstances, we review the functional analysis of SE products as alternative medicines, citing the literatures of other groups and ours, focusing on the following points: (i) component analysis, (ii) spectrum of reported biological activities in comparison with those of Kampo medicines, (iii) possibility of complex formation between the compo-

nents, (iv) inhibition of CYP3A4 activity and (v) the clinical application for the treatment of oral diseases.

2. Component analysis

Components of SE are listed in Table 3. Dietary fibre was the major component of SE. Water-soluble and water-insoluble dietary fibres are present approximately at the 1: 2 ratio.

	mg/100 ml	mg/100 g*		mg/100 ml	mg/100 g*
Protein	1500	22700	Glycine		
Lipid	200	3030	Proline	84	1270
Ash content	900	13600	Glutamic acid	186	2800
Sugar	1200	18200	Serine	21	320
Glucose	90	1360	Threonine	13	200
Arabinose	380	5700	Aspartic acid	159	2400
Xylose	1060	16000	Tryptophan	28	420
Galactose	180	2700			
Dietary fibre	2100	31800	Folic acid	0.008	0.12
Water-soluble	1400	21200	Lutein	0.3	4.5
Water-insoluble	700	10600			
			Sodium	395	5980
Arginine	19	290	Iron	1.02	15
Lysine	59	890	Calcium	1.0	15
Histidine	23	350	Potassium	4.9	74
Phenylalanine	86	1300	Magnesium	0.5	8
Tyrosine	63	950	Zinc	0.08	1.2
Leucine	135	2040			
Isoleucine	53	800	Vitamin A	0.003	0.05
Methionine	32	480	β-Carotene	0.032	0.5
Valine	95	1440	Vitamin K1	0.006	0.09
Alanine	105	1590	Glycine	99	1500

Table 3. Composition of SE. *corrected, assuming that 1 ml contains 66.1 mg SE. Cited from [19], with permission.

According to this information, we have fractionated the LCC into the following three fractions Fr I, II and III by repeated acid precipitation and solubization with $NaHCO_3$ or NaOH

solution, and polysaccharide fraction was recovered as Fr. IV by addition of equal volume of ethanol in Figure 2.

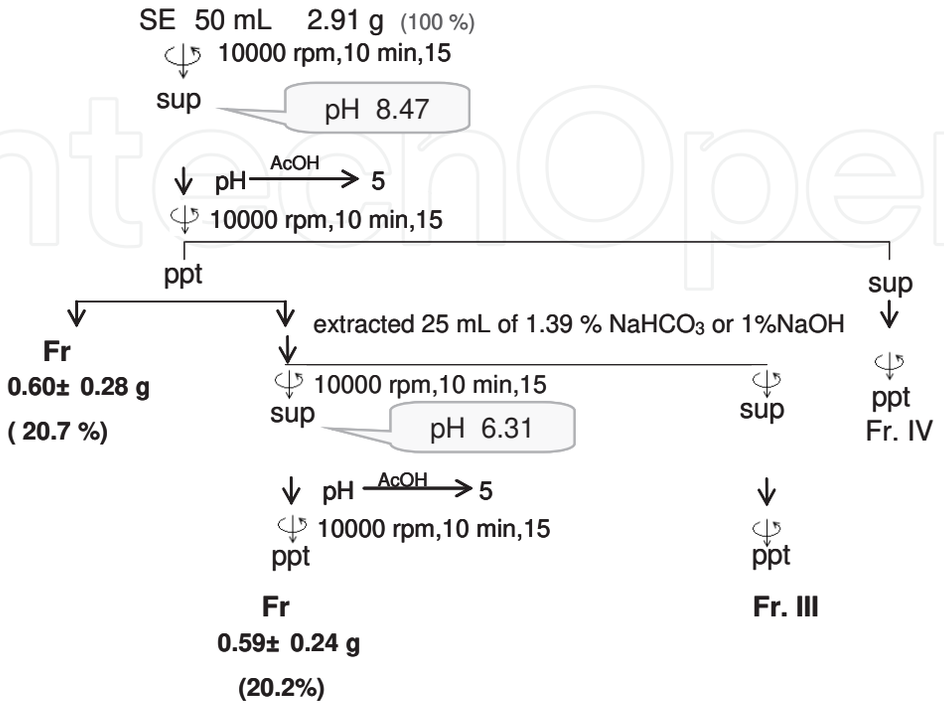

Figure 2. Fractionation of lignin-carbohydrate complex (LCC) fractions Fr I-III and polysaccharide fraction Fr IV. *Yield of Frs. I and II represents mean±S.D. from three independent experiments*. Cited from [18], with permission

Luteolin glycosdes are isolated from the leaves of *Sasa senanensis* Rehder and their structures were identified as decribed below (Figure 3) [32]. Luteolin 6-*C*-β-$_D$-glucoside [compound 1]: yellow amorphous powder, $[\alpha]^{25}_D$ +30.7° (*c*=0.12, CH_3OH), mp 232° (dec.), ultraviolet (UV) λmax (MeOH) nm (ε): 348 (22,200), 270 (17,600) and 258 (17,400). Electrospray ionization time of flight mass spectra (ESI-TOF-MS) *m*/*z*: 448 ([M+H]$^+$), high-resolution mass spectra (HR-MS) *m*/*z*: 449.1094 (calcd. for $C_{21}H_{21}O_{11}$, 449.1084).

Luteolin 7-*O*-β-$_D$-glucoside [compound 2]:yellow amorphous powder, $[\alpha]^{25}_D$ -81.1° (*c*=0.10, CH_3OH), mp 261° (dec.), UV λmax (MeOH) nm (ε): 346 (20,500) and 270 (18,400). ESI-TOF-MS *m*/*z*: 448 ([M+H]$^+$), 287 ([aglycon+H]$^+$), HR-MS *m*/*z*: 449.0976 (calcd. for $C_{21}H_{21}O_{11}$, 449.1084).

Luteolin 6-*C*-α-L-arabinoside [compound 3]:yellow amorphous powder, $[\alpha]^{25}_D$ +66.0° (*c*=0.11, CH_3OH), mp > 300° (dec.), UV λmax (MeOH) nm (ε): 348 (22,100), 270 (17,600) and 258 (17,400). ESI-TOF-MS *m*/*z*: 419 ([M+H]$^+$), HR-MS *m*/*z*: 419.1027 (calcd. for $C_{20}H_{19}O_{10}$, 419.0978).

Tricin [compound 4]: yellow amorphous powder, UVλmax (MeOH) nm (ε): 349 (41,000) and 269 (27,200). ESI-TOF-MS *m*/*z*: 331 ([M+H]$^+$): HR-MS *m*/*z*: 331.0837 (Calcd. for $C_{17}H_{15}O_7$, 331.0818).

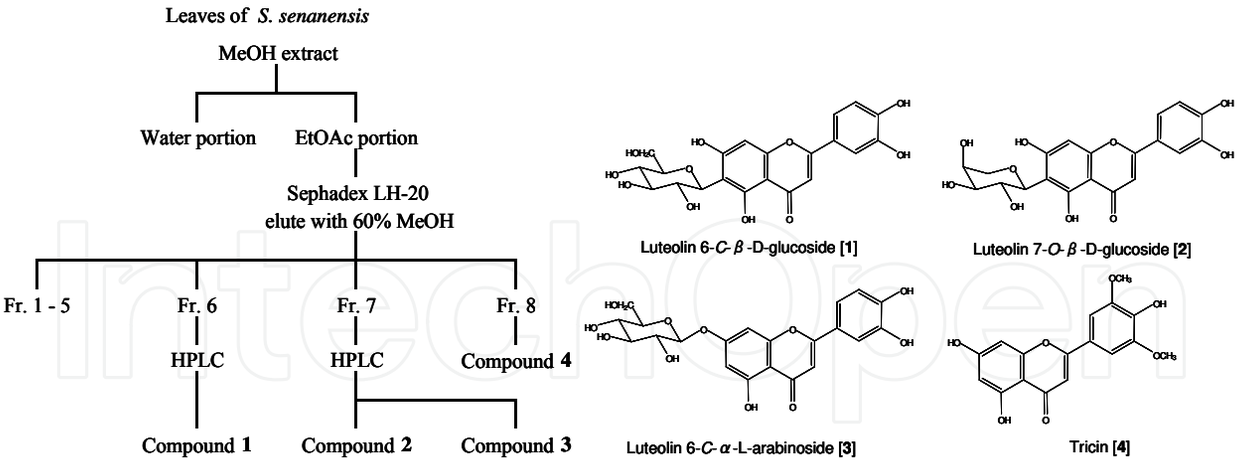

Figure 3. Purification of luteolin 6-*C*-β-$_D$-glucoside [compound 1], luteolin 7-*O*-β-D-glucoside [compound 2], luteolin 6-*C*-α-$_L$-arabinoside [compound 3] and tricin [compound 4] from the leaves of *Sasa senanensis* Rehder. Cited from [32], with permission.

