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Lignin-like Activity of *Lentinus edodes* Mycelia Extract (LEM)

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Abstract. *Background: In order to investigate the physiological role of lignin carbohydrate complex present in Lentinus edodes mycelia extract (LEM), this material was separated into seven fractions. Materials and Methods: Three high molecular weight fractions (Frs. I-III) were prepared from the water extract by successive ethanol fractionation, dialysis and lyophilization. Four higher molecular weight fractions were prepared from the NaOH extract of the residue, followed by acid precipitation (Fr. IV) and stepwise ethanol precipitation (Frs. V-VII). Results: All fractions showed higher anti-HIV activity than the water extract. Fr. IV showed the highest anti-HIV activity and most potently inhibited the NO production by LPS-stimulated mouse macrophage-like cells (RAW264.7, J774.1). ESR spectroscopy demonstrated that all fractions scavenged superoxide anion and hydroxyl radical. These properties are similar to those displayed by lignin carbohydrate complex, but not by glucans. HPLC analysis demonstrated the presence of lignin precursors, but not that of tannins, flavonoids and their related compounds. Conclusion: These results suggest a significant role of lignin-like substances in the expression of several important biological properties displayed by LEM.*

Oral intake of *Lentinus edodes* mycelia extract (LEM) has shown diverse pharmacological activity including anti-hepatopathic (1-3), antitumor (4-8), immunopotentiating (9), anti-vascularization (10) and anti-arteriosclerosis activities (11). Application of LEM cream liniment to animal skin stimulated blood flow in the skin (12), suggesting its possible moisturing and anti-wrinkle effects on the skin. LEM prevented the pigmentation induced by UVB irradiation in the three-dimensional skin cell culture model of epidermal keratinocytes and melanocytes (13), suggesting possible skin-conditioning properties. Many people are aware that LEM improves hepatic function (assessed by the reduction of AST, ALT, γ-GTP), menorrhagia, melancholia, nausea and vomiting, but there was no scientific documentation. Mushroom products have previously been reported to display immunopotentiating and antitumor activities (14). Undocumented clinical data indicating that the application of LEM cream significantly reduced melanin and erythema, suggest its potential anti-aging properties. Several polysaccharides, such as lentinan (15) and KS-2 (16), isolated from *Lentinus edodes* have shown immunopotentiating and antitumor activities.

On the contrary, only a limited number of studies have focused on the anti-HIV (17) and immunopotentiating (18) activity of lignin fractions of the hot water extract of LEM. We demonstrate here that lignin carbohydrate fractions in the alkaline extracts of LEM show higher anti-HIV activity than those of water extract, and other lower molecular weight polyphenols, using the parameter of selectivity index. We also report on the radical scavenging activity of these fractions and their effects on the production of nitric oxide (NO) by lipopolysaccharide (LPS)-activated mouse macrophage-like cells.

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Key Words: LEM, *Lentinus edodes* mycelia, lignin carbohydrate complex, anti-HIV, NO, macrophage, radical, ESR.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL, Gland Island, NY, USA); fetal bovine serum (FBS) (JRH, Bioscience, Lenexa, KS, USA), RPMI1640, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), LPS from *Escherichia coli* (Serotype 0111:B4), hypoxanthine (HX),

xanthine oxidase (XOD), azidothymidine (AZT), 2',3'-dideoxycytidine (ddC) (Sigma Chem Co., St Louis, MO, USA); dimethyl sulfoxide (DMSO) (Wako Pure Chem. Ind., Osaka, Japan); diethylenetriaminepentaacetic acid (DETAPAC), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) (Dojin, Kumamoto, Japan).

Preparation of LEM. *Lentinus edodes* mycelia was sterilely inoculated on solid media composed of bagasse (sugar cane fiber) and defatted rice bran, and cultured for 7 months under constant temperature, humidity and luminous intensity. Before sprouting, the culture medium was ground, digested with enzymes, extracted with hot water and then purified (Figure 1). LEM is a brownish powder, and certified as non-toxic material, based on the toxicity test after repeated oral administration and chromosomal aberration test. LEM contains polyphenols, lignin, amino acid, β-glucan, eritadenine and vitamin as functional components.

