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Synergism of Alkaline Extract of the Leaves of *Sasa senanensis* Rehder and Antiviral Agents

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Abstract. *Background: Previous studies have shown a much greater antiviral activity of alkaline extract of the leaves of Sasa senanensis Rehder (SE) against human immunodeficiency virus (HIV), compared to lignin precursors, tannins and flavonoids, suggesting its possible application to oral diseases. Systematic comparative study with herpes simplex virus (HSV) has been limited compared to that with HIV. In the present study, we investigated whether combination of SE with other popular antiviral agents further enhances their individual activity. Materials and Methods: Cell viability of mock-infected, HIV-infected and HSV-infected cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The antiviral activity was evaluated by the selectivity index, defined as the ratio of 50% cytotoxic concentration to 50% effective concentration. Synergy between SE and antiviral agents was evaluated by MacSynerg and CompuSyn software. Results: SE showed potent anti-HIV activity, although its activity was two-orders lower than that of azidothymidine, 2',3'-dideoxycytidine dextran sulfate and curdlan sulfate. Combination of SE with these antiviral agents produced synergistic effects. Using a newly established MTT assay system for anti-HSV activity, SE and acyclovir were found to have synergistic anti-HSV activity. Conclusion: The present study suggests the possible efficacy of the clinical application of SE combined with antiviral agents.*

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Key Words: Sasa senanensis Rehder leaf, alkaline extract, HIV, HSV, combination, MTT assay.

Alkaline extract of the leaves of *Sasa senanensis* Rehder (SE) ("SASA-Health"), that is a Group III over-the-counter drug in Japan (1), is recognized as being effective in treating fatigue, loss of appetite, halitosis, body odor and stomatitis by oral administration. SE has shown *in vitro* antiseptic (2), membrane-stabilizing (3), anti-inflammatory (4-6), antibacterial (7, 8), anti-human immunodeficiency virus (HIV) (7, 8), anti-UV (9, 10) and radical-scavenging (5, 10, 11) activities, and synergistic action with vitamin C (7). SE has several common biological properties (*i.e.*, prominent anti-HIV, anti-UV and synergistic activity with vitamin C) with lignin–carbohydrate complex (LCC), which is also extracted by alkaline solution (12). We have identified the anti-UV substances of SE as *p*-coumaric acid derivative(s), precursors of lignin (13). Both SE and LCC had much higher anti-HIV activity than tannins and flavonoids (7, 8, 14). We also performed a small-scale clinical study with SE and LCC products. Long-term oral uptake of SE by a patient with lichenoid dysplasia significantly reduced the size of white streaks in the oral mucosa and the salivary concentrations of interleukin (IL)-6 and IL-8 (15). Brushing the teeth with toothpaste containing 26.2 (w/v%) of SE significantly suppressed halitosis in human volunteers (16). Likewise, oral intake of LCC–vitamin C tablet significantly improved the symptom of patients infected with herpes simplex virus (HSV) (17, 18).

In order to further pursue the possibility of clinical application of SE, we investigated here whether combination of SE and four anti-HIV agents, azidothymidine (AZT), 2',3'-dideoxycytidine (ddC), curdlan sulfate (CRDS) and dextran sulfate (DS), and one anti-HSV agent, acyclovir (ACV), had a synergistic antiviral activity in two different *in vitro* assay systems.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle's medium (DMEM) was from Gibco BRL (Grand Island, NY, USA); fetal

bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RPMI-1640 medium, AZT, and ddC were from Sigma Chemical Co. (St Louis, MO, USA); dimethyl sulfoxide (DMSO), and DS (5 kDa) were from Wako Pure Chemical Ind., Ltd. (Osaka, Japan); CRDS (79 kDa) from Ajinomoto Co. Inc., (Tokyo, Japan); and ACV from Tokyo Chemical Industry Co. (Ltd, Tokyo). All antiviral agents were dissolved in DMSO, and cytotoxicity induced by DMSO was subtracted from data.

SE was manufactured by Daiwa Biological Research Institute Co., Ltd., Kawasaki, Kanagawa, Japan. One milliliter of SE contained 66.1 mg freeze-dried powder.