We also isolated substances (SEE-1) that protected the cells from the UV-induced cytotoxicity, by ethanol extraction, Wakosil 40C18 chromatography (H_2O elution) and preparative HPLC (Shimadzu LC-10AD pump, Shimadzu SPD-M10AVP photodiode array detector, separation column: Inatsil ODS-3, eluted with H_2O : acetonitrile : formic acid (90:10:0.1), and proposed the putative structures as *p*-coumaric acid derivative(s) (Figure 4) [31].

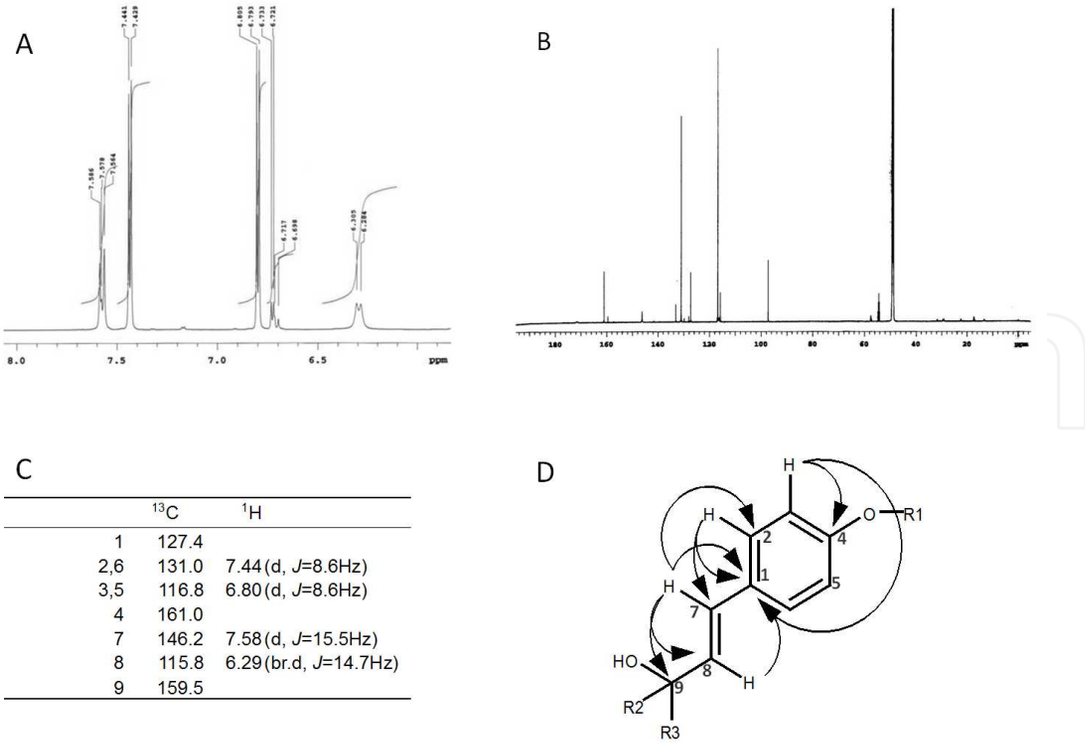

Figure 4. Identification of anti-UV substance(s) as *p*-coumaric acid derivative(s). Cited from [31] with permission.

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3. Biological activities

3.1. Antiviral activity

Anti-human immunodeficiency virus (HIV) activity was assessed quantitatively by a selectivity index ($SI=CC_{50}/EC_{50}$, where CC_{50} is the 50% cytotoxic concentration against mock-infected MT-4 cells, and EC_{50} is the 50% effective concentration against HIV-infected cells). Products A, B and C all effectively and dose-dependently reduced the cytopathic effect of HIV infection (closed symbols in Figure 5), although their anti-HIV activity was much lower than that of positive controls [dextran sulfate (SI=1378), curdlan sulfate (SI=5606), azidothymidine (SI=17746), 2′,3′-dideoxycytidine (SI=5123)] (Table 4). The potency of anti-HIV activity was in the order of product A (Sasa-Health®, SE) (SI=607) > product C (SI=117) > product B (SI=111) (Exp. I, Table 4) [4]. A granulated powder of *Sasa senanensis* Rehder leaf extract (SE-10) (Figure 1D) (SI=54) showed slightly higher anti-HIV activity than SE (SI=45) (Exp. 2, Table 4) [19]. Among the components of SE, LCC fractions prepared as described in Figure 3 (SI=37~62) showed comparable or slightly higher activity anti-HIV activity than unfractionated SE (SI=36) (Exp. 3, Table 4) [28]. Luteolin glycosides, luteolin 6-*C*-β-D-glucoside, luteolin 7-*O*-β-D-glucoside, luteolin 6-*C*-α-$_L$-arabinoside and tricin from *Sasa senanensis* Rehder leaf extract showed somewhat lower anti-HIV activity (SI=2~24) (Exp. 4, Table 4) [32]. The anti-HIV activity of LCCs isolated from SE was comparable with that of LCCs from pine cone, catuaba bark [33], cacao husk [34], cacao mass [35], cultured extract of *Lentinus edodes* mycelia extract [36] and mulberry juice [37, 38], and synthetic lignin (dehydrogenation polymers of phenylpropanoids) [39], and was generally higher than that of tannins [40], flavonoids [41], gallic acid, (-)-epigallocatechin 3-*O*-gallate (EGCG), curcumin, and chemically modified glucans [42] (Exp. 5, Table 4) and Kampo medicines and its constituent plant extracts [43] (Exp. 6, Table 4).

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 6). Tricin showed potent anti-human cytomegalovirus activity [24].

3.2. Anti-bacterial activity

Product B (BLE) significantly reduced the bacterial growth and lactate production *in vitro* in the total saliva [9].

Product A (SE) showed a bacteriostatic, but not a bactericidal effect on *Fusobacterium nucleatum* and *Prevotella intermedia* (Figure 7A, 7B). The MIC_{50} for the *Fusobacterium nucleatum* and *Prevotella 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 $(CH_3)_2H$. SE more efficiently reduced the production of H_2S in *Fusobacterium nucleatum*, with a 50% inhibitory concentration (IC_{50}) of 0.04% (Figure 7C). On the other hand, SE more efficiently reduced the production of CH_3SH in *Prevotella intermedia*, with an IC_{50} of 0.16% (Figure 7D). A higher concentration of SE (2.5%) completely eliminated both H_2S and CH_3SH [2].

	S I		**SI**
Exp. 1 (Alkaline extract)		**Exp. 5 (other plant extracts)**	
Product A (SE)	607	LCC from pine trees (n=2)	27
Product B	111	LCC from pine seed shell	12
Product C	117	LCC from catuaba bark	43
Dextran sulfate	1378	LCC from cacao husk	311
Curdlan sulfate	5606	LCC from cacao mass	46
AZT	17746	LCC from cultured LEM	94
ddC	5123	LCC from mulberry juice	7
Exp. 2 (SE product)		Phenylpropenoid polymers (n=23)	105
SE	45		
SE-10	54	Neutral polysaccharide from pine cone	1
Dextran sulfate	160	*N*,*N*-dimethylaminoethyl paramylon	<1
Curdlan sulfate	781	*N*,*N*-diethylaminoethyl paramylon	<1
AZT	6931	*N*,*N*-dimethylaminoethyl curdlan	<1
ddC	905	Hydrolyzable tannins monomer (n=21)	<1
		Hydrolyzable tannins dimer (n=39)	<1
Exp. 3 (SE component)		Hydrolyzable tannins trimer (n=4)	3
SE	36	Hydrolyzable tannins tetramer (n=3)	11
LCC Fr I (acid precipitation)	37	Condensed tannins (n=8)	<1
LCC Fr II (acid precipitation ×2))	58		
LCC Fr III (acid precipitation × 2)	62	Flavonoids (n=160)	<1
Polysaccharide fraction Fr IV	><1	Gallic acid	<1
Butanol extract	<1	(-)-Epigallocatechin 3-*O*-gallate	<1
		Curcumin	<1
Exp. 4 (SE component)		Chlorophyllin	5
SE	40		
Luteolin 6-*C*-β-D-glucoside [**1**]	>2	**Exp. 6 (Plant extracts)**	
Luteolin 7-*O*-β-D-glucoside [**2**]	7	Kampo medicines (n=10)	<1.0
Luteolin 6-*C*-α-L-arabinoside [**3**]	>7	Constituent plant extracts (n=25)	1.3
Tricin [**4**]	24	AZT	17850

Table 4. Anti-HIV activity of polyphenols.