Fractionation of LEM. The water extract accounted for approximately 74±6% (w/w) of LEM, and this value was almost the same, regardless of the temperature (0 or 90˚C) during the water extraction (data not shown). Therefore, LEM was first suspended in cold water briefly, and centrifuged at 14,400 ×g for 10 min to collect the supernatant (referred to as water extract) (Figure 2). To this water extract, 1-, 2- and 5-fold volumes of ethanol were added successively to precipitate fractions I, II and III. These fractions were extensively dialyzed against water and lyophilized to dryness at the yield of 5.9, 7.8, and 2.8% (mean of two independent experiments). The residue was extracted with 1% NaOH at room temperature. The NaOH extract was acidified to pH 5 with drop wise addition of acetic acid to precipitate Fraction IV, and the resultant supernatant was mixed successively with 1-, 2- and 5-fold volumes of ethanol to precipitate fractions V, VI and VII. These fractions (IV, V, VI, VII) were dialyzed and lyophilized to dryness at the yield of 0.54, 0.82, 1.4, and 0.51%, respectively (mean of two independent experiments) (Figure 2).

Assay for anti-HIV activity. Human T-cell leukemia MT-4 cells were infected with HIV-1_{IIIB} at a multiplicity of infection (m.o.i.) of 0.01. HIV- or mock- infected 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. All data represent the mean values of triplicate measurements. The anti-HIV activity was evaluated by selectivity index (SI), which was calculated by the following equation: $SI=CC_{50}/EC_{50}$ (19).

Radical-scavenging activity. The radical intensity of samples was determined at 25˚C, using electron-spin resonance (ESR) spectroscopy (JEOL JES REIX, X-band, 100 kHz modulation frequency) (20). For the determination of hydroxyl radical (in the form of DMPO-OH), produced by 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 50 μl in H_2O, 1 mM H_2O_2, 30 μl], the gain was changed to 400 (20).

Assay for NO production. Mouse macrophage-like RAW264.7 (21) or J774.1 (22) cells were inoculated at 0.8×10^6/ml (100 μl) in a 96-microwell plate and incubated for 1-2 hours. Near confluent cells were treated for 24 hours with the indicated concentrations of text samples (0-1000 μg/ml) in phenol red-free DMEM supplemented with 10% FBS in the presence or absence of LPS (0.1 μg/ml). The NO released into the culture medium was quantified by Greiss reagent, using the standard curve of NO_2^-. The concentration that inhibited the LPS-stimulated NO production by 50% (50% inhibitory concentration: IC_{50}) was determined from the dose–response curve (21). The efficacy of inhibition of NO production was evaluated by the selectivity index (SI), which was calculated using the following equation: $SI=CC_{50}/IC_{50}$.

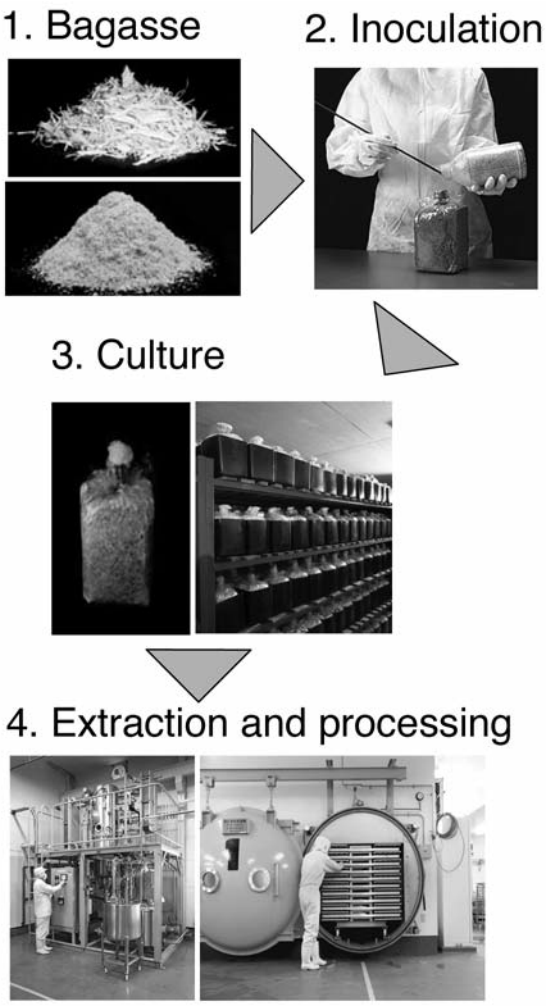

Figure 1. *Flow chart of LEM preparation*.

Analysis of composition of LEM. Chemical composition of LEM was analyzed by HPLC (Otsuka Pharmaceutical Co., Ltd, Tokyo).