Assay for anti-HIV activity. Human T-cell leukemia virus I (HTLV-I)-bearing CD4$^-$ positive human T-cell line, MT-4 (supplied by Dr. Naoki Yamamoto), was cultured in RPMI-1640 medium supplemented with 10% FBS and infected with HIV-1_{IIIB} at a multiplicity of infection (MOI) of 0.01. HIV- and mock-infected (control) MT-4 cells (3×10^4 cells/96-microwell) were incubated for 5 days with different concentrations of products 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 50% that of the control (mock-infected, untreated) cells (EC_{50}) were determined from the dose–response curve. The anti-HIV activity was evaluated by the selectivity index (SI), which was calculated by the following equation: $SI=CC_{50}/EC_{50}$ (19).

Assay for anti-HSV activity. Plaque assay was performed according to the modification of our previous article (20). Confluent monolayer Vero cells [3×10^5 cells in 6-well tissue culture plate (NUNC Labware Product-Sigma Aldrich Inc, Tokyo, Japan] were infected with HSV-1 (F strain) (supplied by the National Institute of Infectious Diseases, Tokyo, Japan) at 300 plaque-forming unit (PFU) per well for 1 h at 37ºC in the absence or present of the test agents during infection. After allowing 1 h for virus adsorption, the infected cells were washed once with minimum essential medium (MEM), and overlaid with 2 ml MEM-10% fecal calf serum (FCS) containing 0.5% Bacto agar (Difco, Bacto Laboratory Pty Ltd, Mt. Pritchard, Australia) without or with test agents. After incubation for 3 days, the agar overlay was removed, and the attached cells were fixed with 50% ethanol and stained with 0.25% crystal violet. The number of visible plaques was then counted under light microscopy.

We also performed the MTT assay to quantify the anti-HSV activity of agents. Vero cells (10,000 cells) were inoculated on a 96-well plate (NUNC). After 24 h, the cells were infected with HSV-1 (strain F) (MOI=0.01). HSV-1 was mixed first with test sample and stood for 20 min, then mixed with ACV and added to the cells. After incubation for 4 or 5 days, the cells were added with MTT reagent (BioAssay Systems, Hayward, CA, USA) and incubated for 4 h. Cells were dissolved with 10%SDS in 0.01 M HCl, and the absorbance at 595 nm was measured.

Statistical treatment. Experimental data are the mean±standard deviation (SD). The statistical differences between control and treated groups were evaluated by Student's *t*-test. A value of $p<0.05$ was considered to be significant. Significance of synergy between SE and four anti-HIV agents was evaluated by MacSynergy II, an analytical tool which describes and quantifies drug interaction. (21). CompuSyn software program (ComboSyn, Inc., Paramus, NJ, USA) was used to calculate the combination index (CI), where the combination effect was judged "synergistic" when CI was less than 1 (22).

Results

Anti-HIV activity. We first compared the anti-HIV activity of SE and four representative anti-HIV agents: AZT, ddT, CRDS and DS (Figure 1). Using uninfected cells, the viable cell number was reduced dose-dependently. From the dose–response curve, we determined the CC_{50} of SE, AZT, ddC, CRDS and DS as 1.42%, 64.10 μM, 2451 μM, >1000 μg/ml and >1000 μg/ml, respectively (Figure 1).

When cells were infected with HIV, all the cells were killed by its cytotoxic effect. By addition of increasing concentrations of antiviral agents, the viability of cells returned to the level of the control (uninfected and untreated). From the dose–response curve, we determined the EC_{50}, the concentration that restored cell viability to 50% of the control level. The EC_{50} of SE, AZT, ddC, CRDS and DS was 0.0178%, 0.0028 μM, 0.206 μM, 0.098 μg/ml and 0.021 μg/ml, respectively (Figure 1).

Anti-HIV activity (defined as the SI value) was determined from the CC_{50} and EC_{50} values, using the equation: $SI=CC_{50}/EC_{50}$. SE had potent anti-HIV activity (SI=80), however, the anti-HIV activity of SE was two-orders lower than that of the four ant-HIV agents: AZT (SI=22893), ddC (SI=11898), CRDS (SI≥10204) and DS (SI≥47619) (Figure 1).

Combination effects of SE and anti-HIV agents. We found that the combination treatment with 32 μM AZT and 0.00064, 0.0032, 0.016 or 0.08% SE, and that with 160 μM AZT and 0.00064 or 0.0032% SE had synergistic anti-HIV activity (Figure 2A). When 0.016% SE was present, 6.4 μM AZT was as effective as 32 μM AZT (without SE) (*i.e.* requiring only 1/5th the amount of AZT), and 32 μM AZT had anti-HIV activity comparable with 160 or 800 μM AZT (without SE) (*i.e.* requiring only 1/5-1/25th the amount of AZT) (Figure 2A). This indicates that supplementation with SE can reduce the concentration of AZT needed to treat HIV-infected cells to 1/5th or 1/25th.

