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Anti-HIV and Immunomodulation Activities of Cacao Mass Lignin–Carbohydrate Complex

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Abstract. *Background: Recently, a prominent antiviral and macrophage stimulatory activity of cacao lignin-carbohydrate complex (LCC) has been reported. However, the solubility and sterility of LCC have not been considered yet. In the present study, complete solubilisation and sterilisation was achieved by autoclaving under mild alkaline conditions and the previously reported biological activities were re-examined. Materials and Methods: LCCs were obtained by 1% NaOH extraction and acid precipitation, and a repeated extraction-precipitation cycle. Nitric oxide (NO) and cytokine productions were assayed by the Griess method and ELISA, respectively. Inducible NO synthase (iNOS) expression was determined by Western blot analysis. Superoxide anion, hydroxyl radical and nitric oxide radical-scavenging activity was determined by ESR spectroscopy. Results: Cacao mass LCC showed reproducibly higher anti-HIV activity than cacao husk LCC. Cacao mass LCC, up to 62.5 μg/ml, did not stimulate mouse macrophage-like cells (RAW264.7 and J774.1) to produce NO, nor did it induce iNOS protein, in contrast to lipopolysaccharide (LPS). Cacao mass LCC and LPS synergistically stimulated iNOS protein expression, suggesting a different point of action. Cacao mass LCC induced tumour necrosis factor-α production markedly less than LPS, and did not induce interleukin-1β, interferon-α or interferon–γ. ESR spectroscopy showed that cacao mass LCC, but not LPS, scavenged NO produced from NOC-7. Conclusion: This study demonstrated several new biological activities of LCCs distinct from LPS and further confirmed the promising antiviral and immunomodulating activities of LCCs.*

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Cocoa bean, the main raw material of chocolate, has been reported to display antioxidant (1), anti-arteriosclerotic (2), antibacterial (3) and antiviral (4) activities. The chemical analysis of the components of cacao, such as catechin, epicatechin, proanthocyanidin glycosides and related polyphenols (5) and lignin as food fibres (6), has been reported. Lignin–carbohydrate complexes (LCCs) have 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 LCCs has not been well characterized. In order to explore the novel functionality of cacao components, LCC was prepared from cacao husk (the shell of the cacao bean) and cacao mass (paste with cacao husk and germ removed). It was unexpectedly found that cacao husk LCC has higher HIV activity than cacao mass lignin fractions, synergistically enhances the superoxide anion and hydroxyl radical-scavenging activity of vitamin C, and stimulates nitric oxide (NO) generation by mouse macrophage-like cells (RAW264.7) (8). However, previous studies (8) have not considered the solubility and sterility of LCCs. The solubility of LCC generally decreases with increased molecular weight and the decreased solubility may make the sterilisation through a Millipore filter difficult. To deal with these problems, the solubility of LCCs was improved by suspension in 1.39% $NaHCO_3$ solution, and sterilisation by autoclaving, with subsequent re-investigation of biological activities. The present study

Table I. *Anti-HIV activity of cacao husk and cacao mass lignin fractions. Data are reported as mean±standard deviation.*

	Yield (%)	LPS contamination (%)	Maximum hormetic response (%)	Anti-HIV activity (SI)
Cacao mass lignin				
1st precipitation	9.6±1.1		11.3±5.1	61.3±24.1
2nd precipitation (repeated washing)	8.2±2.0	0.00035	7.2±4.8	39.0±23.6
Cacao husk lignin				
1st precipitation	4.8±1.8		15.1±0.6	44.5±22.1
2nd precipitation (repeated washing)	0.57±0.57	0.16	2.7±4.7	28.0±16.4
Dextran sulfate				1196
Curdlan sulfate				3292
AZT				10120
ddC				1536
LPS				><1.0

Each value represents the mean±S.D. from four different experiments.