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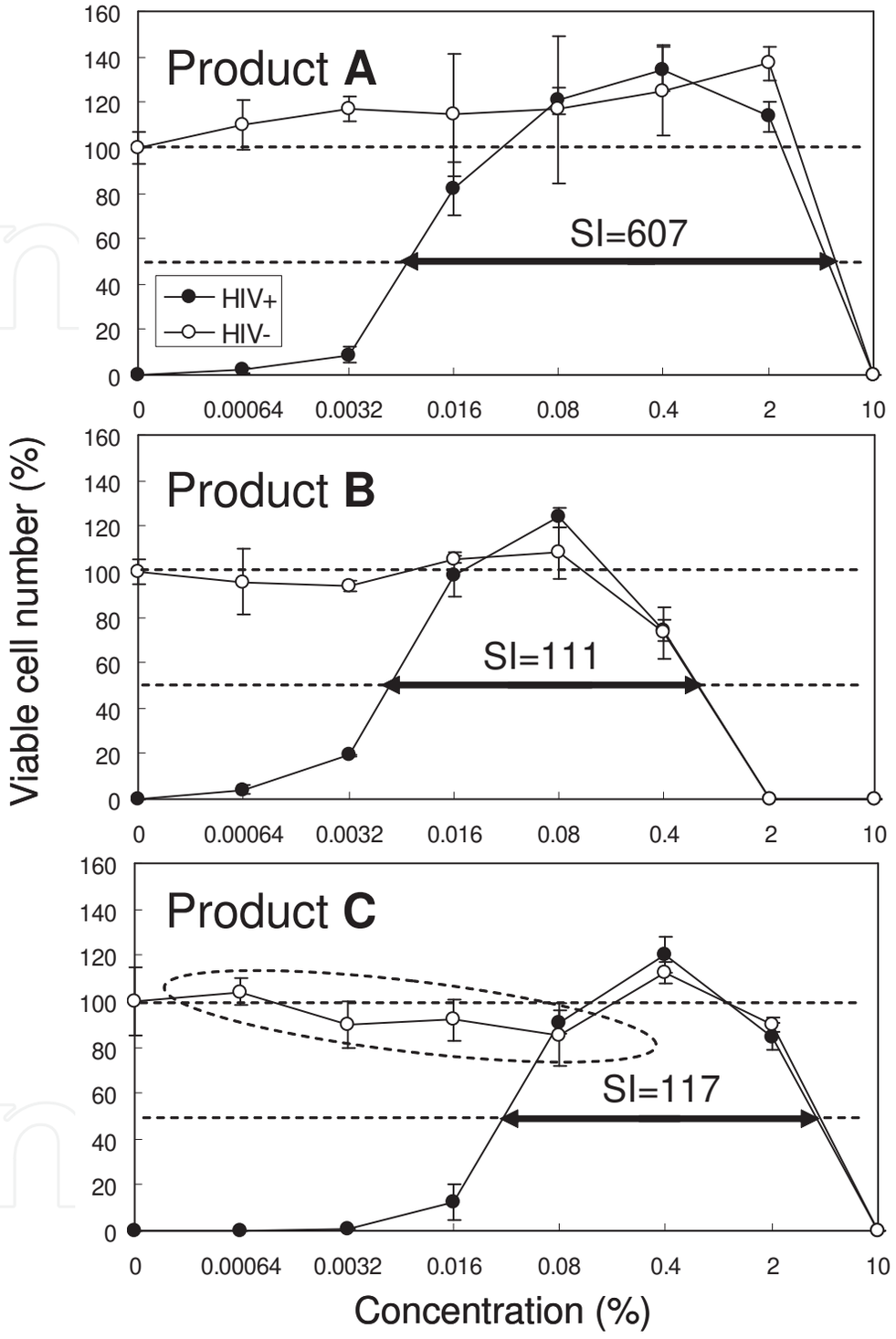

Figure 5. Figure 1. Anti-HIV activity of three commercial products of *Sasa senanensis* Rehder extract. HIV-1_{IIIB}-infected (HIV+) and mock-infected (HIV–) MT-4 cells were incubated for 5 days with the indicated concentrations of products A (upper panel), B (center panel) and C (lower panel) and the 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). Cited from [4], with permission.

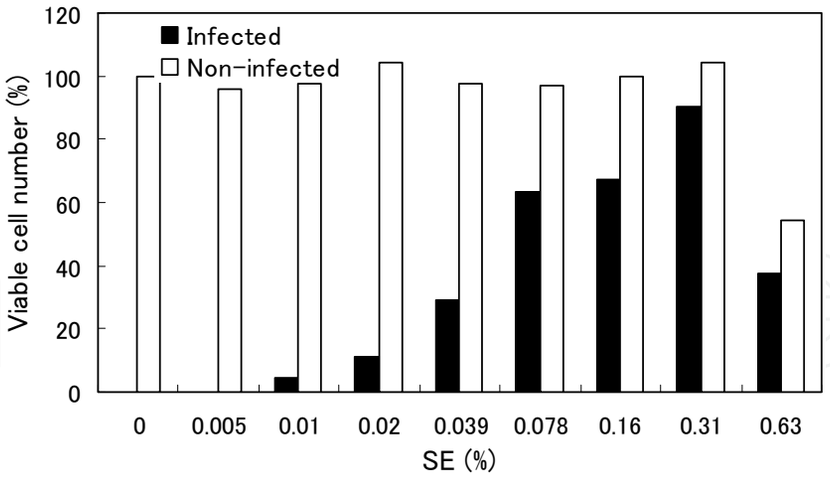

Figure 6. Anti-influenza virus activity of SE. Influenza virus-infected or mock-infected MDCK cells were incubated for 3 days with the indicated concentrations of SE, and the viable cell number was determined by MTT method. Each value represents a mean from triplicate assays. Cited from [2] with permission.

