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Anti-HIV and Vitamin C-synergized Radical Scavenging Activity of Cacao Husk Lignin Fractions

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Abstract. *Cacao husk lignin fractions, prepared by acid precipitation and 50% ethanol precipitation showed unexpectedly higher anti-human immunodeficiency virus (HIV) activity, as compared with the corresponding fractions from the cacao mass, amounting to the level comparable with that of popular anti-HIV compounds. The cacao husk lignin fractions also showed anti-influenza virus activity, but did not show antibacterial activity. The cacao husk lignin fractions synergistically enhanced the superoxide anion and hydroxyl radical scavenging activity of vitamin C. The cacao husk lignin fractions stimulated nitric oxide generation by mouse macrophage-like cells, to a level higher than that attained by lipopolysaccharide (LPS). The present study suggests the functionality of cacao husk lignin fractions as complementary alternative medicine.*

Cocoa bean, a main raw material of chocolate, has been reported to display antioxidant (1), anti-arteriosclerosis (2), antibacterial (3) and antiviral activities (4). The chemical analysis of the components of cacao, such as catechin, epicatechin, proanthocyanidin glycosides and related polyphenols (5) and lignin as food fibers (6), has been reported. Lignin carbohydrate has displayed several unique activities such as anti-human immunodeficiency virus (HIV) activity and synergistic actions with vitamin C (7). However, the physiological role of cacao-derived lignins has not been well characterized. In order to explore the novel functionality of components of cacao, the lignin fractions prepared from cacao husk (the shell of the cacao bean) and cacao mass (paste with cacao husk and germ removed) were investigated for their possible anti-HIV, anti-influenza virus, antibacterial, radical scavenging, and macrophage stimulating activities.

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Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle's medium (DMEM), phenol-red free DMEM, fetal bovine serum (FBS) (suppliers are listed in each section), Eagle's MEM (Nissui, Tokyo, Japan); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), hypoxanthine (HX), xanthine oxidase (XOD), diethylenetriaminepentaacetic acid (DETAPAC), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), 3'-azido-2', 3'-dideoxythymidine (AZT), dideoxycytidine (ddC), lipopolysaccharide (LPS) from *E. coli*, Serotype 0111:B4 (Sigma Chem. Co., St. Louis, MO, USA); dimethyl sulfoxide (DMSO) (Wako Pure Chem. Ind., Ltd., Osaka, Japan); curdlan sufate (79 kD, Ajinomoto Co., Inc., Tokyo, Japan) and dextran sulfate (8 kD, Kowa, Tokyo, Japan).

Preparation of lignin fractions. Cacao husk that contains lignin fractions [**1, 2, 3, 7, 8. 9, 13, 14, 15**], or cacao mass that contains lignin fractions [**4, 5, 6, 10, 11, 12, 16, 17, 18**] were extracted for 2 h with 1% NaOH at room temperature and the insoluble materials were removed by centrifugation at 8,000xg at 4˚C for 10 min (Figure 1). The pH of the NaOH extract was adjusted to 5.0 by drop-wise addition of acetic acid to precipitate the lignin carbohydrate complex fractions [**1, 4, 7, 10, 13, 16**]. Addition of ethanol (final concentration:

50%) to the supernatant precipitated the polysaccharide-rich fractions [**2, 5, 8, 11, 14, 17**]. Further addition of ethanol (final concentration: 83%) to the supernatant precipitated the low molecular weight fractions [**3, 6, 9, 12, 15, 18**]. All these fractions were extensively dialyzed against water, and lyophilized (Figure 1).