Results

Anti-HIV activity. The water extract of LEM showed some anti-HIV activity. From the dose–response curve in mock-infected cells, 50% cytotoxic concentration (CC_{50}) of LEM was calculated to be >500 μg/ml. Infection with HIV-1

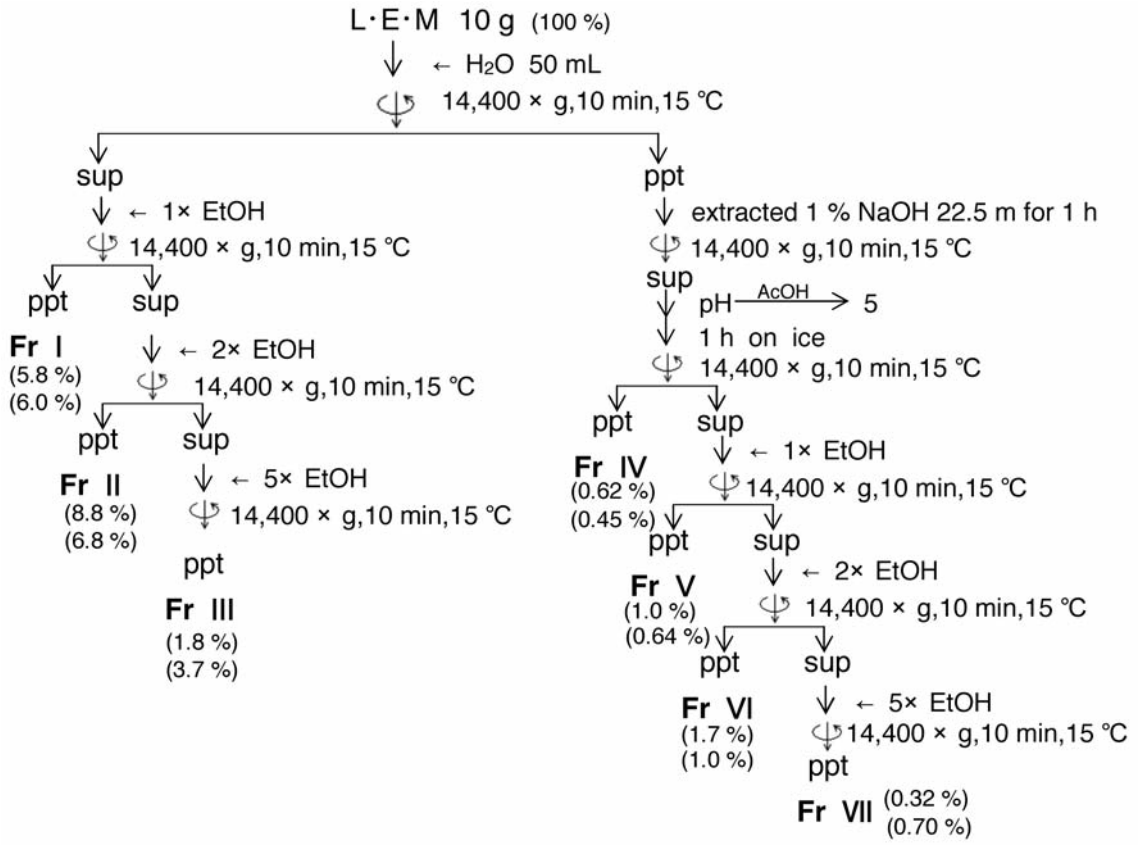

Figure 2. *Fractionation of LEM. Numbers in parentheses are the yield of dried powder in two independent experiments. Sup and ppt refer to the supernatant and precipitate after centrifugation, respectively.*

resulted in the complete cell death. With increasing concentrations of LEM, the cytopathic effect of HIV-1 was gradually diminished. The concentration that increased the viability to 50% (EC_{50}) was 50.7 μg/ml. The selectivity index (SI) was >9 (Figure 3, Experiment 1 in Table I).

The anti-HIV activity of high molecular weight fractions of water extracts (Fractions I, II, III) was investigated next. These fractions showed higher anti-HIV activity (SI=>61>32>14) than the water extract. Four fractions of the NaOH extract (Fractions IV, V, VI, VII) showed much higher anti-HIV activity (SI=>94>48>63>46) (Figure 3, Experiment 1 in Table I). Similar results were obtained in the fractions prepared on different occasion (Experiment 2 in Table I). Although the anti-HIV activity of the LEM fractions was much lower than that of popular anti-HIV agents [curdlan sulfate (300 kDa), dextran sulfate (8 kDa), 3'-azido-2',3'-dideoxythymidine (AZT), dideoxycytidine (ddC)] (SI=>13681, >1765, 6598, 2516) (Table II), it was much higher than that of tannins (SI=1-10) (19) and flavonoids (SI=1)(23, 24). The anti-HIV activity of the LEM fractions was comparable with that of natural lignin carbohydrate complex prepared from other plants (SI=10-100) (25, 26) and synthetic lignin (dehydrogenation polymers of phenylpropenoids) (SI=100) (26). It should be noted that all LEM fractions failed to induce the growth stimulating effects observable at lower concentrations, the so-called "hormetic effect" (27).