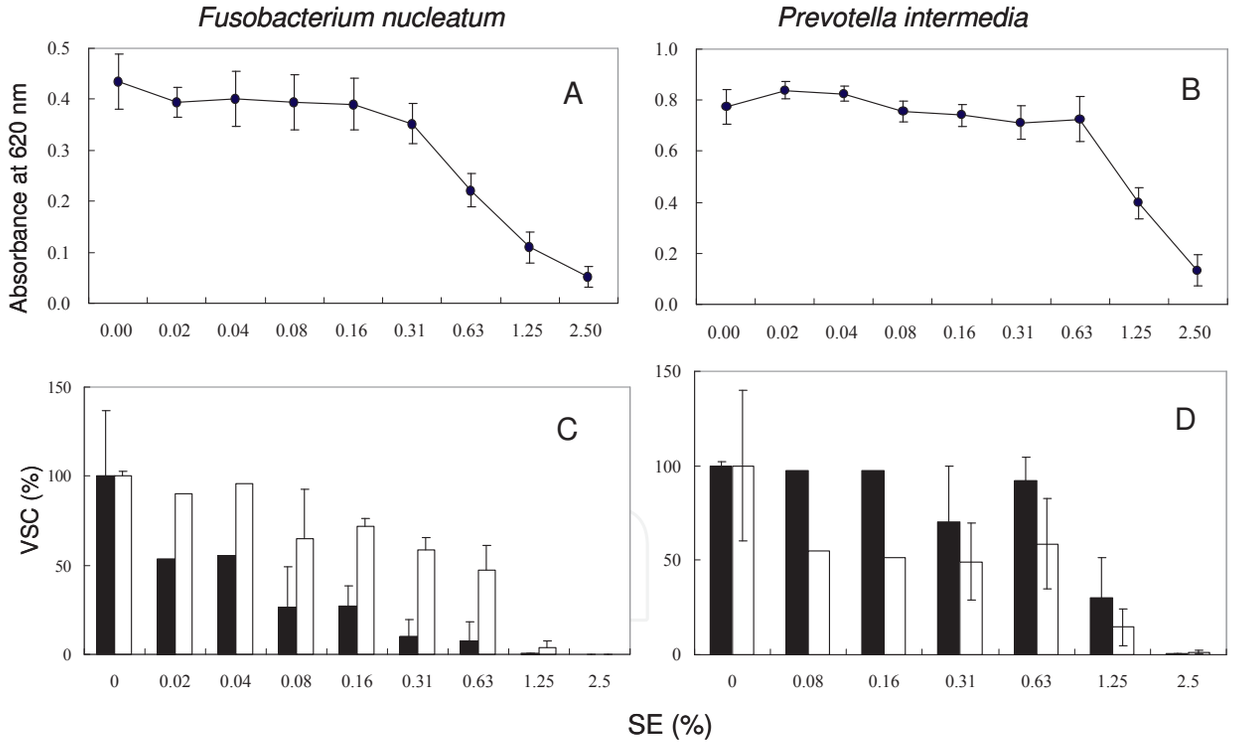

Figure 7. Antibacterial activity of SE. *Fusobacterium nucleatum* (A, C) and *Prevotella intermedia* (B, D) were cultured anaerobically for 24 hours at 37°C with the indicated concentrations of SE in capped 15-cm centrifugation tubes. The VSC released into the culture medium (black bar: H_2S, white bar, 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. Each bar represents mean ±S.D. from triplicate assays. Without bar (0.02 and 0.04%SE (C), and 0.08 and 0.16% SE (D)) means the value from a single assay. Cited from [2] with permission.

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3.3. Antitumor activity

Oral administration of SE (*ad lib.*) significantly delayed the development and growth of mammary tumors in a mammary tumor strain of virgin SHN mice [25]. Oral administration of SE (*ad lib.*) significantly inhibited spontaneous mammary tumorigenesis, reduced tumor multiplicity, inhibited the mammary duct branching, side bud development and angiogenesis in another mouse model of human breast cancer, transgenic FVB-Her2/NeuN mouse model [26].

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 [gingival fibroblast (HGF), pulp cell (HPC), periodontal ligament fibroblast (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 5). 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 [2].

3.4. Membrane stabilizing activity

In order to investigate whether SE contains membrane -stabilizing activity, SE was defatted with hexane, and fractionated on Silica gel chromatography, according to the polarity, into Fr. 1 (eluted with *n*-hexane: CH_2Cl_2), Fr. 2 (CH_2Cl_2), Fr. 3 (acetone), Frs 4 and 5 (methanol) and Fr. 6 (residue). SE inhibited the hemolysis of rat red blood cells in hypotinic buffer by 13%. Frs. 3, 4 and 5 ihibited the homolysis approximately 35, 20 and 35%, respectively [12], suggesting the membrane-stabilization activity of SE and its fractions.

Membrane stability can be evaluated by the extracellular leakage of glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transamiase (GPT) from the hepatocytes. Control hepatocytes released 85.1± 5.4 (mean±SD) K.U./ml GOT into the culture medium. SE (1~5 μi=60~300 μg/ml) significantly inhibited the release of GOT. Among the SE fractions, Frs. 1, 4 and 6 showed the inhibitory effects (Figure 8A). Control hepatocytes released 37.0 ± 3.6K.U./ml GPT inoto the culture medium. SE (1~2 μl=60, 120 μg/ml) significantly inhibited the GTP release (Figure 8B) [13].

SE, Fr. 3 and Fr. 5 showed the surfactant action by reducing the surface tension. These substances did not significantly affect the phase-transtion temperature of dipalmitoyl phosphatidylcholine (DPPC)-liposome bilayer nor the membrane-fluidity. These data suggest that the membrane-stabilizing activity of SE may be generated by polysaccharide, lignin, or chlorophyll present in Fr. 3, 4 and 5.

	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	

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) + CC_{50} (KG-1) + CC_{50} (MT-4)] x (11/3). Cited from [2] with permission.

Table 5. Cytotoxic activity of SE against human normal and tumor cells.

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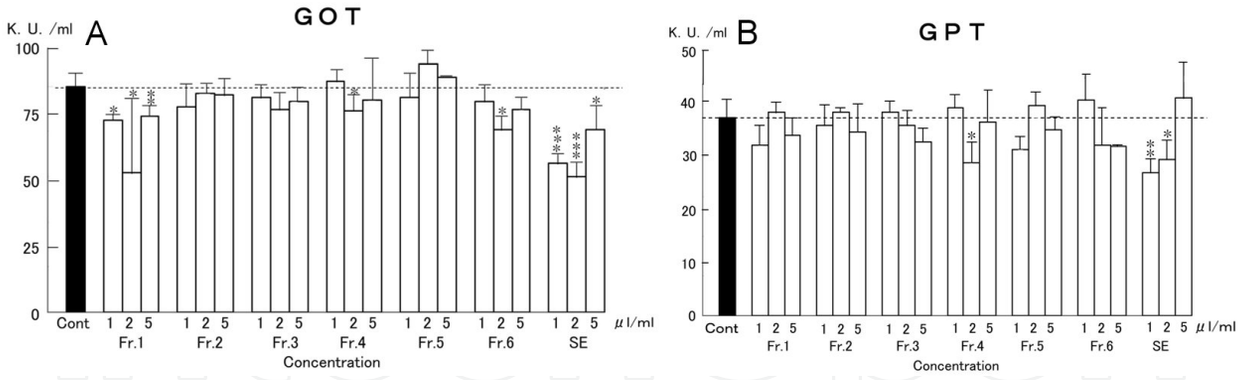

Figure 8. Effect of SE and each fraction on the leakage of GOT (A) and GPT (B) from rat cultured hepatocytes. Hepatocytes were suspended in Williams' E medium for 24 h at 37ºC and culture was continued in the same medium with SE or each fraction for 24 h at 37ºC. Each value was mean±S.D. of five experiments, and expressed as Karmen Units/ml of medium. Values are significant: $*p<0.05$, $**p<0.01$, $***p<0.001$; control *vs* experiments (students *t*-test). Cited from [13] with permission.

We next compared the hepatocyte protective effect of SE, other Herbal extracts and tinctures (Aloe, Gambir, Swertiae, Plantaginis, Geranii, Houttuyniae extracts). Aloe extract rather enhanced the leakage of liver enzymes, whereas SE and Gambir extract were inhibitory. SE more significantly inhibited the enzyme leakage, as compared with other herbal extracts and tinctures, suggesting that the hepatocyte protective activity of SE may be more potent that other herbal extracts [13].

3.5. Anti-inflammatory activity

Oral administration of hot water extract of leaves of bamboo of genus *Sasa* spp (HSBE) inhibited the carrageenan-induced edema and 12-*O*-tetradecanoylphorbol-13-acetate-induced ear swelling in mice, possibly by inhibiting the production of proinflammatory substances [prostaglandin E_2 (PGE_2), serotonin) and expression of 5-lipoxygenase, cycooxygenase-2 (COX-2), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and IL-10. Although the anti-inflammatory activity of HSBE was much less than that of dexamethasone, the major activity was concentrated into lower molecular weight, dialyzable and methanol eluted fraction [16].

Single oral administration of SE (10~20 ml =0.6~1.2 mg/kg) slightly reduced the vasopermeability in ddY male mice (assessed by Whittle method). Single oral administration of SE (5 ml=0.3 mg/kg) slightly inhibited the formation of carrageenin-induced edema in SD male rats at 1 h, but rather enhanced the formation of edema at 3 h and thereafter (Figure 9A). Single oral administration of SE (5 ml=0.3 mg/kg) inhibited the formation of formalin-induced edema at 3 h (Figure 9B). Repeated oral administration of SE (1, 5, 10 ml/kg/day×7 or 9days) stimulated the growth of fibroblasts and neovascularization, in contrast to the enhanced formation of collagen fiber. This suggests that SE may stimulate the regeneration of normal tissue during the restoration process of inflammatory tissues [12].

Oral administration of SE slightly increased the phagocytic index (assessed by carbon clearance method) after 3~5 h, but did not affect the phagocytic index at 7 days, suggesting that SE does not reduce the function of reticuloendothelial system.