Assay for anti-HIV activity. Human T-cell leukemia virus I (HTLV-I)-bearing CD4 positive human T cell line, MT-4 cells (kindly supplied by Prof. Yamamoto, Tokyo Medical Dental University, Japan), were cultured in RPMI1640 medium supplemented with 10% FBS (GIBCO BRL, Grand Island, NY, USA), and infected with HIV-1_{IIIB} at a multiplicity of infection (m.o.i.) of 0.01. The 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 (8). All the data represent the mean values of triplicate measurements. The anti-HIV activity was evaluated by the selectivity index (SI), which was calculated by the following equation: $SI=CC_{50}/EC_{50}$

Assay for anti-influenza A (H1N1) virus activity. Madin-Darby canine kidney (MDCK) cells were cultured in Eagle's minimum essential medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum (Hyclone Ltd., UT, USA) and infected with influenza A (H1N1) virus at 100 $TCID_{50}$ (50% tissue culture infectious dose). The virus- or mock-infected (control) MDCK cells were incubated for 5 days with a serial twofold dilutions of the test samples starting at 2.5 mg/mL. During the incubation, 3 days after infection, the maintenance medium in each well was exchanged for fresh medium which contained the same concentration of test sample as before the exchange. The relative viable cell number was determined by MTT assay. The CC_{50} and EC_{50} were determined from the dose-response curve with mock-infected or virus-infected cells, respectively. The antiviral activity was evaluated using a selectivity index (SI), calculated as above.

Antimicrobial activity. Streptococcus mutans were grown anaerobically at 37˚C in Todd Hewitt Broth (Difco Laboratories, Detroit, MI, USA). *Actinomyces viscosus*, *Fusobacterium nucleatum* and *Porphyromonas gingivalis* were grown anaerobically at 37˚C in Brain Heart Infusion Broth (Difco Laboratories) supplemented with hemin (5 μg/mL) and menadione (50 μg/mL). These bacteria were cultured for 2 days with 1 mg/mL of the test sample, and the antibacterial activity was determined by their growth in each medium.

Radical scavenging activity. The radical intensity was determined at 25˚C in 0.1 M Tris-HCl (pH 7.0), 0.1 M Na_2CO_3/$NaHCO_3$ (pH 11.0) or 0.1 M KOH (pH 13.5), using electron spin resonance (ESR) spectroscopy (JEOL JES REIX, X-band, 100 kHz modulation frequency) (9). The instrument settings were: center field, 336.0±5.0 mT; microwave power, 8 mW; modulation amplitude, 0.1 mT: gain, 500, time constant, 0.1 s and scanning time, 2 min.

For the determination of the superoxide anion (in the form of DMPO-OOH), produced by the HX-XOD reaction (total volume: 200 μL) (2 mM HX in 0.1 M phosphate buffer [pH 7.4] [PB] 50 μL, 0.5 mM DETAPAC 20 μL, 8% DMPO 30 μL, sample (in PB) 40 μL, H_2O 30 μL, XOD (0.5 U/mL in PB) 30 μL), the time constant was changed to 0.03 s (9).

For the determination of the hydroxyl radical (in the form of DMPO-OH), produced by the Fenton reaction (200 μL) (1 mM $FeSO_4$ [containing 0.2 mM DETAPAC] 50 μL, 0.1 M phosphate buffer [pH 7.4] 50 μL, 92 mM DMPO 20 μL, sample [in H_2O] 50 μL, 1 mM H_2O_2, 30 μL), the gain was changed to 400 (9).

Effect on NO production by macrophages. Mouse macrophage-like cells RAW264.7 were incubated in DMEM supplemented with 10% heat-inactivated FBS (JRH Bioscience, Lenexa, KS, USA). The RAW264.7 cells ($6x10^4$/mL) were inoculated into 96-microwell plates, and incubated for 24 h. The medium was then replaced with phenol red-free DMEM medium containing 10% FBS and the indicated concentrations of lignin sample or LPS. After incubation for 24 h, the NO released into the culture supernatant was measured by the Griess method.