Combination treatment with 1,600 μM ddC and 0.016% SE, and that with 8,000 μM ddC and 0.00064% SE had synergistic anti-HIV activity (Figure 2B). When 0.016% SE was present, 320 μM ddC had anti-HIV activity comparable with 8,000 μM AZT (without SE) (requiring only 1/25th the amount of AZT) (Figure 2B).

Combination treatment with 1,600 ng/ml CRDS and 0.00064, 0.016, 0.08 or 0.4% SE, and that with 8000 ng/ml CRDS and 0.016 or 0.4% SE had synergistic anti-HIV activity (Figure 2C). In the presence of 0.00064% SE, 1600 μM CRDS had anti-HIV activity comparable with 8,000 μM CRDS (without SE) (*i.e.* requiring only 1/5th the amount of CRDS) (Figure 2C).

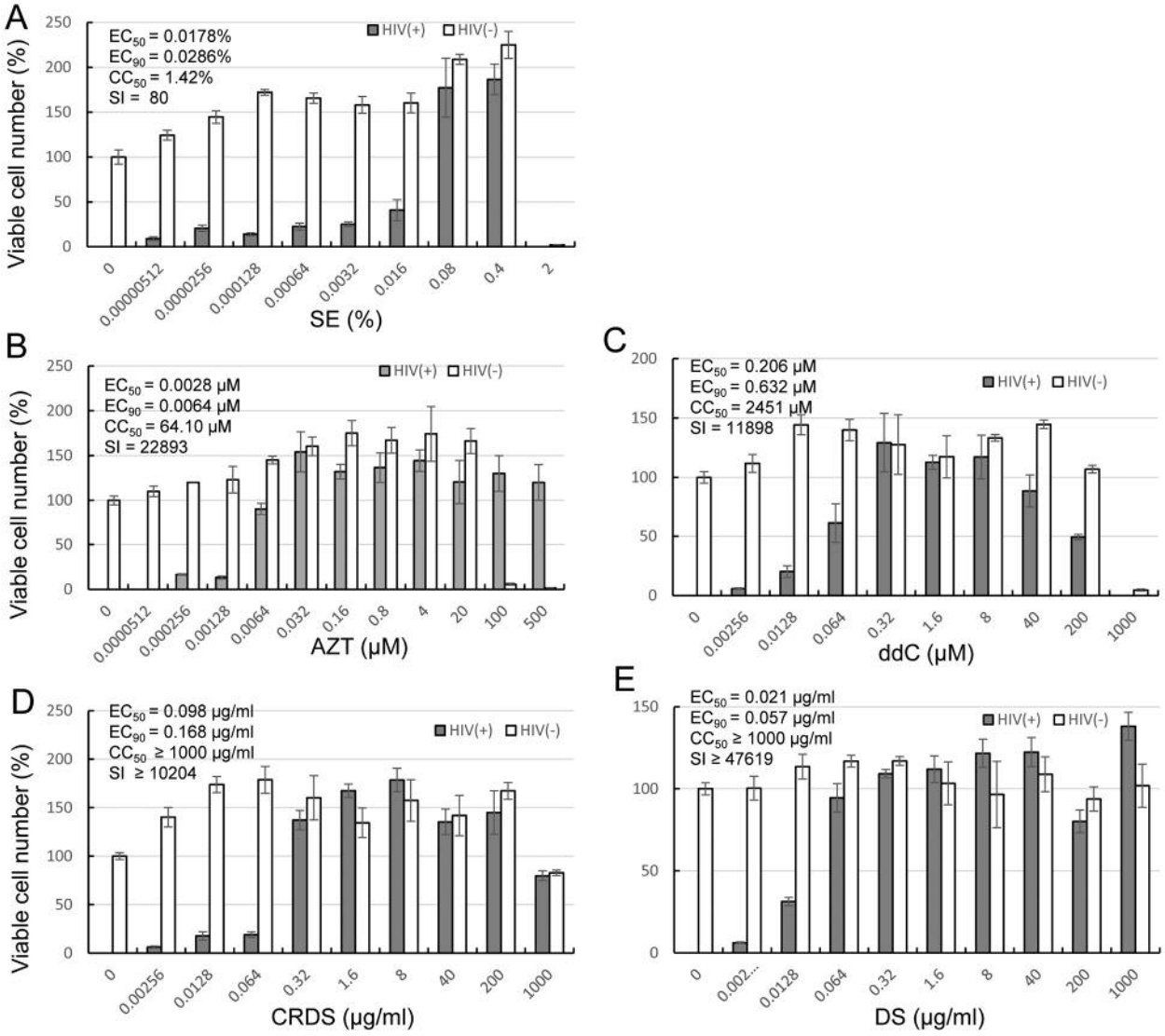

Figure 1. *Anti-HIV activity of Sasa senanensis Rehder extract (SE) and chemotherapeutic agents. HIV-1_{IIIB}-infected (HIV^+) (gray bars) and mock-infected (HIV^-) (white bars) MT-4 cells were incubated for 5 days with the indicated concentrations of SE (A), azidothymidine (AZT) (B), 2',3'-dideoxycytidine (ddC) (C), curdlan sulfate (CRDS) (D) on dextran sulfate (DS) (E), 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. EC_{50}: Concentration that increased the viable cell number of HIV-infected cells to 50% that of the control (mock-infected, untreated) cells; CC_{50}: concentration that reduced the viable cell number of uninfected cells by 50%; SI: selectivity index, $=CC_{50}/EC_{50}$.*

Combination treatment with 2.56 ng/ml DS and 0.016% SE, that with 12.8 ng/ml DS and 0.032 or 0.016% SE, or that with 64 ng/ml DS and 0.08 or 0.4%SE produced synergistic anti-HIV activity (Figure 2D). In particular, the combination of 2.56 ng/ml DS and 0.032% SE increased the cell viability of HIV-infected cells to nearly 50% (99.9% synergy), while single treatment of DS or SE had no anti-HIV activity (Figure 2D).

Anti-HSV activity. Plaque assay demonstrated that a high concentration of SE (above 0.3%) exhibited cytotoxicity against Vero cells (Figure 3A, left column), and that infection with HSV-1 (300 PFU) induced cytopathic effect, killing all cells (Figure 3A, right column). Mixing of HSV-1 with SE for 20 min rapidly reduced the infectivity, restoring the cellular viability completely (Figure 3A, right column).

To quantify the anti-HSV activity of SE, we newly established the MTT assay for anti-HSV activity. Infection of HSV-1 significantly reduced the cellular viability to 25% or 10% after 4 and 5 days, respectively (Figure 3B). The addition of more than 0.03 to 0.01% SE nearly completely eliminated