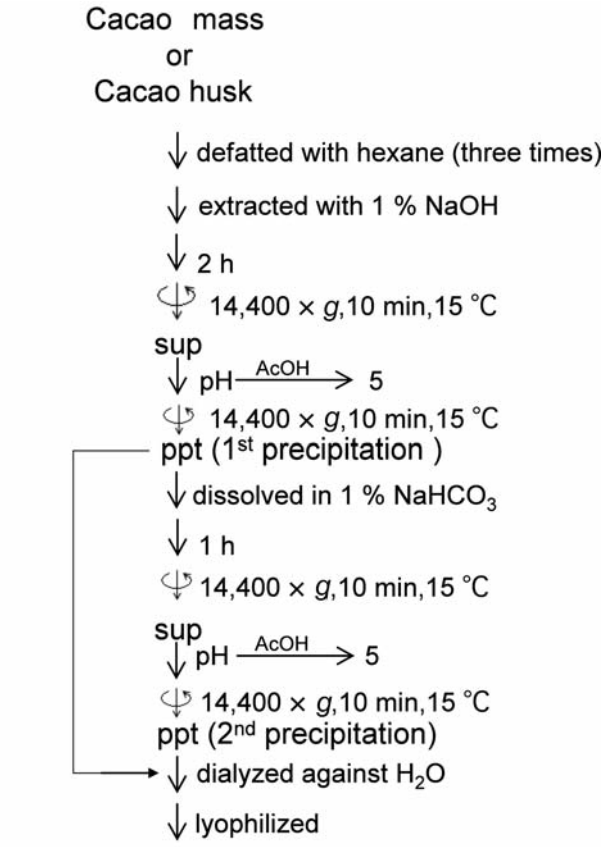

Figure 1. *Preparation of cacao mass and husk lignin fractions. ppt: Precipitate, sup: supernatant.*

confirmed the previous findings and further demonstrated several new biological activities of cacao mass LCC, distinct from those of lipopolysaccharide (LPS).

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 (GIBCO BRL, Grand Island, NY, USA); foetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA), RPMI-1640, 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), LPS from *Escherichia coli*, Serotype 0111:B4, phenylmethylsulfonyl fluoride (PMSF) (Sigma Chem. Co., St. Louis, MO, USA); dimethyl sulfoxide (DMSO) (Wako Pure Chem. Ind., Ltd., Osaka, Japan); curdlan sulfate (79 kD; Ajinomoto Co., Inc., Tokyo, Japan) and dextran sulfate (8 kD; Kowa, Tokyo, Japan).

Preparation of lignin fractions. Cacao mass or husk was defatted three times with hexane and then extracted for 2 h with 1% NaOH at room temperature (25˚C). After removal of the insoluble materials by centrifugation at 14,400×*g* at 15˚C for 10 minutes (Figure 1), the pH of the NaOH extract was adjusted to 5.0 by dropwise addition of acetic acid to precipitate the crude LCC (1st precipitate). Aliquots of crude LCC were dissolved in 1% $NaHCO_3$ and the insoluble materials were removed by centrifugation. The obtained supernatant was acidified by acetic acid to precipitate the washed LCC fraction (2nd precipitate). These two precipitates were dissolved in 1% $NaHCO_3$, dialysed against excess water and then lyophilised (Figure 1). The yield of LCC from cacao mask and husk was 9.6±1.1% (1st) and 8.2±2.0% (2nd), and 4.8±1.8% (1st) and 0.57±0.57% (2nd), respectively (Table I).

Assay for endotoxin contamination. LPS concentration was measured using the kinetic-chromogenic endotoxin-specific LAL reagent (Endospecy; Seikagaku Biobusiness Co., Tokyo, Japan), according to the Endotoxin Test in Japanese Pharmacopoeia, edition XV (9). Japanese Pharmacopoeia Standard Endotoxin (JPSE) 10000 was used as a standard in the assay. After confirming that LCC did not contain interfering factors with the LAL reaction, endotoxin contamination in the extracts was measured. Briefly, 50 μl of different concentrations of LLC or JPSE and 50 μl of the endotoxin-specific LAL reagent prepared according to the manufacturer instructions were added to each well in 96-well plates. The mixtures were incubated for 30 min at 37°C and during the incubation period, changes in the absorbance at 405 nm (reference at 492 nm) were monitored using a microplate reader (Wellreader SK603; Seikagaku Biobusiness Co., Tokyo, Japan).

Assay for anti-HIV activity. Human T-cell leukaemia virus I (HTLV-I)-bearing CD4-positive human T cell line, MT-4 cells, were cultured in RPMI-1640 medium supplemented with 10% FBS and