Antioxidant activity. Radical generation by LEM fractions was investigated by ESR spectroscopy. The water extract of LEM (final concentration: 3 mg/ml) produced radicals under alkaline conditions [0.1 M $NaHCO_3/Na_2CO_3$ buffer (pH 11)] (g value=1.96) (data not shown). All LEM fractions produced higher intensity of radical. The order of radical intensity (expressed as the ratio to the height of MnO, an extrinsic standard) is the following order: Fraction III (2.42)>VII (1.92)>II (1.73)>VI (0.90)>IV (0.86)>V (0.52)>I (0.38).

The radical scavenging activity of LEM was investigated. Since radical species are unstable, and have a short half-life, the radical was trapped by 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), a spin-trapping agent. In the presence of DMPO, the superoxide anion radical (O_2^-) and the hydroxyl radical are detectable by ESR as DMPO-OOH and DMPO-OH radicals, respectively. First, the scavenging activity of O_2^-

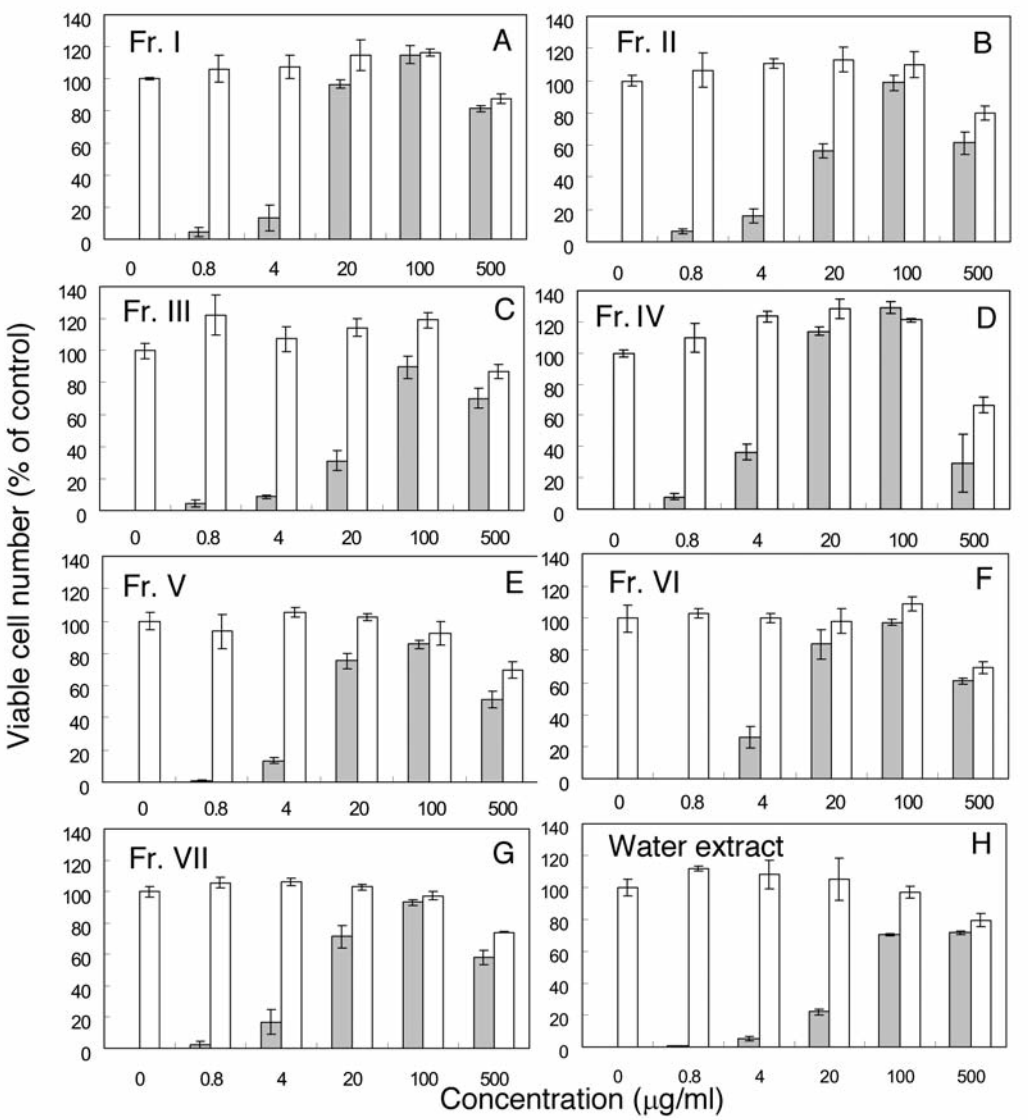

Figure 3. *Anti-HIV activity of LEM fractions. HIV-1_{IIIB}-infected (gray color) and mock-infected (white color) MT-4 cells were incubated for 5 days with the indicated concentrations of LEM fractions, and the viability was determined by MTT method and expressed as % of control. Each value represents mean±S.D. of triplicate determinations.*