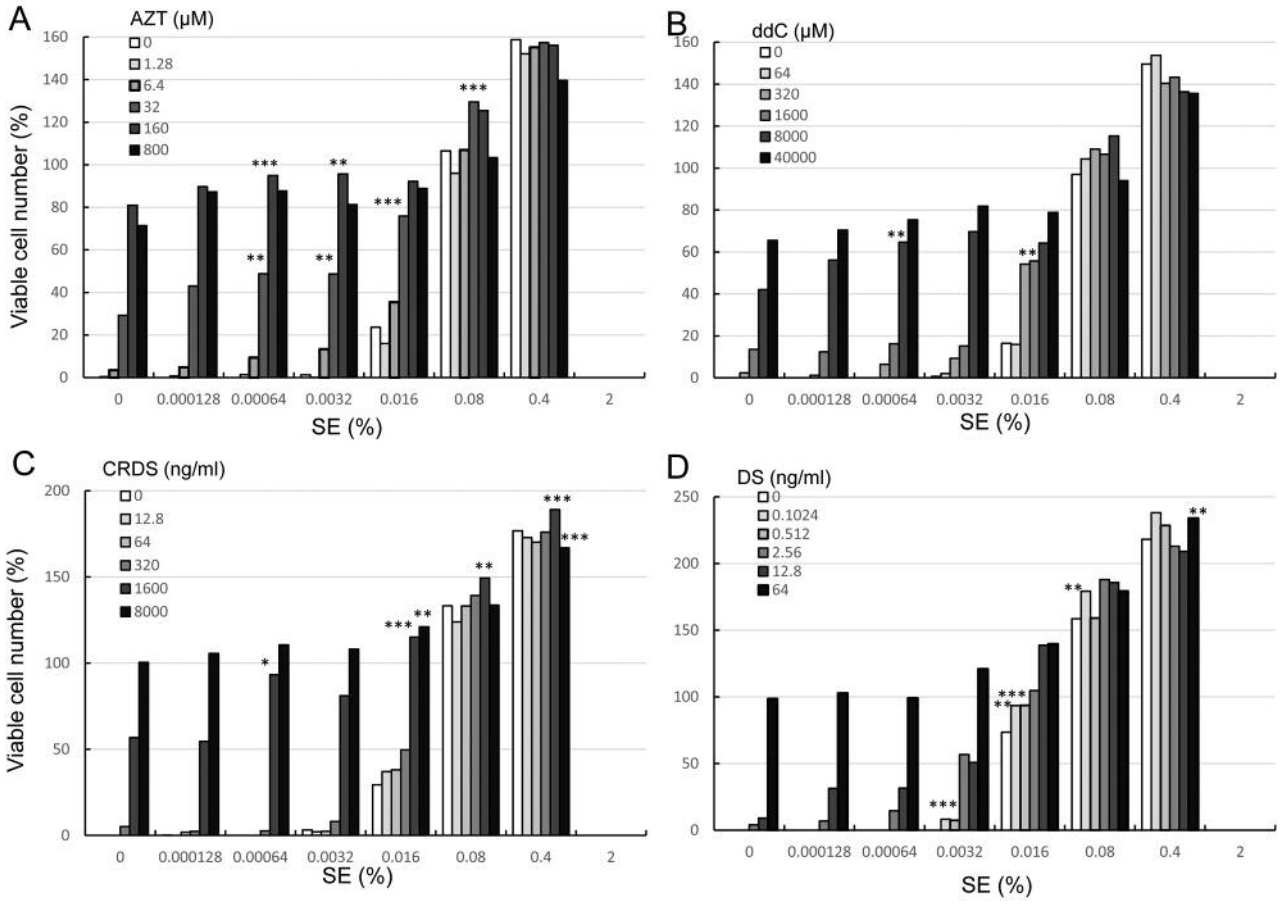

Figure 2. *Synergistic anti-HIV activity of Sasa senanensis Rehder extract (SE) and chemotherapeutic agents. HIV-1_{IIIB}-infected (HIV^+) and mock-infected (HIV^-) MT-4 cells were incubated for 5 days with 0, 0.000128, 0.00064, 0.0032, 0.016, 0.08, 0.4 or 2% SE in the presence of the indicated concentrations of azidothymidine (AZT) (A), 2',3'-dideoxycytidine (ddC) (B), curdlan sulfate (CRDS) (C) or dextran sulfate (DS) (D), 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. *95% Synergy, **99% synergy, ***99.9% synergy.*

the cytopathic effect of HSV-1 infection. ACV (300 nM) was also partially effective. The CI calculated using an isobologram analysis indicated that addition of 0.003 or 0.01% SE together with ACV produced synergy of anti-HSV activity (CI=0.8 and 0.5, respectively) (Figure 3B). When the incubation time was prolonged to 5 days, synergic anti-HSV activity was observed at a wider range of SE concentrations (0.001, 0.003, 0.01, and 0.03%; CI=0.8, 0,6, 0,5 and 0.3, respectively) (Figure 3B).

Discussion

The present study demonstrated for the first time that SE can synergistically enhance the anti-HIV activity of four established chemotherapeutic agents (AZT, ddC, CRDS, DS). Such synergistic action was more pronounced when SE was used with nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), such as, AZT and ddT (23), rather than with sulfated polysaccharides such as CRDS or DS that prevent viral adsorption through binding to the viral envelope glycoprotein (24). This is reasonable since LCC, a putative active component of SE, was found to have very strong affinity to cells (25), similar to sulfated polysaccharides, but had a different site of action from NRTIs.