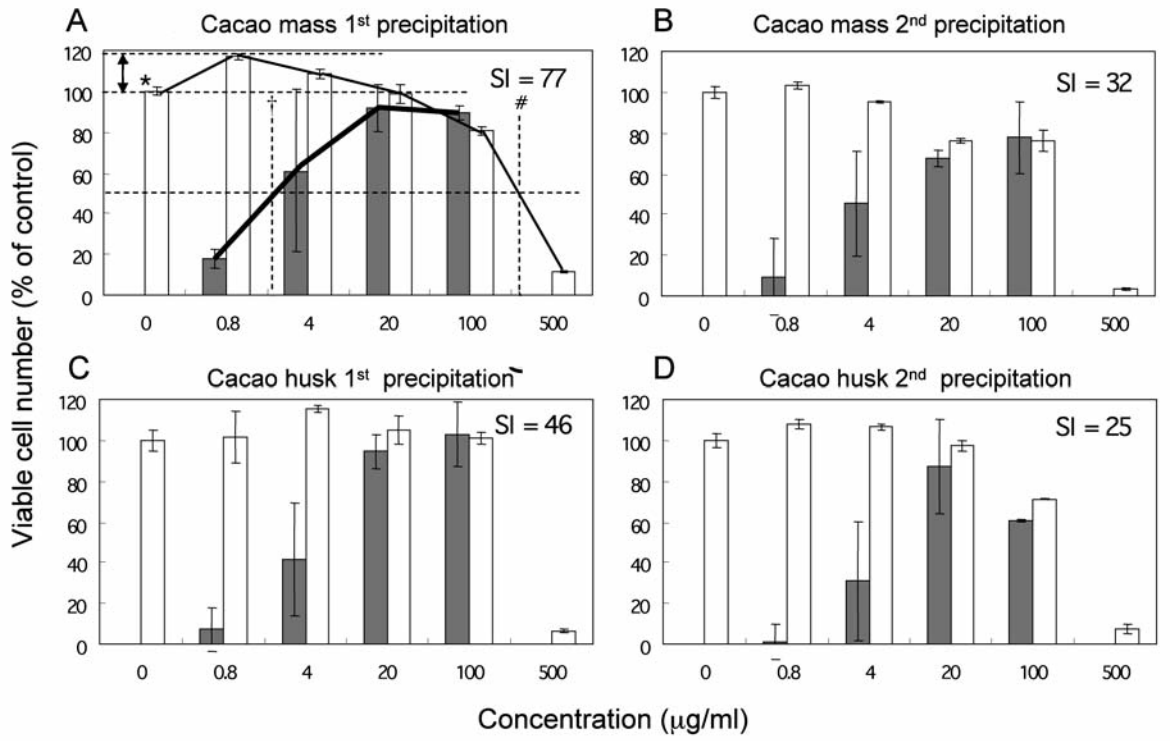

Figure 2. *Anti-HIV activity of cacao mass and husk LCCs. HIV-1_{IIIB}-infected (gray bars) and mock-infected (white bars) MT-4 cells were incubated for 5 days with the indicated concentrations of LCCs and the viable cell number was determined by the MTT method and expressed as a % of the control. Data represent the mean±standard deviation of three determinations. Data derived from four independent experiments with different batches of samples are summarised in Table I. †EC_{50}; #CC_{50}; *maximum hormetic response (%).*

infected with HIV-1_{IIIB} at a multiplicity of infection of 0.01. The HIV- or mock-infected (control) MT-4 cells (3×10^4 cells/96-microwell) were incubated for five days with different concentrations of test samples and the relative viable cell number was determined by MTT assay (10). 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 (10) (Figure 2A). The anti-HIV activity was evaluated by the selectivity index (SI), which was calculated by the following equation: $SI=CC_{50}/EC_{50}$ (10).

Assay for hormesis. The hormetic response was evaluated by the maximum response in each dose–response curve (Figure 2A), as described previously (11-12).

Effect on NO production by macrophages. Mouse macrophage-like cells RAW264.7 (13) or J774.1 (14) (6×10^4/ml) were inoculated into 96-microwell plates (Becton Dickinson, Labware, NJ, USA), and incubated for 24 h in DMEM supplemented with 10% heat-inactivated FBS. The medium was then replaced with phenol red-free DMEM containing 10% FBS and the indicated concentrations of test samples. After incubation for 24 h, the NO released into the culture supernatant was measured by the Griess method (15).

Assay for iNOS protein expression. RAW264.7 cells were inoculated at 3×10^5/ml in 24-well plates (Becton Dickinson) and incubated for 1-2 h. Near-confluent cells were treated for 24 h with different sample concentrations. The cell pellets were lysed with 50 μl of lysis buffer (10 mM Tris-HCl (pH 7.6), 1% Triton X-100, 150 mM NaCl, 5 mM EDTA-2Na, 2 mM PMSF and 1× Protease Inhibitor Cocktail Set I (Merck KGaA, Darmstadt, Germany)) for 10 min on ice. The cell lysates were centrifuged at 16,000×*g* for 20 min at 4˚C to remove the insoluble materials and the supernatant was collected. The protein concentrations of supernatant were measured by Protein Assay Kit (Bio Rad, Hercules, CA, USA). Equal amounts of the protein from cell lysates (10 μg) were mixed with 2×sodium dodecyl sulfate (SDS) sample buffer (0.1 M Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 0.01% bromophenol blue, 1.2% 2-mercaptoethanol), boiled for 10 min, and applied to the SDS-8% polyacrylamide gel electrophoresis, and then transferred to polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% non-fat skimmed milk in phosphate-buffered saline (PBS (–)) plus 0.05% Tween 20 for 90 min and incubated for 90 min at room temperature with anti-iNOS (dilution: 1:1,000; Santa Cruz Biotechnology, Delaware, CA, USA) or anti-actin antibody (dilution: 1:2,000; Sigma Chem. Co.), and then incubated with horseradish peroxidase-conjugated anti-rabbit (dilution: 1:2,000) or anti-mouse (dilution: 1:4,000) IgG for 60 min at room temperature. Immunoblots were detected by Western Lighting™ Chemilu minescence Reagent plus (PerkinElmer Life Sciences, Boston, MA, USA) (15).