generated by hypoxanthine (HX)-xanthine oxidase (XOD) reaction was investigated. The O_2^- scavenging activity [expressed as the decrease (%) of DMPO-OOH radical intensity by addition of LEM fraction (150 μg/ml)] was in the following order: I (66.1)>water extract (65.1)>III (64.2)>II (62.8)>VII (62.3)>VI (60.1)>V (42.3)>IV (41.0). On the other hand, glucans such as schizophyllan (MW: 6000,000) (28), paramylon sulfate (substitution ratio=0.07%), dimethylaminoethyl paramylon (substitution ratio=10.3%)(29) were not able to scavenge O_2^- even at 2000 μg/ml [decrease (%)=5.5, 0, 24.9) (Table II).

The scavenging activity of the hydroxyl radical generated by the Fenton reaction was investigated next. The hydroxyl radical scavenging activity [expressed as the decrease of DMPO-OH radical intensity (%) by addition of LEM fraction (1500 μg/ml)] was in the following order: III (72.6)>water extract (69.4)>VII (66.9)>II (64.9)>I (59.0)>VI (58.3)>IV 41.9)>V (38.3) (Table II).

Anti-inflammatory activity. The LEM fraction alone did not stimulate the production of nitric oxide (NO) by mouse macrophage-like cells RAW264.7. Addition of lipopolysaccharide (LPS) (0.1 μg/ml) elevated the NO production (determined by NO released into culture medium) from 1-3 μM (base line) to 10-20 μM. The water extract of LEM inhibited the LPS-induced NO production in dose-

Table I. *Anti-HIV activity of LEM fractions.*

Fractions	CC_{50} (μg/ml)	EC_{50} (μg/ml)	SI
Exp. 1			
I	>500	8.1	>61
II	>500	15.5	>32
III	>500	33.5	>14
IV	>500	5.3	>94
V	>500	10.3	>48
VI	>500	7.9	>63
VII	>500	10.6	>46
Water extract	>500	50.7	>9
Exp. 2			
I	>500	9.4	>52
II	>500	7.8	>64
III	>500	37.2	>13
IV	>500	6.6	>75
V	>500	11.2	>44
VI	>500	6.9	>71
VII	>500	5.9	>85
Positive controls			
Curdlan sulfate	>1000	0.07	>13681
Dextran sulfate	>1000	0.57	>1765
AZT (μM)	98.2	0.015	6598
ddC (μM)	1895	0.75	2516

50% Cytotoxic concentration (CC_{50}) in mock-infected cells and the concentration that increased the viability to 50% in HIV-1-infected cells (EC_{50}) were determined from Figure 3. Selective index (SI) was determined by dividing the CC_{50} by EC_{50}.

Table II. *Radical scavenging activity of LEM fractions.*

	Superoxide radical (DMPO-OOH) intensity (%)	Hydroxyl radical (DMPO-OH) intensity (%)	
Fractions	150 μg/ml	150 μg/ml	1500 μg/ml
Exp. 1			
I	33.9±2.0	84.4±4.0	41.0±2.4
II	37.2±2.1	87.8±4.1	35.1±1.8
III	35.8±1.6	71.3±4.8	27.4±3.2
IV	59.0±5.1	87.4±2.9	58.1±4.0
V	57.7±1.7	90.6±1.1	61.7±2.5
VI	39.9±1.6	84.3±4.7	41.7±1.3
VII	37.7±1.1	75.2±3.3	33.1±2.2
Water extract	34.9±1.0	74.3±2.4	30.6±1.2
Exp. 2			
I	34.8±1.5	88.9±2.0	41.8±2.5
II	46.8±0.7	80.9±2.5	34.6±2.7
III	45.9±3.0	83.1±2.7	42.7±0.6
IV	78.2±2.6	88.5±1.7	66.3±5.2
V	62.8±2.7	86.0±9.9	55.4±1.6
VI	57.5±5.0	84.7±4.2	47.0±5.5
VII	47.3±0.7	80.7±3.2	53.3±2.7
Glucans	(2000 μg/ml)		
Schizophyllan	94.7		
Paramylon sulfate	100.9		
Dimethylaminoethyl paramylon	75.1		