Sub-Saharan Africa has the highest number of patients with HIV but a low ability to purchase expensive drugs, therefore it is important to establish therapeutic methods that further enhance the therapeutic potential of conventional anti-HIV drugs. In 2014, this area had 25.8 million HIV-positive patients, 1.4 million newly-infected patients, and 790 thousand fatalities (26). The present study demonstrated that the addition of SE can reduce the amount of anti-HIV agent

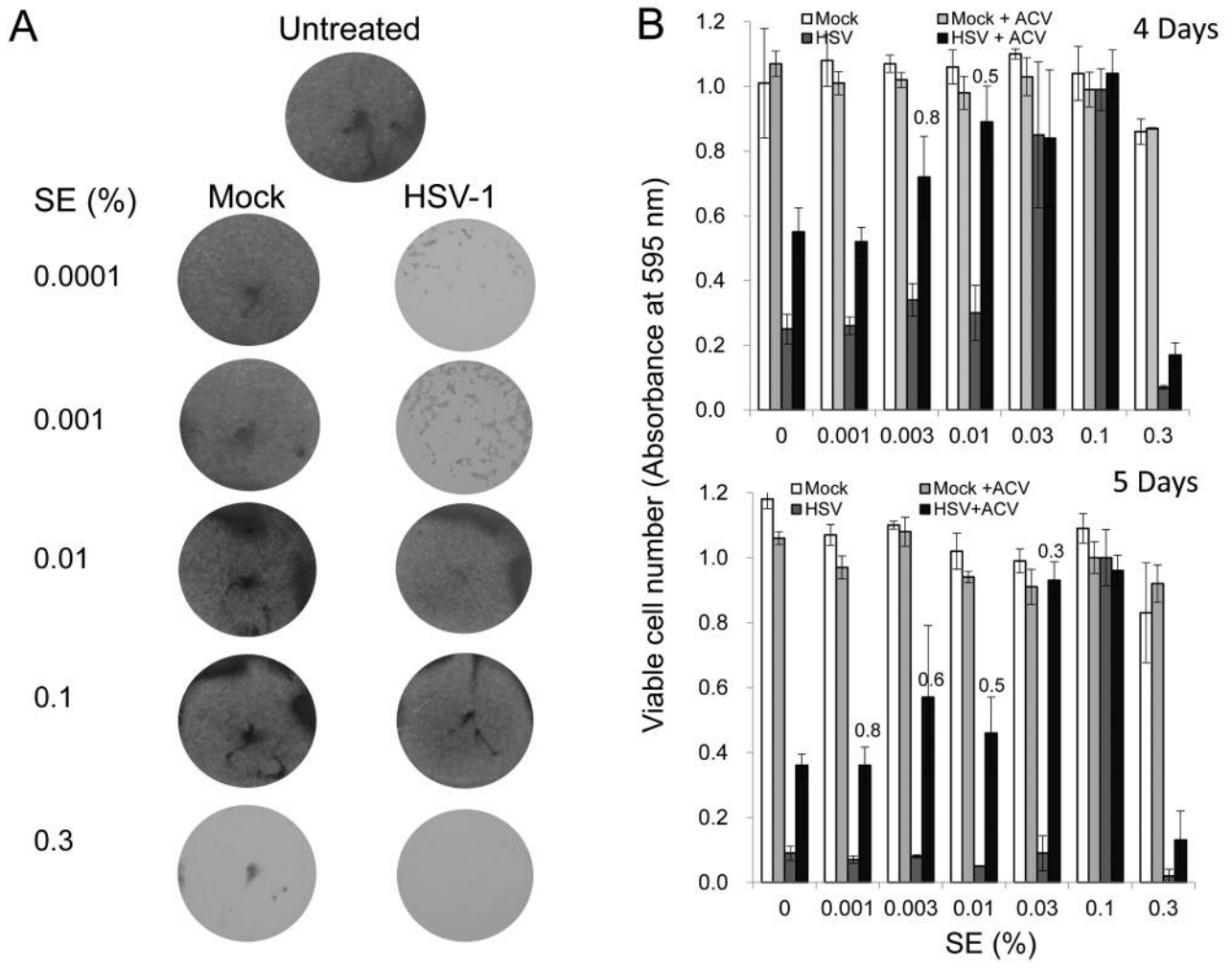

Figure 3. *A: Anti-herpes simplex virus (HSV) activity of alkaline extract of the leaves of Sasa senanensis Rehder (SE). HSV-1 and SE were mixed and pre-incubated for 20 min. Vero cells were mock-infected or infected with HSV-1 (300 plaque forming unit/well), and subjected to plaque assay. Plaques formed during 3 days incubation were stained and photographed under light microscopy. B: Combination effect of SE and acyclovir (ACV). Vero cells were infected with HSV-1 that had been mixed with the indicated concentrations of SE, and the viable cell number was determined 4 or 5 days later. Each value represents mean±S.D. of triplicate assays. The numbers given above bars are the combination index (CI).*

necessary for antiviral action (only 4-20% are needed under optimal conditions). Such supplementation with SE may achieve more economical use of the same supplies.