Results

Yield. The yield of the lignin carbohydrate complex fractions [**1, 7, 13**] from the cacao husk was the greatest (3.2±1.1% (n=3)), followed by that of the polysaccharide-rich fractions [**2, 8, 14**] (2.8±0.2%) and then that of the low molecular fraction [**3, 9, 15**] (0.74±0.15%) (Table I).

The yield of the lignin carbohydrate complex fractions [**4, 10, 16**] from the cacao mass was the greatest (7.9±3.4% (n=3)), followed by that of the polysaccharide-rich fractions [**5, 11, 17**] (1.4±0.6%) and then that of the low molecular weight fraction [**6, 12, 18**] (0.73±0.04%) (Table I).

Anti-HIV activity. The anti-HIV activity of the lignin carbohydrate complex fractions [**1, 7, 13**] from the cacao husk (SI=311±210 (n=3)) was one order higher than that of the corresponding fractions from the cacao mass [**4, 10, 16**] (SI=46±39) (Table I). The anti-HIV activity of the carbohydrate-rich fractions [**2, 8, 14**] from the cacao husk (SI=30~100,000) was the greatest, one to three orders higher than that of the corresponding fractions from the cacao mass [**5, 11, 17**] (SI=3.3±1.6) (Table I). The anti-HIV activity of the low molecular weight fractions [**3, 9, 15**] from the cacao husk (SI=115±98) was one order higher than that of the corresponding fractions from the cacao mass [**6, 12, 18**] (SI=17±21) (Table I). These results indicated that the lignin fractions from the cacao husk had higher anti-HIV activity than the corresponding fractions from the cacao mass, and that the lignin carbohydrate fractions and the polysaccharide-rich fractions showed higher anti-HIV activity than the low molecular weight fractions.

Anti-influenza virus activity. The cacao husk lignin fractions [**7, 8**] also potently inhibited the cytopathic effect induced in the MDCK cells by the influenza virus infection. The cacao husk polysaccharide fraction [**8**] (EC_{50}=0.009 mg/mL; CC_{50}=1.447 mg/mL, SI=155) (Figure 2B) showed higher anti-influenza virus activity than the lignin carbohydrate complex fraction [**7**]

Table I. *Anti-HIV activity of cacao husk and cacao mass lignin fractions.*

	CC_{50} (μg/mL)	EC_{50} (μg/mL)	SI	Yield (%)
1. Cacao husk (acid precipitation)	342	3.7	92	2.10
2. Cacao husk (50% ethanol precipitation)	110	3.7	30	3.00
3. Cacao husk (83% ethanol precipitation)	361	299	1.2	0.75
4. Cacao mass (acid precipitation)	107	22	4.9	4.00
5. Cacao mass (50% ethanol precipitation)	>1000	542	> 1.8	0.85
6. Cacao mass (83% ethanol precipitation)	494	115	4.3	0.77
7. Cacao husk (acid precipitation)	28	0.08	331	4.20
8. Cacao husk (50% ethanol precipitation)	>1000	0.01	>100000	2.80
9. Cacao husk (83% ethanol precipitation)	>1000	5.7	>175	0.58
10. Cacao mass (acid precipitation)	618	12	50	9.90
11. Cacao mass (50% ethanol precipitation)	535	104	5	1.30
12. Cacao mass (83% ethanol precipitation)	>1000	24	>42	0.72
13. Cacao husk (acid precipitation)	21	0.04	511	3.40
14. Cacao husk (50% ethanol precipitation)	450	0.12	3800	2.70
15. Cacao husk (83% ethanol precipitation)	>1000	5.9	>168	0.88
16. Cacao mass (acid precipitation)	776	9.3	83	9.80
17. Cacao mass (50% ethanol precipitation)	376	97	3	2.00
18. Cacao mass (83% ethanol precipitation)	395	61	6	0.70
Positive controls				
Dextran sulfate (μg/mL)	361	0.077	4716	
Curdlan sulfate (μg/mL)	625	0.032	20143	
AZT (μM)	193	0.0053	36401	
ddC (μM)	1340	0.3	4412	

Each value represents mean from triplicate determinations.