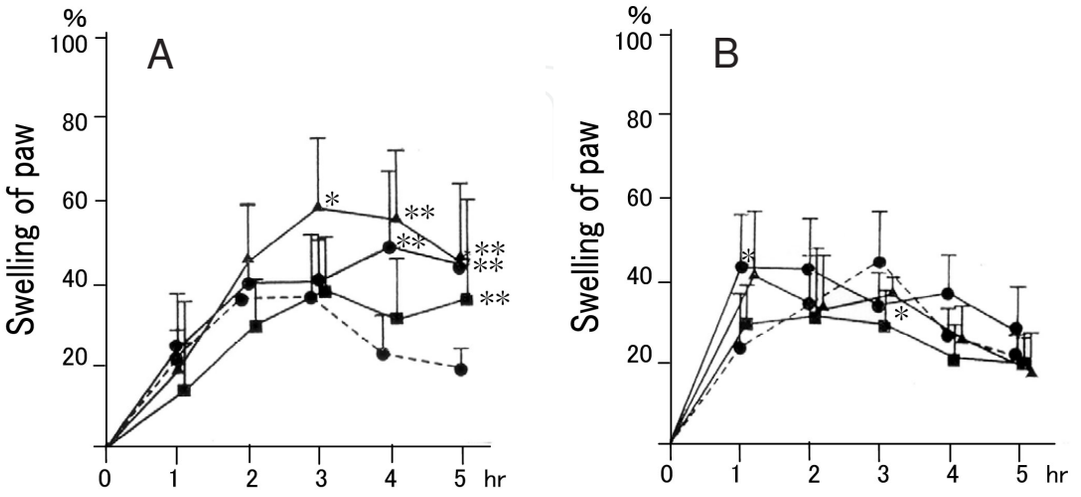

Figure 9. Effect of SE on carrageenin (A) and formalin (B)-induced hind paw edema in rats.Each point represents the mean of eight rats with S.D. ●---● : control, ●———● : SE 1ml/kg, ■———■ : SE 5 ml/kg,▲———▲ : SE 10 ml/kg,. ＊,＊＊ : significantly different from the control value with $p<0.05$, $p<0.01$ (Student's t-test). Cited from [12] with permission.

SE also inhibited the production of nitric oxide (NO) and prostaglandin E_2 (PGE_2) from the LPS-activated mouse macrophage-like cells RAW264.7 *via* inhibition of the expression of iNOS and COX-2 at protein and mRNA levels [14].

IL-1β induced one to two order higher production of proinflammatory substances (PGE_2, IL-6, IL-8, MCP-1), but not NO and TNFα by human gingival fibroblast (HGF). SE also inhibited the production of IL-8 production by IL-1β-stimulated human gingival fibroblast (Figure 10)[15].

3.6. Radical scavenging activity

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 [28], appeared (RI=0.14) [2].

Products A, B and C dose-dependently reduced the intensity of superoxide anion (O_2^-) (detected as DMPO-OOH) generated by hypoxanthine and xanthine oxidase reaction. The potency of O_2^- scavenging activity of the three products was comparable: product A (IC_{50}=0.46 mg/ml), product B (IC_{50}=0.52 mg/ml) and product C (IC_{50}=0.54 mg/ml) (Table 6) [4].

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

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was comparable with each other (IC_{50}=2.1 and 1.9 mg/ml, respectively), but 4-fold higher than that of product B (IC_{50}=8.0 mg/ml) (Table 6) [4].

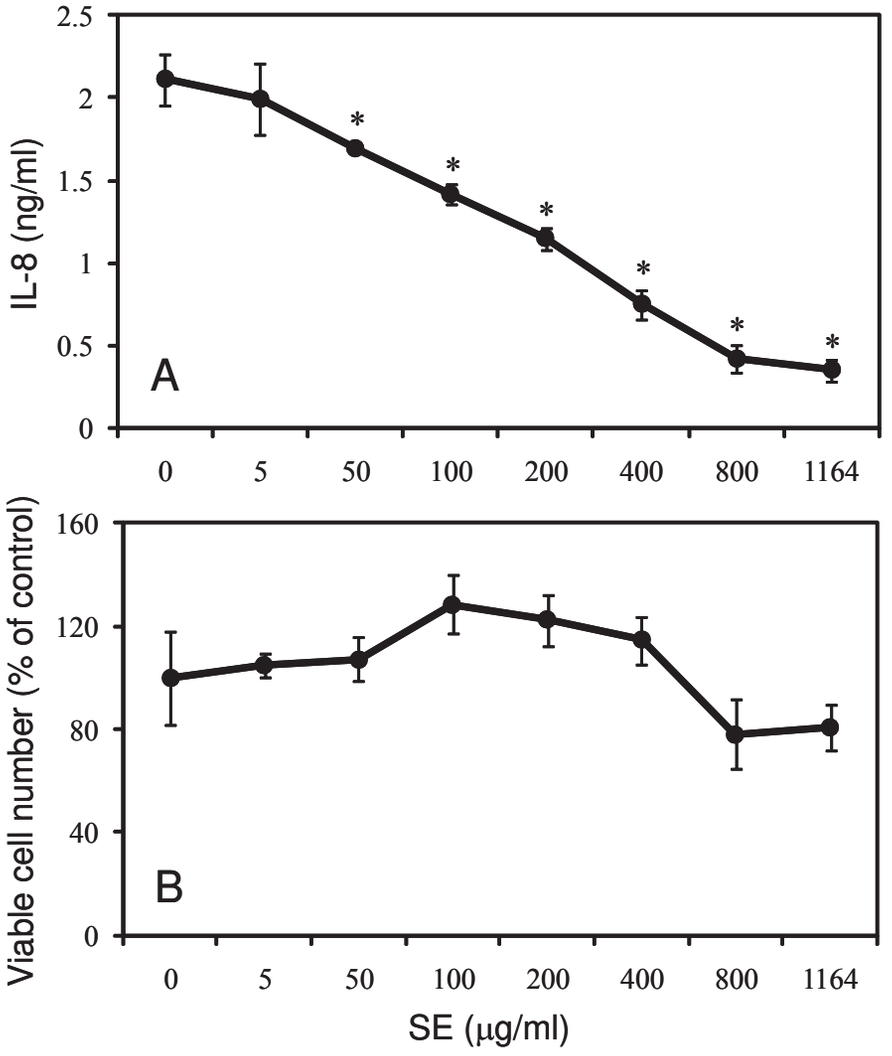

Figure 10. SE inhibited the IL-1β-stimulated IL-8 production by HGF cells. HGF cells were incubated for 48 h with the indicated concentrations of SE in the presence of IL-1β (1 ng/ml), and the extracellular IL-8 concentration (A) and viable cell number (B) were determined. mean± S.D (n=3). *<0.01. Cited from [15] with permission.

	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)

Table 6. Radical scavenging activity of three commercial products of *Sasa senanensis* Rehder extract. Data was cited from [2], with permission.

3.7. Anti-UV activity

UV irradiation (6 J/m^2/min) for 1 min followed by 48 h culture resulted in extensive cell death (closed circles in Figure 11). Popular antioxidants, *N*-acetyl-L-cysteine (NAC) and catalase (enzyme that degrades hydrogen peroxide), could not prevent the UV-induced cellular damage, suggesting that hydrogen peroxide may not be involved in the UV-induced cytotoxicity, but the type of radical species produced by UV irradiation remains to be identified (Exp. 1, Table 7). SE dose-dependently inhibited the UV-induced cytotoxicity in a bell-shaped fashion (Figure 11). The viability of the cells was recovered to 50% by the addition of 0.53 mg/ml SE (=EC_{50}). From the dose-response curve without UV irradiation, CC_{50} of SE was calculated to be 22.24 mg/ml. From these values, selectivity index SI (CC_{50}/EC_{50}) was calculated to be 41.96. Similar experiments were repeated three times to yield the mean value of SI=19.7±15.1 (mean of four independent experiments) (Exp. I, Table 7). The ant-UV activity of SE was slightly less than that of sodium ascorbate (SI=30.2±13.4) (mean of five independent experiments), but higher than that of luteolin 6-*C*-β-$_D$-glucoside [1] (SI>8), luteolin 7-*O*-β-$_D$-glucoside [2] (SI>6), luteolin 6-*C*-α-$_L$-arabinoside [3] (SI>6), Tricin [4] (TS>3), gallic acid (SI=17.1), EGCG (SI=7.7), chlorophyllin (SI=0.53) and chlorophyll a (SI<0.24) (Exp. I, Table 7) [3].