We have established a new simplified method for quantification of anti-HSV activity with MTT reagent. Using this method, we found that SE and ACV also had synergistic anti-HSV activity. Repeated experiments revealed that the SI value of SE for anti-HSV activity was 6-7 (unpublished data), approximately one order lower than that for anti-HIV activity (SI=80) (Figure 1A). This low SI may be explained by the difference of cell types we used. MT-4 cells cultured in suspension can divide 32-times over 5 days, while the growth of adherent cells, such as Vero, are much slower due to contact inhibition, possibly reducing the difference of viable cell number of mock-infected cells and that of HSV-infected cells, thus yielding an unexpectedly lower SI value.

Using this anti-HSV assay method, we found that SE had much higher anti-HSV activity than other molecular polyphenols (unpublished data). Further study is underway to identify the active components in SE, and searching more potent natural and synthetic anti-HIV substances.

Conflicts of Interest

The first Author (HS) was supported by Daiwa Biological Research Institute Co., Ltd., Kanagawa, Japan. The Authors wish to confirm that such financial support has not influenced the outcome or the experimental data.

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References

1 The Pharmaceutical Affairs Law in Japan. Pharmaceuticals and Medical Safety Bureau, Ministry of Health, Labour and Welfare, Tokyo, 2009.
2 Chuyen NY, Kurata T and Kato H: Anti-septic activity of *Sasa senanensis* Rehder extract. J Antibac Antifung Agents *11*: 69-75, 1983 (in Japanese).
3 Ohizumi T, Kodama K, Tsuji M and Oguchi K: The effect of *Sasa senanensis* Rehder extract and crude herb medicine extract on the membrane. Showa Med J *49*: 315-321, 1989 (in Japanese).
4 Ohizumi T, Shirasaki K, Tabata T, Nakayama S, Okazaki M and Sakamoto K: Pharmacological studies of *Sasa senanensis* Rehder extract on anti-inflammatory effect and phagocytic activity. Showa Med J *48*: 595-600, 1988 (in Japanese).
5 Zhou L, Hashimoto K, Satoh K, Yokote Y, Kitajima M, Oizumi T, Oizumi H and Sakagami H: Effect of *Sasa senanensis* Rehder extract on NO and PGE2 production by activated mouse macrophage-like RAW264.7 cells. In Vivo *23*: 773-778, 2009.
6 Ono M, Kantoh K, Ueki J, Shimada A, Wakabayashi H, Matsuta T, Sakagami H, Kumada H, Hamada N, Kitajima M, Oizumi H and Oizumi T: Quest for anti-inflammatory substances using IL-1β-stimulated gingival fibroblasts. In Vivo *25*: 763-768, 2011.
7 Sakagami H, Amano S, Kikuchi H, Nakamura Y, Kuroshita R, Watanabe S, Satoh K, Hasegawa H, Nomura A, Kanamoto T, Terakubo S, Nakashima H, Taniguchi S and Ohizumi T: Antiviral, antibacterial and vitamin C-synergized radical-scavenging activity of *Sasa senanensis* Rehder extract. In Vivo *22*: 471-476, 2008.
8 Sakagami H, Zhou Li, Kawano M, Thet MM, Takana S, Machino M, Amano S, Kuroshita R, Watanabe S, Chu Q, Wang QT, Kanamoto T, Terakubo S, Nakashima H, Sekine K, Shirataki Y, Hao ZC, Uesawa Y, Mohri K, Kitajima M, Oizumi H and Oizumi T: Multiple biological complex of alkaline extract of the leaves of *Sasa senanensis* Rehder. In Vivo *24*: 735-744, 2010.
9 Matsuta T, Sakagami H, Kitajima M, Oizumi H and Oizumi T: Anti-UV activity of alkaline extracts of the leaves of *Sasa senanensis* Rehder. In Vivo *25*: 751-755, 2011.