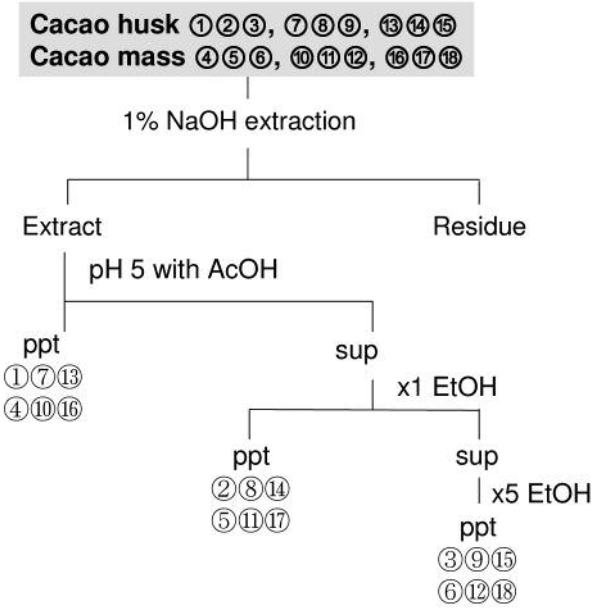

Figure 1. *Preparation of cacao husk and cacao mass lignin fractions. Ppt: precipitate, sup: supernatant.*

(EC_{50}=0.042g/mL; CC_{50}=1.093/mL, SI=26) (Figure 2A), consistent with the results observed for the HIV-infection experiments (Table I).

Table II. *Radical production by cacao husk lignin fractions.*

	Radical intensity		
Fraction	pH 7.0	pH 11	pH 13.5
Fr. 7	0.34	0.38	0.76
Fr. 8	0.31	0.31	0.54
Fr. 13	0.31	0.42	0.86
Fr. 14	0.27	0.33	0.41

0.1 M buffer (pH 7.0, 11.0 or 13.5): sample final 2 mg/mL.

Antibacterial activity. The lignin fractions from the cacao husk [**7, 8**] and the cacao mass [**10**] (1 mg/mL) showed no direct antibacterial activity against *Streptococcus mutans*, *Actinomyces viscosus*, *Fusobacterium nucleatum* and *Porphyromonas gingivalis* (data not shown).

Synergistic radical scavenging activity of lignin fraction and vitamin C. Lignin fractions produced typical broad peak, and the radical intensity was increased with the increase in pH (Table II). The lignin carbohydrate complex fractions from the cacao husk [**7, 13**] showed higher radical intensity than the polysaccharide-rich fractions [**8, 14**] (Figure 3).