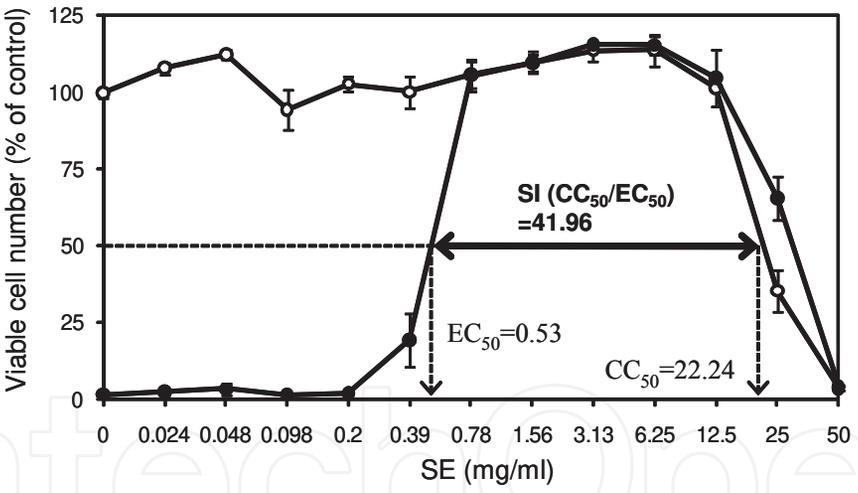

Figure 11. Anti-UV activity of SE. HSC-2 cells were treated without (○) or with UV (●) irradiation (6 J/m^2/min) for 1 min in PBS(–) containing SE. Viable cell number determined by MTT method 48 h after irradiation, and expressed as percent of control (without UV irradiation). EC_{50}: 50% effective concentration, CC_{50}: 50% cytotoxic concentration.Mean ± S.D. of triplicate determinations. Cited from [3] with permission.

SE (product A) showed higher anti-UV activity (SI=20) than other *Sasa senanensis* Rehder leaf products B (SI=4) (that has lower amounts of LCC) and C (SI=13) (that contains ginseng extract and pine (*Pinus densiflora*) leaf extract) [4], suggesting the importance of LCC for the anti-UV activity. A granulated powder of *Sasa senanensis* Rehder leaf extract (SE-10) (SI=129) showed approximately three-fold higher anti-UV activity than SE (Exp. 2, Table 7)[19], suggesting some synergistic effect of SE and other components present in SE-10.

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LCCs from pine cones, pine seed and cultured LEM (SI=26-42) showed comparable anti-UV activity with SE (SI=39). Lignin precursor, vanillin, showed higher anti-UV activity comparable with that of sodium ascorbate (SI=64) (Exp. 3, Table 7) [44, 45]. On the other hand, chemically-modified glucans, such as *N*,*N*-dimethylaminoethyllaminarin, *N*,*N*-dimethylaminoethylpullulan, *N*,*N*-dimethylaminoethyldextran and paramylon sulfate (SI<1) [45] (Exp. 4, Table 7), hot water extract (Kampo medicines and constituent plant extracts) [43] (SI=1~2) (Exp. 5, Table 7) and tea extracts (green tea, black tea, oolong tea, burley tea, Jasmine tea) [44] (Exp. 6, Table 7) were also inactive (SI=1~2). These data suggests the alkaline extracts (such as SE and LCCs) show higher anti-UV activity than hot-water extracts (such as Kampo medicines, tea extracts).

	S I		**SI**
Exp. 1		Exp. 3 (LCCs)	
SE	20	LCC from pine cones (n=3)	33
Luteolin 6-*C*-β-$_D$-glucoside [**1**]	>8	LCC from pine seed	26
Luteolin 7-*O*-β-$_D$-glucoside [**2**]	>6	LCC from cultured LEM	42
Luteolin 6-*C*-α-$_L$-arabinoside [**3**]	>6	SE	39
Tricin [**4**]	>3	Vanilline	64
Chlorophyllin	<1	Sulfated lignin (n=2)	>8
Chlorophyl a	<1	Sodium acorbate	64
Sodium ascorbate	30	Exp. 4 (polysaccharides)	
Gallic acid	17	*N*,*N*-Dimethylaminoethyllaminarin	<1
EGCG	8	*N*,*N*-Dimethylaminoethylpullulan	<1
Curcumin	<1	*N*,*N*-Dimethylaminoethyldextran	<1
Ar-turmerone	<1	Paramylon sulfate	<1
		Sodium ascorbate	89
N-Acetyl-L-cysteine	<1		
Catalase	<1	Exp. 5 (Plant extracts)	
		Kampo medicines (n=10)	2
Exp. 2 (SE products)		Constituent plant extracts (n=25)	1
Product A (SE)	20		
Product B (BLE)	4	Exp. 6 (Tea extract)	
Product C (KS)	13	Green tea	3
Sodium acorbate	33	Black tea	<1
		Oolong tea	<1
SE	39	Burley tea	<1
SE-10	129	Jasmine tea	<1
Sodium ascorbate	90	Sodium ascorbate	30

Table 7. Anti-UV activity of various natural products.

3.8. Synergistic action with vitamin C

Vitamin C exhibited either antioxidant or prooxidant activity, depending on the concentration [46]. We have reported that ascorbate derivatives that produced the doublet signal of ascorbate radical (sodium-$_L$-ascorbate, $_L$-ascorbic acid, $_D$-isoascorbic acid, 6-β-$_D$-galactosyl-$_L$-ascorbate, sodium 5,6-benzylidene-$_L$-ascorbate) induced apoptosis (characterized by internucleosomal DNA fragmentation and an increase in the intracellular Ca^{2+} concentration) in HL-60 cells. On the other hand, ascorbate derivatives that did not produce radicals ($_L$-ascorbic acid-2-phosphate magnesium salt, $_L$-ascorbic acid 2-sulfate and dehydroascorbic acid) did not induce apoptosis [47, 48]. This suggests the possible involvement of the ascorbate radical in apoptosis-induction by ascorbic acid-related compounds.

We accidentally found that LCCs from the pine cone of *Pinus parviflola* Sieb et Zucc, pine cone of *Pinus elliottii* var. Elliotti, leaf of *Ceriops decandra* (Griff.) Ding Hou and, thorn apple of *Crataegu Cuneata* Sieb. et Zucc modulated the radical intensity of ascorbate bi-phasically, depending on the concentrations. At higher concentration, LCCs strongly enhanced the radical intensity of sodium ascorbate, which rapidly decayed, possibly due to the breakdown of ascorbic acid or to the consumption of ascorbyl radical. LCCs, not only from pine cones (Fr. VI), but also from Catuaba bark, pine seed shell, *A. nikoense* Maxim. and *C. Cuneata Sieb*. et Zucc. enhanced the radical intensity and cytotoxic activity of sodium ascorbate [27]. On the other hand, tannins such as gallic acid, EGCG, and tannic acid counteracted the radical intensity and cytotoxic activity of sodium ascorbate [49].

Sodium ascorbate rapidly reduced the oxygen concentration in the culture medium, possibly due to oxygen consumption *via* its pro-oxidation action. Simultaneous addition of LCCs further enhanced the ascorbate-stimulated consumption of oxygen [50]. These data suggest that the synergistic enhancement of the cytotoxic activity of LCCs and ascorbate might be due at least in part to the stimulated induction of hypoxia.

Lower concentration of LCC (pine cone Fr. VI) and sodium ascorbate showed radical scavenging activity. LCC further stimulated the superoxide anion (O_2^-) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of sodium ascorbate. LCCs from *Ceriops decandra* (Griff.) Ding Hou. and cacao husk scavenged O_2^- and hydroxyl radical, and synergistically enhanced the radical scavenging activity of sodium ascorbate [27, 34].

Similarly, SE and vitamin C synergistically enhanced the activity that scavenging superoxide anion radical (determined by the intensity of DMPO-OOH) and hydroxyl radical (determined by the intensity of DMPO-OH radical) (Table 8) [2].

	DMPO-OOH radical intensity (% of control)		DMPO-OH radical intensity (% of control)	
	0.5% VC	0.25% + 5 μM VC	1% VC	0.5% + 5 μM VC
SE	48.3	47.1 < 60.4 [(48.3+72.4)/2]	65.4	75.5 < 87.3 [(65.4+109.1)/2]
10 μM vitamin C	72.4		109.1	

Table 8. Synergistic radical scavenging activity of SE and vitamin C. Cited from [2] with permission.