10 Matsuta T, Sakagami H, Satoh K, Kanamoto T, Terakubo S, Nakashima H, Kitajima M, Oizumi H and Oizumi T: Biological activity of luteolin glycosides and tricin from *Sasa senanensis* Rehder. In Vivo *25*: 757-762, 2011.
11 Komatsu M and Hiramatsu M: Free radical scavenging activity of *Sasa senanensis* Rehder extract. KISO TO RINSHO *31*: 3321-3324, 1997 (in Japanese).
12 Sakagami H, Kushida T, Oizumi T, Nakashima H and Makino T: Distribution of lignin–carbohydrate complex in the plant kingdom and its functionality as alternative medicine. Pharmacol Ther *128*: 91-105, 2010.
13 Matsuta T, Sakagami H, Sugiura T, Kitajima M, Oizumi H and Oizumi T: Structural characterization of anti-UV components from *Sasa senanensis* Rehder extract. In Vivo *27*: 77-84, 2013.
14 Sakagami H, Iwamoto S, Matsuta T, Satoh K, Shimada C, Kanamoto T, Terakubo S, Nakashima H, Morita Y, Ohkubo A, Tsuda T, Sunaga K, Kitajima M, Oizumi H and Oizumi T: Comparative study of biological activity of three commercial products of *Sasa senanensis* Rehder leaf extract. In Vivo *26*: 259-264, 2012.
15 Matsuta T, Sakagami H, Tanaka S, Machino M, Tomomura M, Tomomura A, Yasui T, Itoh K, Sugiura T, Kitajima M, Oizumi H and Oizumi T: Pilot clinical study of *Sasa senanensis* Rehder leaf extract treatment on lichenoid dysplasia. In Vivo *26(6)*: 957-962, 2012.
16 Sakagami H, Sheng H, Ono K, Komine Y, Miyadai T, Terada Y, Nakada D, Tanaka S, Matsumoto M, Yasui T, Watanabe K, Junye J, Natori T, Suguro-Kitajima M, Oizumi H and Oizumi T: Anti-halitosis effect of toothpaste supplemented with alkaline extract of the leaves of *Sasa senanensis* Rehder. In Vivo *30(2)*: 107-111, 2016.
17 López BSG, Yamamoto M, Utsumi K, Aratsu C and Sakagami H: Clinical pilot study of lignin-ascorbic acid combination treatment of herpes simplex virus. In Vivo *23*: 1011-1016, 2009.
18 López BSG, Yamamoto M and Sakagami H: Chapter 9. Treatment of Herpes Simplex Virus with lignin–carbohydrate complex tablet, an alternative therapeutic formula. *In*: Antiviral Drugs – Aspects of Clinical Use and Recent Advances. Arbuthnot P (ed.). InTech, Croatia, pp.171-194, 2012.
19 Nakashima H, Murakami T, Yamamoto N, Sakagami H, Tanuma S, Hatano T, Yoshida T and Okuda T: Inhibition of human immunodeficiency viral replication by tannins and related compounds. Antiviral Res *18*: 91-103, 1992.
20 Fukuchi K, Sakagami H, Okuda T, Hatano T, Tanuma S, Kitajima K, Inoue Y, Inoue S, Ichikawa S, Nonoyama M and Konno K: Inhibition of herpes simplex virus infection by tannins and related compounds. Antiviral Res *11*: 285-298, 1989.
21 https://www.uab.edu/medicine/peds/macsynergy
22 Sugimori N, Espinoza JL, Trung LQ, Takami A, Kondo Y, An DT, Sasaki M, Wakayama T and Nakao S: Paraptosis cell death induction by the thiamine analog benfotiamine in leukemia cells. PLoS One *10(4)*: e0120709. doi: 10.1371/journal.pone.0120709. eCollection 2015.
23 De Clercq E: New developments in anti-HIV chemotherapy. Curr Med Chem *8(13)*: 1543-1572, 2001.
24 Esteves AI, Nicolai M, Humanes M, Goncalves J: Sulfated polysaccharides in marine sponges: extraction methods and anti-HIV activity. Mar Drugs *9(1)*: 139-153, 2011.
25 Sakagami H, Takeda M, Kawazoe Y, Nagata K, Ishihama A, Ueda M and Yamazaki S: Anti-influenza virus activity of a lignin fraction from cone of *Pinus parviflora* Sieb. et Zucc. In Vivo *6*: 491-496, 1992.
26 UNAIDS Fact sheet 2015, available at http://www.unaids.org/en/resources/documents/2015/20150714_factsheet (last accessed 06/04/2016).

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