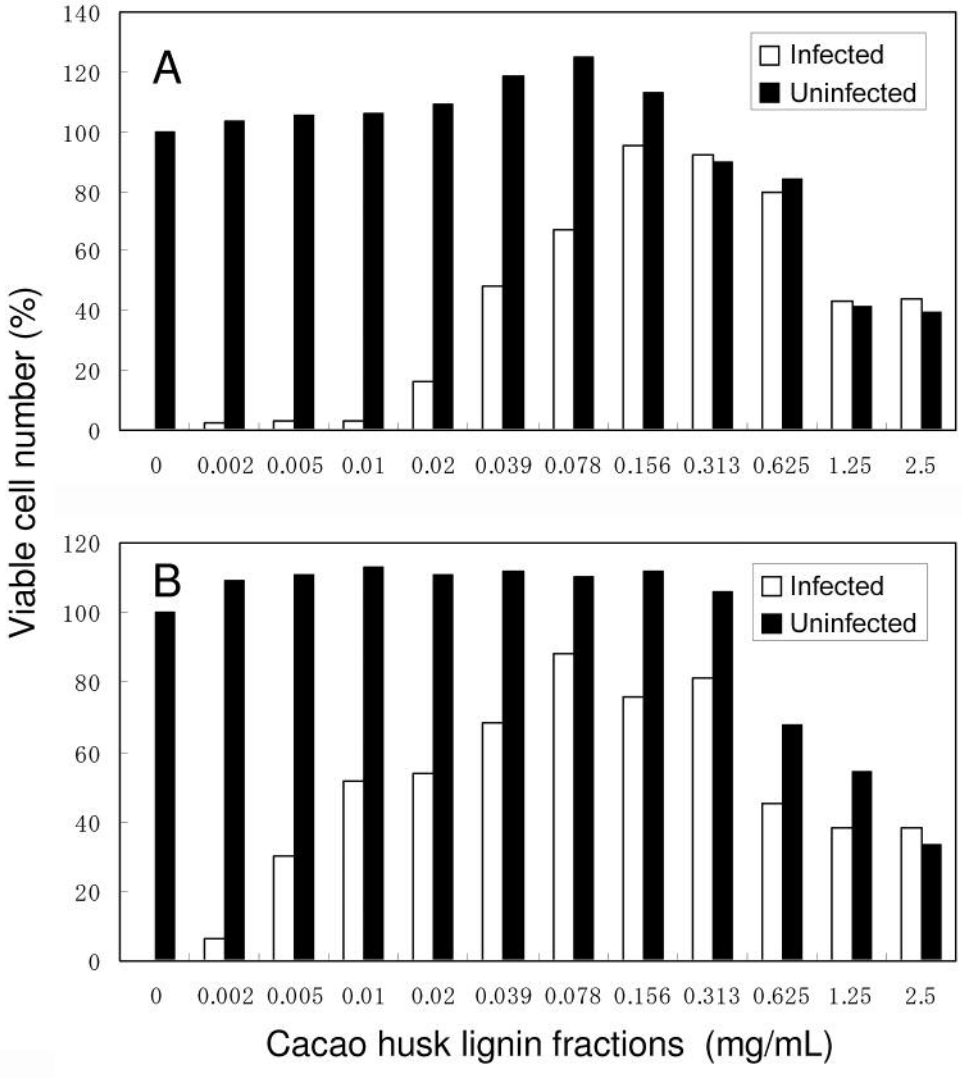

Figure 2. *Anti-influenza virus activity of cacao husk lipopolysahccharide-rich fraction. Influenza virus-infected (white bar) or mock-infected (black bar) MDCK cells were incubated for 3 days with the indicated concentrations of cacao husk lignin carbohydrate complex fraction [7] (A) or polysaccharide-rich fraction [8] (B), and the viable cell number was determined by MTT method. Each value represents mean from triplicate assays.*

Table III. *Synergistic superoxide scavenging activity of cacao husk lignin fractions and vitamin C (VC).*

	DMPO-OOH radical intensity (% of control)	
Fraction	100 μg/mL	50 μg/mL + 1.25 μM VC
Fr. 7	40.8	54.5<70.4 [(100+40.8)/2] synergism
Fr. 8	91.1	49.1<74.6 [(100+91.1)/2] synergism
Fr. 13	36.0	46.7<68.0 [(100+36.0)/2] synergism
Fr. 14	93.6	88.3<96.8 [(100+93.6)/2] synergism
2.5 μM VC	100.0	

Table IV. *Synergistic hydroxyl radical scavenging activity of cacao husk lignin fractions and vitamin C (VC).*

	DMPO-OH raidical intensity (% of control)	
Fraction	100 μg/mL	50 μg/mL + 12.5 μM VC
Fr. 7	72.4	39.2<53.0 [(33.6+72.4)/2] synergism
Fr. 8	82.0	61.4>57.8 [(33.6+82.0)/2]
Fr. 13	70.4	46.2<52.0 [(33.6+70.4)/2] synergism
Fr. 14	91.4	49.1<62.5 [(33.6+91.4)/2] synergism
25 μM VC	33.6	