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3.9. Inhibition of CYP3A4 activity

CYP3A4 activity was measured by β-hydroxylation of testosterone in human recombinant CYP3A4. Products A, B and C dose-dependently inhibited the β-hydroxylation of testosterone, generally used for the assay of CYP3A4 activity. Product C exhibited the highest CYP3A4–inhibitory activity (IC_{50}=58 μg/ml), followed by product B (IC_{50}=124 μg/ml) and then product A (IC_{50}=403 μg/ml). Product B inhibited the CYP3A4 to an extent similar to that attained by Cu (II)- chlorophyllin; product A inhibited CYP3A4 to lower extent than that achieved by grapefruit juice (Figure 12) [4].

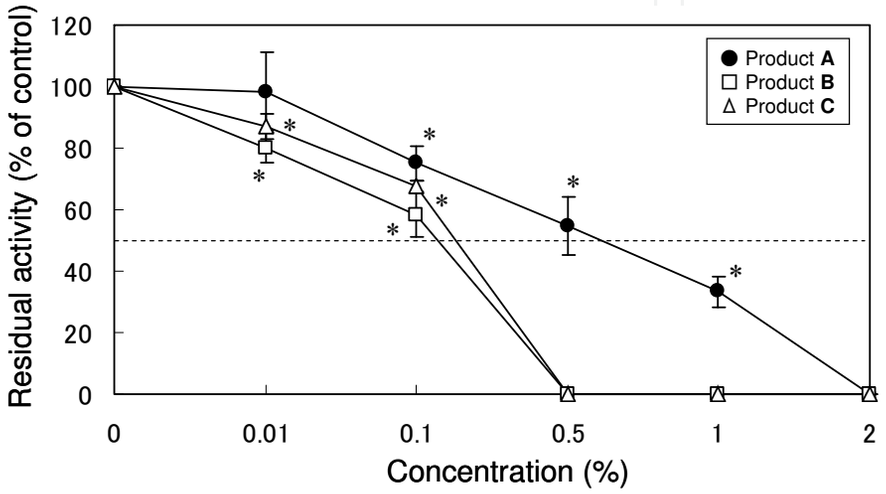

Figure 12. CYP3A4 inhibitory activity of products **A**, **B** and **C**. One millilitre of products **A**, **B** and **C** was freeze dried to produce the powder (66.1, 77.6 and 28.5 mg, respectively).Each value represents the mean ± S.D. (n=3). *p<0.05 relative to the control (0%). Cited from [4] with permission.

SE-10 and SE dose-dependently inhibited the β-hydroxylation of testosterone, generally used for the assay of CYP3A4 activity. SE-10 (IC_{50}= 0.516 μg SE equivalent/ml) had an approximately 16% lower CYP3A4-inhibitory activity (IC_{50}= 0.445 μg/ml) [19].Combined with our recent report [4], 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 more potently.

3.10. Possibility of complex formation between the components

Solvent fractionation of SE demonstrated that the majority of chlorophyllin and the activity that inhibited the NO production by macrophages were recovered from the water layer that contains majority of compounds (more than 81%) [18]. This suggests the possibility that chlorophyllin in SE may be associated with hydrophilic substances, especially LCC in the native state or after extraction with alkaline solution, since the preparative method of SE is the same with that of LCC. This was supported by the observation that LCC isolated from 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 [18]. Upon binding to chlorophyllin, LCC may obtain the activity of inhibiting the NO production by activated macrophages.

3.11. Clinical application for the treatment of oral diseases

Oral intake of product B (BLE) slightly but significantly reduced the gingival crevicular fluid (determined by Periometer®), and tended to reduce gingival index in the experimentally induced gingivitis patients [9].

Lichen planus is a chronic mucocutaneous disease that affects the skin, tongue, and oral mucosa. The most common presentation of oral lichen planus is the reticular form that manifests as white lacy streaks on the mucosa (known as Wickham's striae) or as smaller papules (small raised areas). The cause of lichen planus is not known. Some lichen planus-type rashes occur as allergic reactions to medications and a complication of chronic hepatitis C virus infection [51]. Hepatitis C virus has been reported to occasionally replicate in oral lichen tissue and contribute to mucosal damage [52, 53]. It has been reported that the Epstein-Barr virus is more frequently detected in oral lesions such as oral lichen planus and oral squamous cell carcinoma in comparison with healthy oral epithelium [54].

Potent antiviral, antibacterial, and anti-inflammatory activity of SE prompted us to investigate whether SE is effective on oral lichenoid dysplasia and osteoclastogenesis. A male patient with white lacy streaks in the oral mucosa was orally administered SE three times a day for ten months. Long-term treatment cycle of SE progressively reduced both the area of white steaks (Figure 13) and the base-line levels of salivary interleukin-6 and 8 (Figure 14) [55]. IL-8 concentration after SE treatment was below the initial level throughout the experimental period. This was accompanied by the improvement of patient's symptoms. Before the SE treatment, the patient felt that the mucosa is uneven, rough and cut by touching with his tongue. Three weeks after the treatment, such feeling reduced and the mucosa became much smooth. At four weeks, the rough mucosa was narrowed into smaller area, and the patient could eat without pungent feeling on the oral mucosa. Oral intake of SE also improved the patient's symptom of pollen allergy, and loose teeth, giving an impression that the oral mucosa became much tighter. SE significantly inhibited the RANKL-induced differentiation of mouse macrophage-like RAW264.7 cells towards osteoclasts (evaluated by TRAP-positive multinuclear cell formation). These pilot clinical study suggests the therapeutic potentiality of SE against oral diseases [55].

4. Conclusion

SE (Sasa Health®), alkaline extract of *Sasa senanensis* Rehder extract has shown diverse biological activities including membrane stabilizing, anti-leukemia, anti-inflammatory, radical scavenging, anti-UV, bacteriostatic, antiviral, anti-stomatitis, and anti-lichen planus activity

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(Figure 15). Among these biological activities, antiviral, anti-UV and synergism with vitamin C are unique properties to SE as well as LCC (Figure 15).

Figure 13. Time-dependent effect of SE on the oral lichenoid dysplasia. One 51 years old male patient with lichenoid dysplasia was treated for 0, 1 or 10 months with 13.3 ml of 50% diluted SE (containing 33 mg dried material/ml) at each meal, 3 times a day. Intraoral photographs in the right side (upper panel) and left side (lower panel) of buccal mucosa were taken. It should be noted that the SE treatment progressively reduced the area of the white streaks (a → d → g, b → e → h in the right side of buccal mucosa and c → f → i in the left side of buccal mucosa). Cited from [55] with permission.

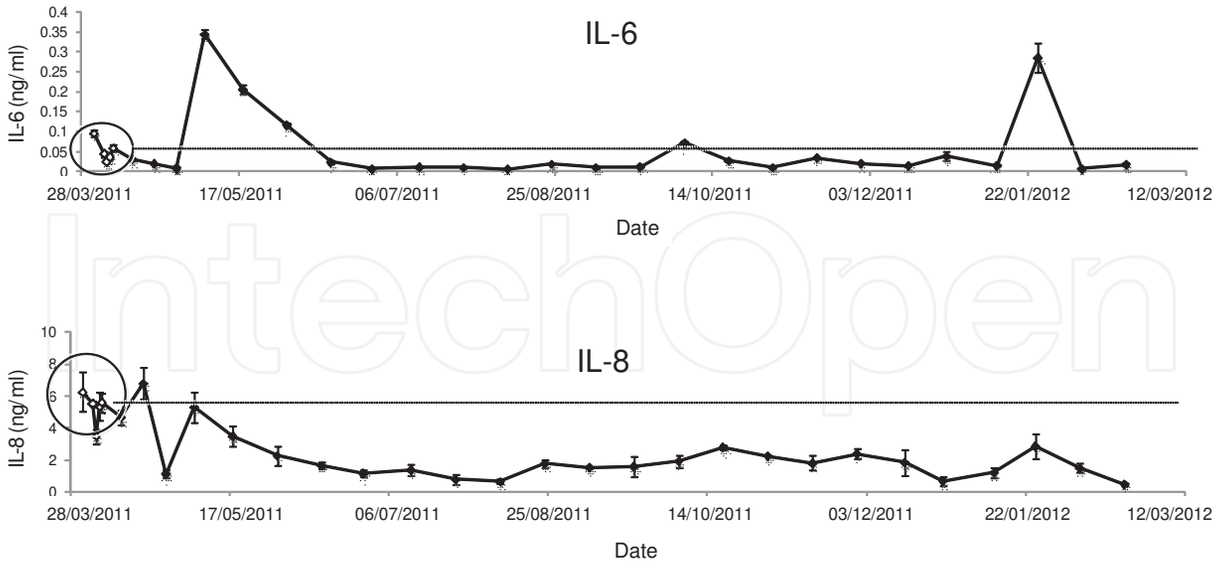

Figure 14. Effect of SE on saliva inflammatory cytokine level. One lichenoid dysplasia patient was treated with SE for the indicated periods, and the salivary IL-6 and IL-8 concentrations were determined by ELISA. Each value represents mean±S.D. of triplicate assays. ○: Control, ●:SE treatment. Cited from [55] with permission.