The O_2^- (produced by HX-XOD reaction) and hydroxyl radical (produced by Fenton reaction) in the presence of increasing concentrations of each sample were trapped by DMPO as DMPO-OOH and DMPO-OH, and the intensity of these radicals were expressed as % of control (without treatment). Each value represents mean±S.D of triplicate determinations.

dependent manners [IC_{50}=343.9 μg/ml; CC_{50}>1000 μg/ml; SI (CC_{50}/IC_{50})=>2.9]. Among LEM fractions, fractions IV and VII most potently inhibited the LPS-stimulated NO production (SI=>11.0, >12.0) (Figure 4, Table III). LEM failed to induce NO production in another mouse-macrophage-like cell line J774.1. Fraction IV most potently inhibited the LPS-stimulated NO production by J774.1 cells (SI=>34.6) (Figure 5, Table III).

Discussion

The present study demonstrated several new biological activities of LEM. These include the anti-HIV activity, radical-scavenging activity, and the inhibition of NO production by activated macrophages. Among them, anti-HIV activity and radical-scavenging activities are common characteristics displayed by lignin carbohydrate complex, but not by glucans. This indicates that pharmacological actions of LEM may be displayed at least in part by lignin carbohydrate complex present. This is confirmed by our findings that lignin precursors such as vanillic acid (785.5 μg/g sample), caffeic acid (12.0), syringic acid (638.0),

Table III. *Inhibition of NO production by LPS-stimulated mouse macrophage-like cell lines by LEM fractions.*

	RAW264.7			J774.1		
Fractions	CC_{50} (μg/ml)	EC_{50} (μg/ml)	SI	CC_{50} (μg/ml)	EC_{50} (μg/ml)	SI
I	>1000	121.6	>8.2	214.7	124.6	1.7
II	>1000	123.1	>8.1	>1000	472.2	>2.1
III	>1000	260.0	>3.8	996.2	674.0	1.5
IV	>1000	91.0	>11.0	>1000	28.9	>34.6
V	803.2	111.7	7.2	872.1	113.0	7.7
VI	>1000	162.9	>6.1	>1000	200.0	>5.0
VII	>1000	83.4	>12.0	>1000	274.5	3.0
Water extract	>1000	343.9	>2.9	>1000	338.9	>3.0

The 50% cytotoxic concentration (CC_{50}) of LEM fractions and the concentration that inhibited the LPS-stimulated NO production by 50% (EC_{50}) were determined from Figures 4 and 5. Selective index (SI) was determined by dividing CC_{50} by EC_{50}.