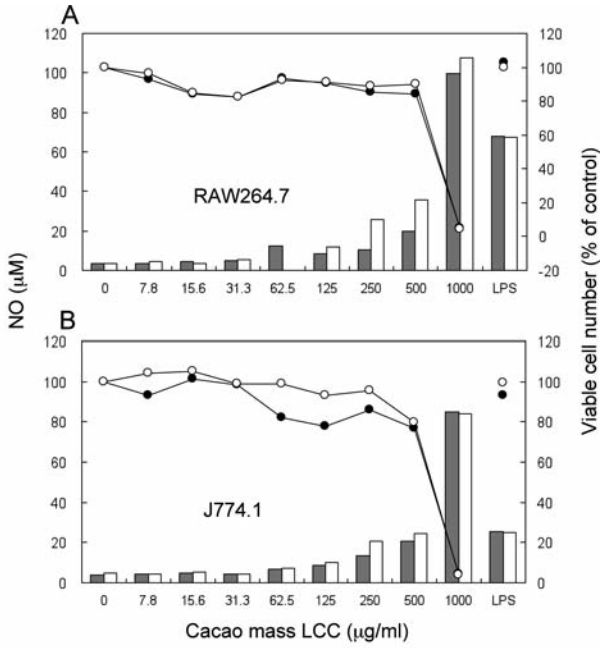

Figure 3. *Effect of cacao LCCs on NO production by mouse macrophage-like cells. RAW264.7 (A), and J774.1 cells (B) were incubated for 24 h with the indicated concentrations of cacao mass LCC. The extracellular NO concentration (bars) and the relative viable cell number (circles) were determined by the Griess and MTT method, respectively. White and gray symbols correspond to 1st and 2nd precipitation samples, respectively. Each value represents the mean from duplicate experiments. Cacao mass LCC had brownish colour and produced pseudopositive reaction (equivalent to 19-20 μmole NO/g) (data not shown), and these values were subtracted from the observed values.*

Assay for cytokine production. Mouse macrophage-like cells (RAW264.7, J774.1) were incubated for 24 h with samples, and the culture supernatants were assayed for the concentration of tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) or interferon-γ (IFN-γ) (R&D Systems Inc, Minneapolis, MN, USA), or IFN-γ (PBL Interferon Source, Piscataway, NJ, USA), using respective ELISA kits, according to manufacturer's instructions.

Radical-scavenging activity. The radical intensity was determined at 25˚C, using electron spin resonance (ESR) spectroscopy (JEOL JES REIX, X-band, 100 kHz modulation frequency) (16). The instrument settings were: centre field, 335.5±5.0 mT; microwave power, 16 mW; modulation amplitude, 0.1 mT: gain, 630; time constant, 0.03 s and scanning time, 2 min.

For the determination of the superoxide anion (in the form of DMPO-OOH), produced by the HX-XOD reaction (total volume: 200 μl) (2 mM HX in 0.1 M phosphate buffer (PB) (pH 7.4) 50 μl, 0.5 mM DETAPAC 20 μl, 8% DMPO 30 μl, sample (in PB) 40 μl, PB 30 μL, XOD (0.5 U/ml in PB) 30 μl), the time constant was changed to 0.03 s (16).

For the determination of the hydroxyl radical (in the form of DMPO-OH), produced by the Fenton reaction (200 μl) (1 mM $FeSO_4$ (containing 0.2 mM DETAPAC) 50 μl, 0.1 M PB (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 160 (16).

The radical intensity of NO, produced from the reaction mixture of 20 μM carboxy-PTIO and 60 μM NOC-7, was determined in 0.1 M PB (pH 7.4) in the presence of 30% DMSO (microwave power and gain were changed to 8 mW and 400, respectively). When NOC-7 and carboxy-PTIO were mixed, NO was oxidised to NO_2 and carboxy-PTIO was reduced to carboxy-PTI, which produces seven-line signals. Samples were added 3 min after mixing. The NO radical intensity was defined as the ratio of the signal intensity of the second peak (indicated by arrows in Figure 5) to that of MnO (16).