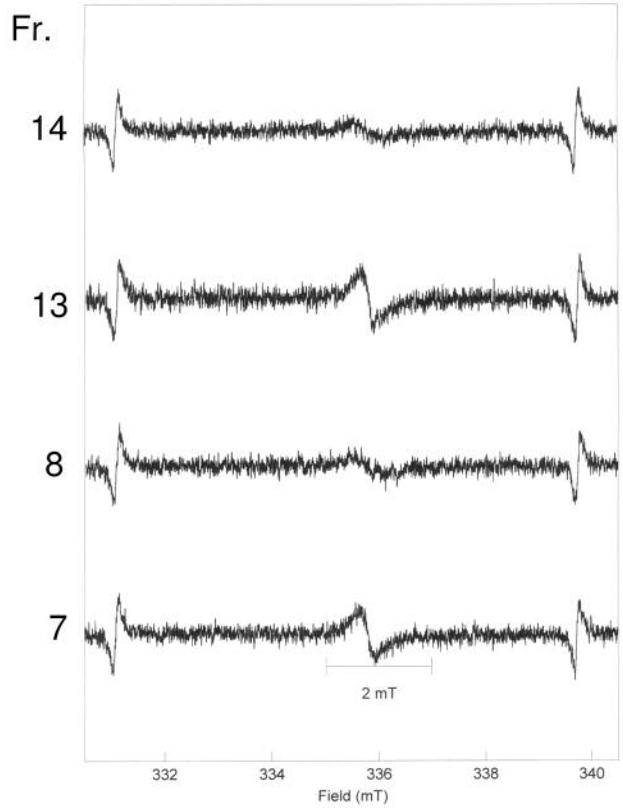

Figure 3. *Radical production by cacao husk lignin fractions. Radical intensity of each husk lignin fraction (2 mg/mL) was measured in 0.1 M KOH (pH 13.5).*

When the superoxide (generated by HX-XOD reaction) was mixed with DMPO, four radical peaks derived from the spin adduct (DMPO-OOH) were detected by ESR spectroscopy (data not shown). Sodium ascorbate (2.5 μM) alone did not show any superoxide scavenging activity, but in combination with the cacao husk lignin fractions [**7, 8, 13, 14**], the superoxide scavenging activity was synergistically enhanced (Table III).

When the hydroxyl radical (generated by the Fenton reaction) was mixed with DMPO, four radical peaks derived from the spin adduct (DMPO-OH) appeared (data not shown). Sodium ascorbate and the cacao husk lignin fractions [**7, 13, 14**] synergistically scavenged the hydroxyl radical (Table IV).

Macrophage activation. The lignin carbohydrate complex fractions from the cacao husk [**7, 13**] (3-50 μg/mL) stimulated the NO production by the RAW264.7 mouse macrophage-like cells, more potently than the corresponding polysaccharide-rich fractions [**8, 14**] (50-400 μg/mL) (Figure 4A, B). It should be noted that the maximum NO production stimulated by LPS (3-800 ng/mL) was only half that attained by the lignin carbohydrate complex fractions (Figure 4C).