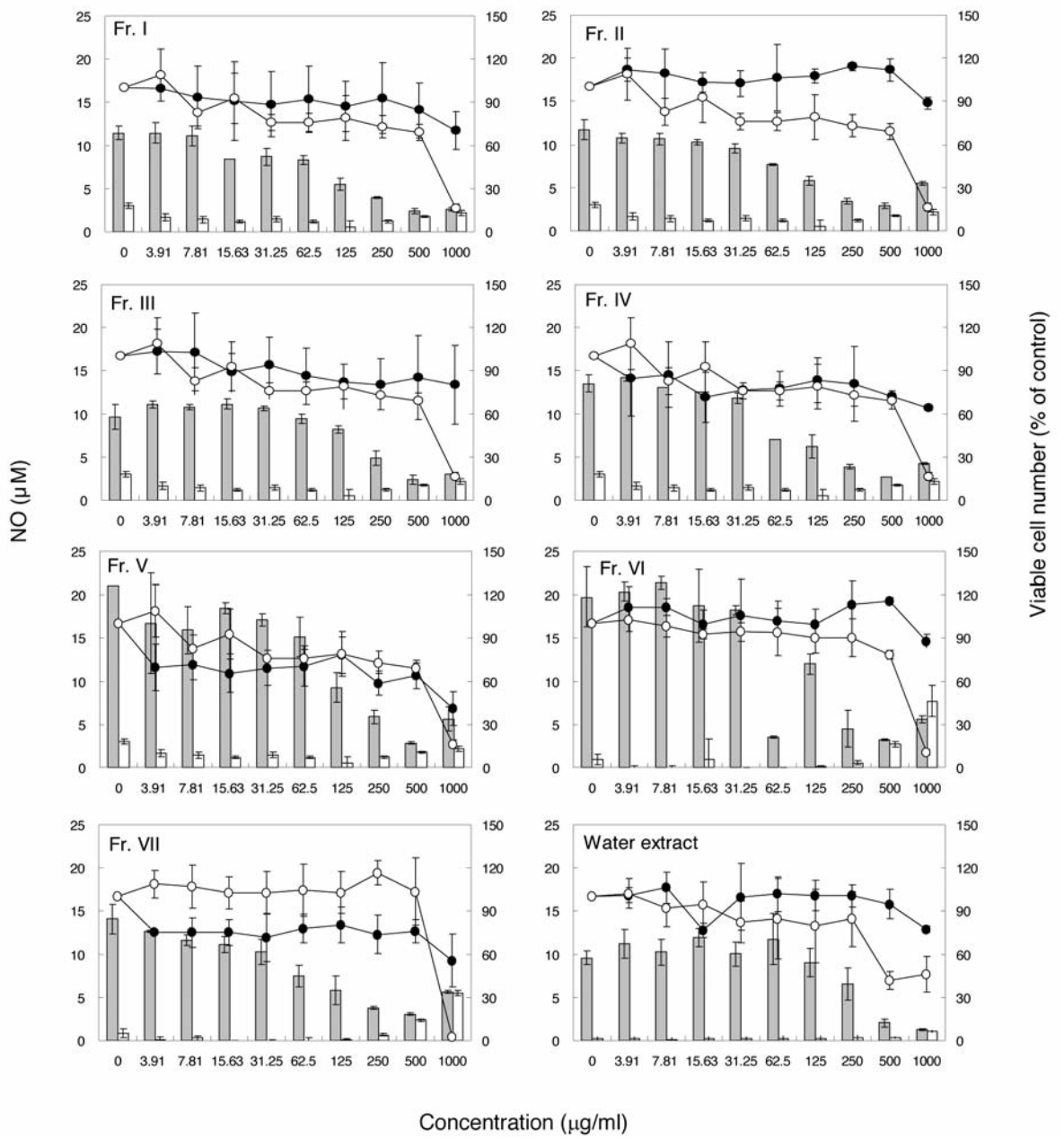

Figure 4. *Inhibition of NO production by LPS-stimulated RAW264.7 cells by LEM fractions. RAW264.7 cells were incubated for 24 hours with the indicated concentrations of each LEM fraction in the presence (color) or absence (no color) of 0.1 μg/ml LPS. The number of viable cells attached to 96-microwell plate (circles) and the NO released into the culture supernatant (bars) were then determined by the MTT and Griess methods, respectively. Each value represents the mean±S.D. of triplicate determinations.*

vanillin (59.5), ferulic acid (74.5), *p*-coumaric acid (277.0) were detected in LEM, whereas flavonoids (fisetin, daizein, genistein, quercetin, kaempferol, apigenin, chrysin), tannins and related compounds (gallic acid, gallo catechin, catechin, epicatechin, epigallocatechin gallate, gallocatechin gallate, epicatechin gallate, catechin gallate, ellagic acid, theaflavin-3-gallate, theaflavin-3'-gallate, theaflavin-3,3'-gallate) and chlorogenic acid and edugenol were undetectable (<0.1 μg/ml) (Table IV).

The anti-HIV activity of the hot water extract of LEM has been previously demonstrated (17). The present study demonstrated for the first time the higher anti-HIV activity of the alkaline extract of LEM compared to the water extract. We further observed that the lignin