Statistical analysis. Data are reported as mean ± standard deviation. The difference between control and treated groups were compared by Student's *t*-test (Microsoft Excel Analysis).

Results

Yield. The yield of LCC prepared from the cacao mass (9.6±1.1%) was almost twice that prepared from the cacao husk (4.8±1.8%) (1st precipitate in Table I). By repeated cycling of alkaline solubilisation and acid precipitation, the yield was reduced only by 15% for cacao mass LCC, but by 88% for cacao husk LCC (2nd precipitate in Table I).

Anti-HIV activity. To accurately evaluate the anti-HIV activity of LCCs, it was essential for them to be completely dissolved and sterilised. Autoclave treatment (121˚C, 20 min) in 1.39% $NaHCO_3$ resulted in complete dissolution, and the molecular weight estimated by gel filtration exceeded 100 kDa (data not shown), suggesting the maintenance of the integrity of higher molecular-weight structures even after autoclave treatment. Autoclaved cacao mass LCC showed higher anti-HIV activity (SI=77) (Figure 2A) than that of autoclaved cacao husk (SI=46) (Figure 2C). Superiority of cacao mass LCC over cacao husk LCC was not changed after repeated cycling of alkaline solubilisation and acid precipitation (Figure 2B, D). This finding was confirmed by another three independent experiments with different batches of LCC preparations (Table I). Cacao mass LCC contained 0.00035% (w/w) of LPS, when the relative activity was assumed as 1 EU=0.1 ng. LPS (0.000256-100 μg/ml) did not show any cytotoxicity (CC_{50}>100 μg/ml) nor anti-HIV activity (EC_{50}>100 μg/ml), yielding the SI value of ><1.0 (Table I). Lower concentrations (0.8-4.0 μg/ml) of LCCs stimulated the growth of MT-4 cells slightly (maximum hormetic response=15.1%) (Table I).

Effect on NO production. Cacao mass LCC, at lower concentration ranges (7.8-125 μg/ml), prepared by either single or repeated cycles of alkaline solubilisation and acid precipitation steps, did not stimulate the NO production by mouse macrophage-like RAW264.7 and J774.1 cells (Figure 3). Western blot analysis demonstrated that cacao mass LCC (0.015-50 μg/ml) alone did not stimulate iNOS protein

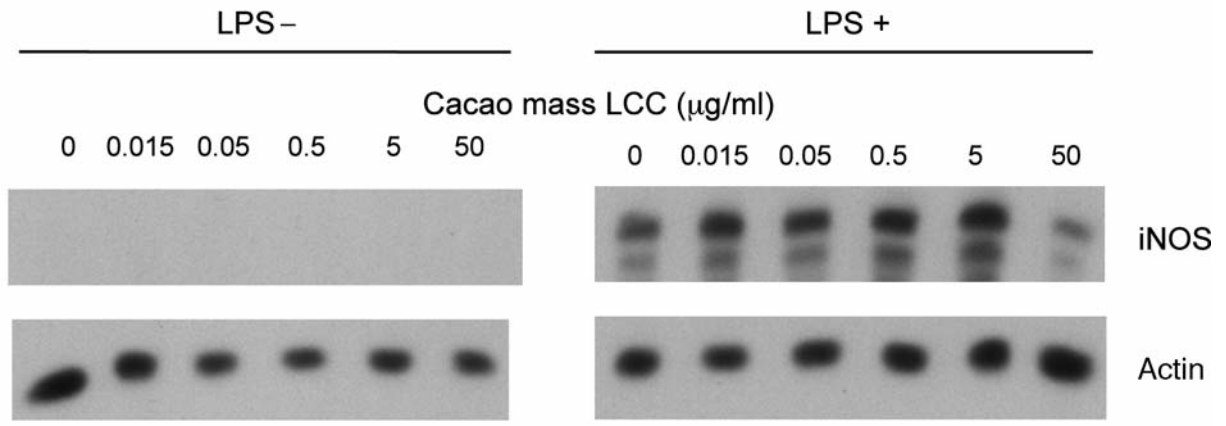

Figure 4. *Stimulation of LPS-induced iNOS protein expression by cacao mass LCC. RAW264.7 cells were incubated for 24 h with the indicated concentrations of cacao mass LCC in the presence or absence of LPS (100 ng/ml), and processed for Western blot analysis as described in the Materials and Methods.*