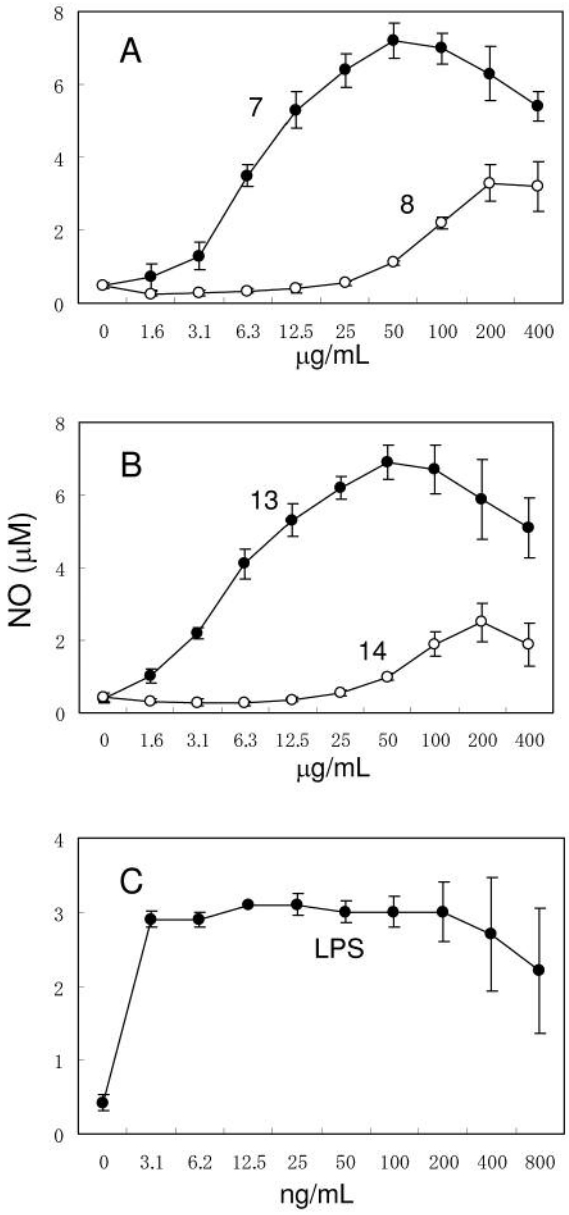

Figure 4. *Macrophage activation by cacao husk lignin fractions. RAW264.7 cells were incubated for 24 h with the indicated concentrations of cacao husk lignin fractions [**7, 8**] (A), [**13, 14**] (B) or LPS (C), and the NO released into the culture supernatant was determined by Griess Method.*

Discussion

The present study demonstrated the potent ant-HIV activity of the cacao husk lignin carbohydrate and polysaccharide-rich fractions. Cacao husk seems to be an attractive source for the mass production of anti-HIV substance, since its carbohydrate-rich fractions (SI=30-10,000) showed

surprisingly higher anti-HIV activity than that of tannins (SI=1-10) (8), flavonoids (SI=1) (10, 11) and natural lignins from other plant sources and dehydrogenation polymers of phenylpropenoids (so-called "synthetic lignin" without the sugar moiety) (SI=10-100) (12), and comparable with that of sulfated polysaccharide (dextran sulfate, curdlan sulfate) and reverse transcriptase inhibitors (AZT, ddC) (Table I). The higher anti-HIV activity of the cacao husk carbohydrate-rich fractions may be due to higher solubility and optimal molecular size. This fraction also had potent anti-influenza virus activity (Figure 2). The chemical analysis of lignin components such as the sugar moieties may be crucial to elucidate the potent antiviral activity of cacao lignin fractions. Whether or not sulfated polysaccharide and other polyphenols show a similar magnitude of anti-influenza virus activity should also be investigated.

The present study demonstrated that cacao husk lignin fractions synergistically enhanced the radical scavenging activity of vitamin C, similarly to lignins from mulberry juice (13) and pine cone extract (14). We have previously reported that pine cone lignin carbohydrate complex enhanced both the radical intensity and the cytotoxic activity of vitamin C (15). The synergism between lignin and vitamin C seems to be a universal phenomenon, suggesting its potential application in cosmetics. Our preliminary experiments showed that the cacao husk lignin carbohydrate complex prevented the cytotoxicity against human gingival fibroblasts induced by smoke (Sakagami *et al*., unpublished data).

The present study also demonstrated that, like LPS, the lignin carbohydrate complex activated mouse macrophages. It remains to be investigated whether lignin acts on the macrophage *via* a similar signaling pathway to LPS.

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