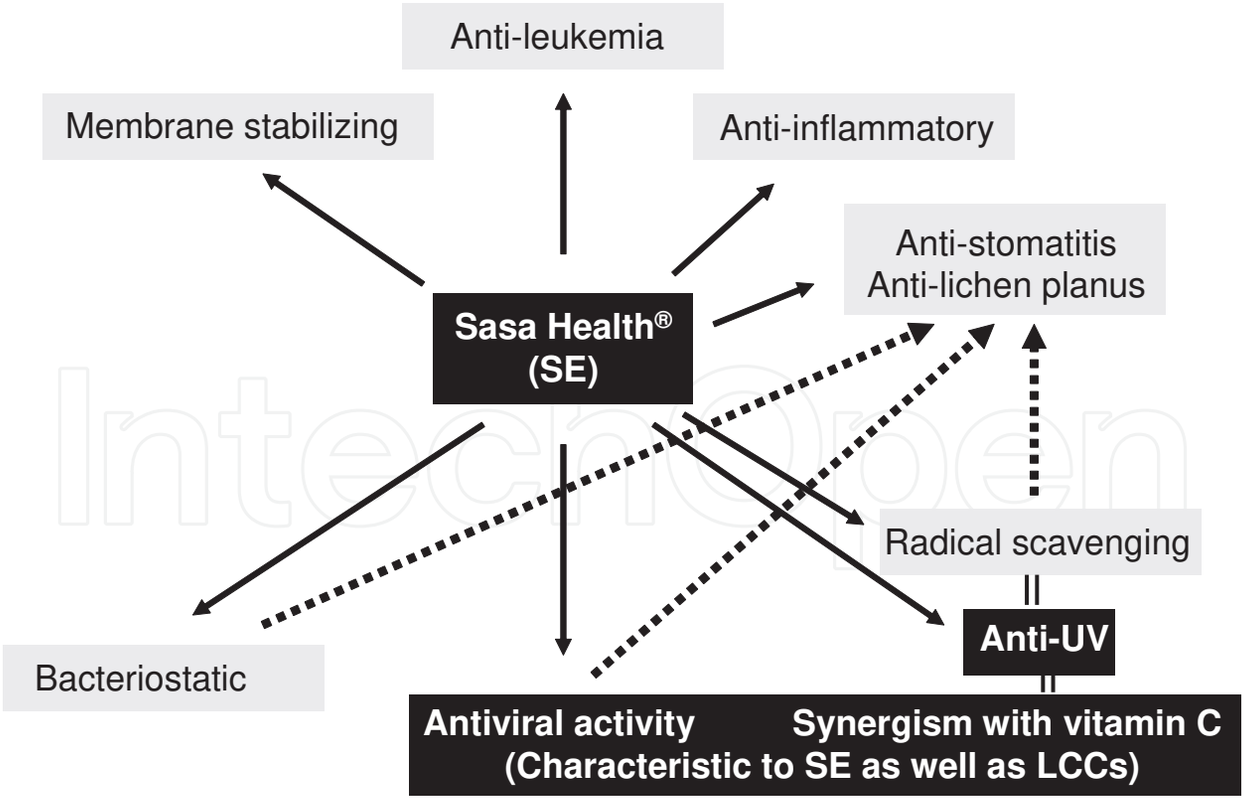

Figure 15. Diverse biological activity of SE..

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SE as well as LCCs, which are efficiently extracted with alkaline solution, showed higher anti-HIV and anti-UV activity, as compared with hot water extract of many plant species including Kampo medicines (Figure 16). Antitumor activity of polysaccharide fractions of pine cone extracts against ascites tumor cells transplanted in mice also increased with acidity (binding strength to DEAE-cellulose column) [56], suggesting the potency of alkaline extract against certain types of diseases.

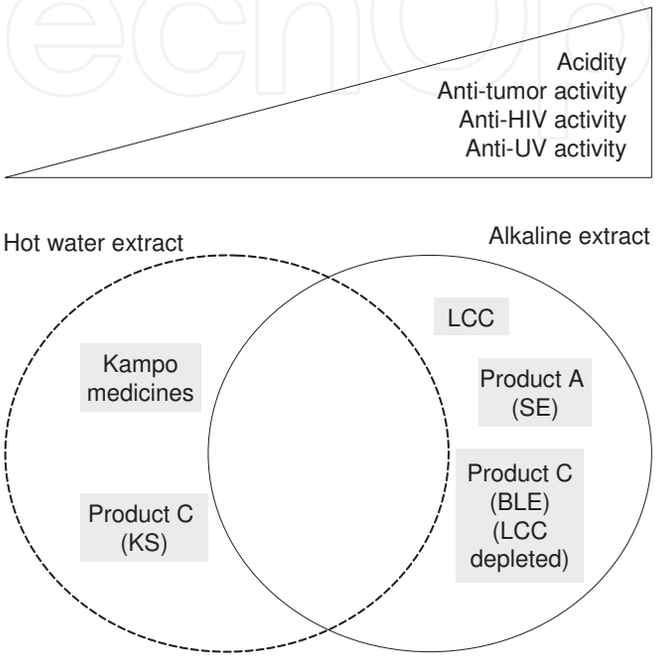

Figure 16. Comparison of several biological activities between hot water and alkaline extracts.

We have reported broad antiviral spectrum of LCC ranging from HIV [57-59], influenza virus [60-62], herpes simplex virus [63-65]. Oral administration of LCC from pine cone extract significantly improved the symptom of HSV-infected patients [66, 67], and lichenoid dysplasia patient [55]. These data suggest the possible application of SE to virally-induced diseases. Considering to low absorption through the intestinal tract [68], the application through the mucosa membrane is recommended. We are now studying the interaction between SE, antibacterial agent and charcoal to optimize the therapeutic potential of SE for the main component of toothpaste.

LCC is composed of two major components: polysaccharide and phenylpropanoide polymer [29, 30, 69, 70]. Limited digestion study demonstrated that anti-viral activity of LCC is generated by its phenylpropanoid portion [58, 61], and immnopotentiation activity possibly by polysaccharide. Using DNA microarray analysis, we have recently reported that treatment of mouse macrophage-like J774.1 cells with LCC fractions isolated from LEM (Fr4) enhanced the expression of dectin-2 (4.2-fold) and toll-like receptor (TLR)-2 (2.5-fold) prominently, but only slightly modified the expression of dectin-1 (0.8-fold), complement receptor 3 (0.9-fold), TLR1, 3, 4, 9 and 13 (0.8- to 1.7-fold), spleen tyrosine kinase (Syk)b, zeta-chain (TCR) associated protein kinase 70kDa (Zap70), Janus tyrosine kinase (Jak)2 (1.0- to 1.2-fold), nuclear fac-

tor (Nf)κb1, NFκb2, reticuloendotheliosis viral oncogene homolog (Rel)a, Relb (1.0- to 1.6-fold), Nfκbia, Nfκbib, Nfκbie, Nfκbi12 Nfκbiz (0.8- to 2.3-fold). On the other hand, LPS did not affect the expression of dectin-2 nor TLR-2. These data suggest the significant role of the activation of the dectin-2 signaling pathway in the action of LCC on macrophages [71]. It is generally accepted that dectin- 2 is the receptor for mannan, whereas dectin-1 is that for glucan [72-76]. It remains to be investigated the signaling pathway of LCC via dectin-2.

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