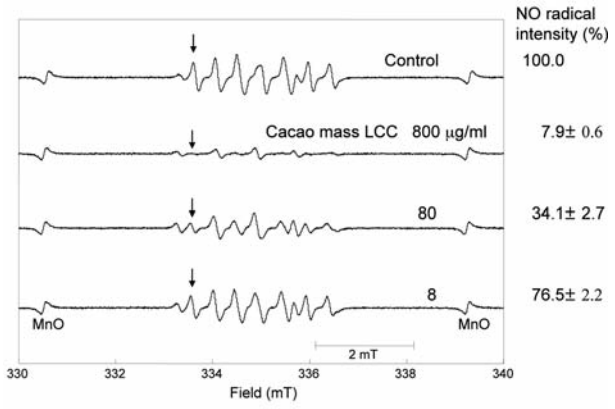

Figure 5. *NO radical-scavenging activity of cacao mass LCC. The radical intensity of NO generated from NOC-7 in the presence of carboxy-PTIO without (control) or with 8, 80 or 800 μg/ml of cacao mass LCC was measured with ESR spectroscopy. The NO radical intensity was determined by the second carboxy-PTI peak indicated by arrows, and expressed as a % of the control. Each value represents the mean±standard deviation from three independent experiments.*

expression in RAW264.7 cells, in contrast to LPS. It should be noted that cacao mass LCC enhanced LPS-induced iNOS protein expression, suggesting that the action points of cacao mass LCC and LPS may be different (Figure 4).

NO radical-scavenging activity. Cacao mass LCC scavenged NO radical generated from NOC-7 in the presence of carboxy-PTIO (Figure 5). The EC_{50} of cacao mass LCC was calculated to be 53 μg/ml. In contrast, LPS (up to 250 μg/ml) did not scavenge superoxide anion (generated by hypoxanthine-xanthine oxidase reaction), hydroxyl radical (produced by Fenton reaction), or NO radical (produced from NOC-7) (Figure 6A-C). Combination of LPS and vitamin C did not produce synergistic superoxide radical-scavenging activity (Figure 6D), in contrast to the combination of cacao LCC and vitamin C (8).

Effect on cytokine production. RAW264.7 and J774.1 cells spontaneously released TNF-α into the culture medium. Compared with RAW264.7 cells (Figure 7A), J774.1 cells spontaneously produced one order higher amounts of TNF-α (Figure 7B), in agreement with a previous report (17). Cacao mass LCC (7.8-500 μg/ml) increased TNF-α production in a dose-dependent manner. The decline of TNF-α production at 1,000 μg/ml was due to cell injury. Cacao mass LCC did not induce the production of IL-1β, IFN-α, or IFN-γ over a wide range of concentrations (0.005-0.5 μg/ml), in contrast to LPS (100 ng/ml) (Figure 8).

Discussion

There is a possibility that cacao mass may be originally contaminated with LPS corresponding to an important component of the outer membrane of Gram-negative bacteria because such bacteria are widely distributed in the natural environment. In addition, contaminated LPS is similarly extracted with alkaline solution and precipitated with acid during the isolation step of LCC. Most previous studies have not considered such LPS contamination in the LCC preparations. The alkaline extraction step that is necessary for the preparation of LCC has advantages and disadvantages. An advantage is the chemical inactivation of LPS. A disadvantage is the degradation of LCC into smaller sizes. Therefore, the conditions for alkaline extraction should be optimised to maximise LPS inactivation and minimise the loss of biological activity.

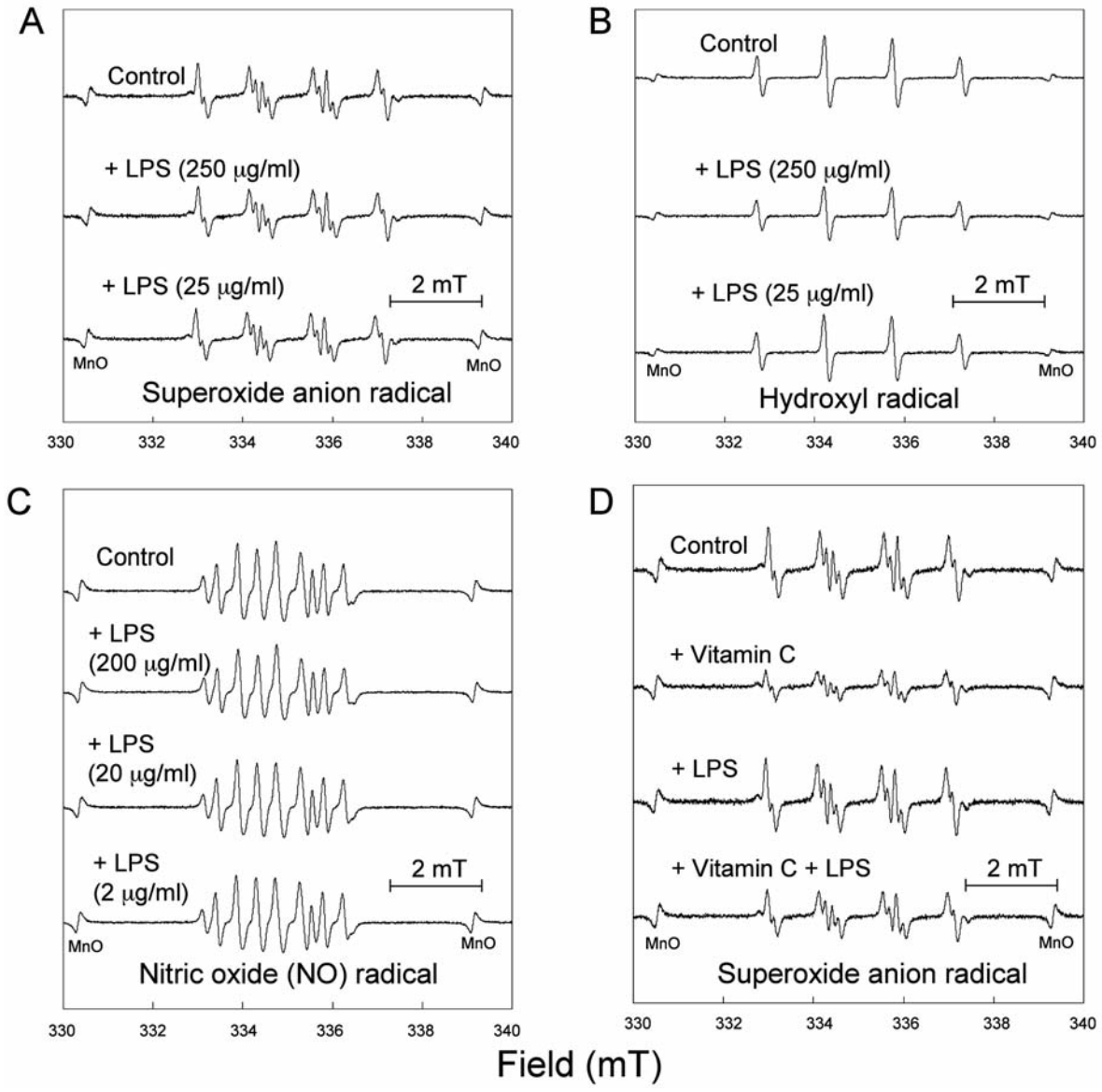

Figure 6. *Effect of LPS on the radical intensity of: (A) superoxide anion radical, measured as DMPO-OOH produced by hypoxanthine–xanthine oxidase reaction; (B) hydroxyl radical, measured as DMPO-OH produced by Fenton reaction; and (C) NO radical, measured as carboxy-PTI produced from NOC-7); (D) combination effect of LPS (2.5 μg/ml) and vitamin C (25 μM) on the superoxide anion radical intensity.*

The present study demonstrated that highly solubilised and sterilised cacao mass LCC, manufactured by autoclave treatment under mild alkaline conditions, shows higher anti-HIV activity (SI=39.0-61.3) than that of LCCs prepared from cacao husk (SI=28.0-44.5) and other eight plant species (SI= 26.8±30.0) (7).

The present study demonstrated that cacao mass LCC has several unique biological properties distinct from LPS, namely higher anti-HIV (Table I) and NO radical-scavenging activity (Figures 5 and 6) and the inability to induce iNOS protein (Figure 4) and cytokines (Figure 8). Furthermore, there was synergism between cacao mass LCC and LPS to induce the expression of iNOS protein (Figure 4). All these data suggest that the action points of cacao mass LCC and LPS may be different.

A recent DNA microarray analysis demonstrated that relatively higher concentrations of LCC from *Lentinus edodes* mycelia extract (LEM) induces the expression of various immune response-related genes, most of which overlap with those induced by LPS (18). LPS has been reported to induce the production of cytokines by the Toll-like receptor (TLR) signalling pathway through TLR4 (19) and to activate Janus kinase 2 (JAK2), which compose the

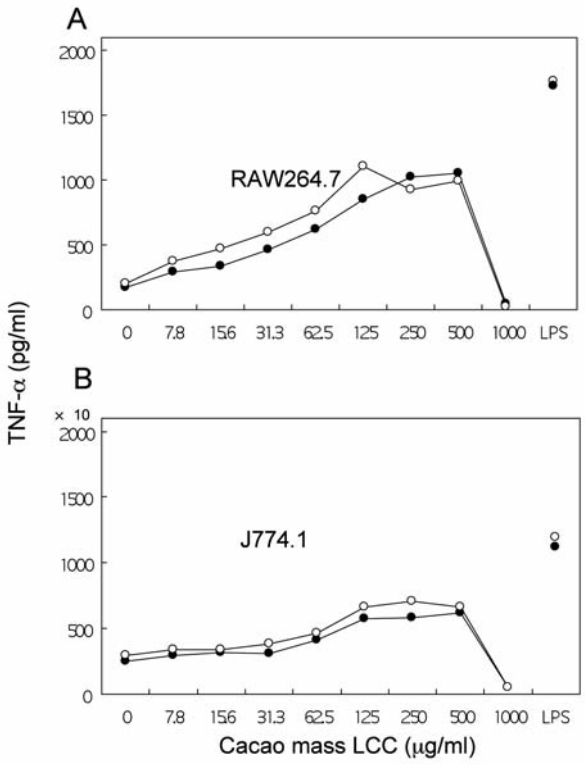

Figure 7. *Effect of cacao mass LCC on TNF-α production by mouse macrophage-like cells. RAW264.7 (A), and J774.1 cells (B) were incubated for 24 h with the indicated concentrations of cacao mass LCC and the extracellular TNF-α concentration was determined by ELISA. White and gray symbols represent the 1st and 2nd precipitation samples, respectively. The data represent the mean from experiments performed in duplicate. Cacao mass LCC produced only a background level of pseudopositive reaction for TNF-α determination (<16 pg/ml TNF-α, respectively) (data not shown).*

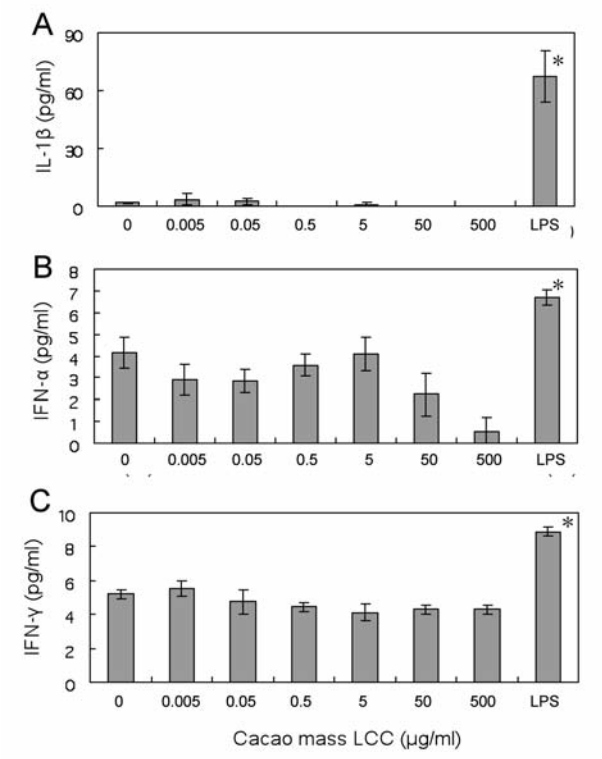

Figure 8. *Effect of cacao mass LCC on production of other cytokines by RAW264.7 cells. RAW264.7 cells were incubated for 24 h with different concentrations of cacao mass LCC or with LPS (100 ng/ml). The extracellular concentrations of IL-1β (A), IFN-α (B) and IFN-γ (C) were determined by ELISA. The data represent the mean ± standard deviation from experiments performed in triplicate. Cacao mass LCC produced only a background level of pseudopositive reaction for IL-1β, IFN-α and IFN-γ determination (data not shown).* * *p<0.05*.

JAK-STAT (signal transducer and activator of transcription) signalling pathway (20). Both LEM-LCC and LPS have similar bioactivity with regard to immune response-related gene expression; however, LPS more strongly affected immune response-related gene expression than LEM-LCC (18). It remains to be investigated whether cacao mass LCC induces similar changes in gene expressions.

It was previously reported that protein-bound polysaccharide, PSK, stimulated the differentiating-inducing activity of TNF-α (21) and IFN-γ (22) against human myelogenous leukaemia cells and towards maturing macrophage-like cells. It remains to be investigated whether cacao mass LCC may stimulate the biological activity of TNF-α and IFN-γ.

The present study revealed that cacao LCCs stimulate the growth of MT-4 cells by inducing hormesis. This hormetic assay may be useful to detect LCC-sensitive cells that may express putative LCC receptor on the cell surface membrane.

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