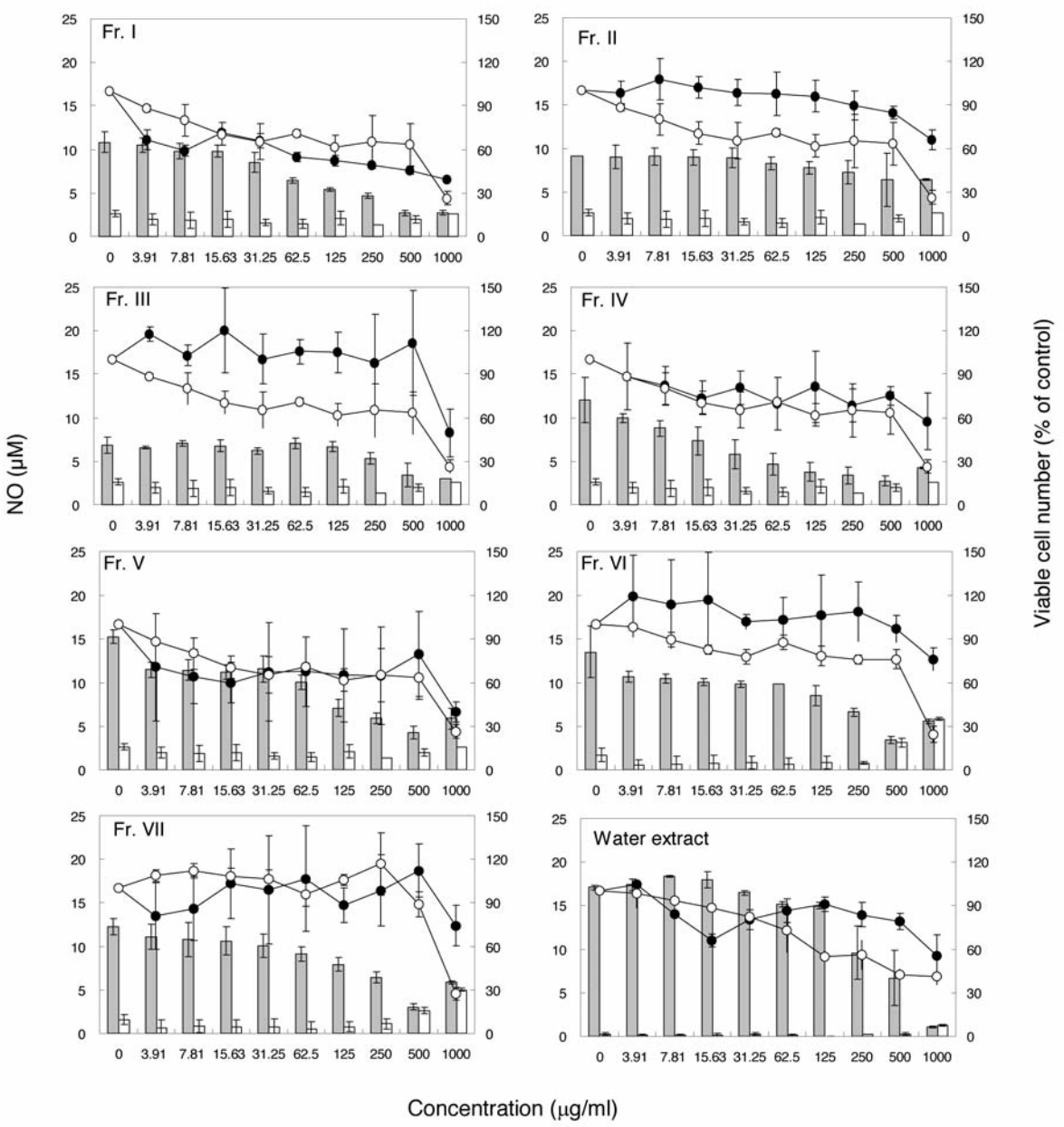

Figure 5. *Inhibition of NO production by LPS-stimulated J774.1 cells by LEM fractions. J774.1 cells were incubated for 24 hours with the indicated concentrations of each LEM fraction in the presence (color) or absence (no color) of 0.1 μg/ml LPS. The number of viable cells attached to 96-microwell plates (circles) and the NO released into the culture supernatant (bars) were then determined by the MTT method and Griess methods, respectively. Each value represents the mean±S.D. of triplicate determinations.*

carbohydrate fraction inhibited NO production by LPS-activated macrophages. Whether this resulted from the inhibition of iNOS expression or not remains to be investigated. Since the lignin carbohydrate complex fractions scavenged the superoxide and hydroxyl radicals, the possibility that the apparent reduction of NO production is simply due to NO-scavenging activity should also be investigated.

Recent publications of the biological activity of lignin carbohydrate complexes have been limited, possibly due to their structural complexity. Our group has also found that LEM fractions stimulated the TNF-α production of RAW264.7 cells (unpublished data). This suggests that LEM fractions modulate NO and TNF-α production by different mechanisms. Further studies are necessary to elucidate the mechanism of action of the lignin carbohydrate complex in LEM.

Table IV. *Detection of lignin precursors in LEM.*

	µg/g		µg/g
Lignin precursors		Tannins and related compounds	
Vanillic acid	785.5	Gallic acid	<0.1
Syringic acid	638.0	Catechin	<0.1
p-Coumaric acid	277.0	Epicatechin	<0.1
Ferulic acid	74.5	Epigallocatechin gallate	<0.1
Vanillin	59.5	Gallocatechin gallate	<0.1
Caffeic acid	12.0	Epicatechin gallate	<0.1
Syringaldehyde	<0.1	Catechin gallate	<0.1
o-Coumaric acid	<0.1	Ellagic acid	<0.1
		Theaflavin-3-gallate	<0.1
Flavonoids		Theaflavin-3'-gallate	<0.1
Fisetin	<0.1	Theaflavin-3,3'-gallate	<0.1
Daizein	<0.1		
Genistein	<0.1	Others	
Kaempferol	<0.1	Eugenol	<0.1
Apigenin	<0.1	Chlorogenic acid	<0.1
Galangin	<0.1	Salicylic acid	<0.1
Chrysin	<0.1	*p*-Hydroxy benzoic acid	737.0
Hesperetin	<0.1	*o*-Aminobenzoic acid	<0.1
Myricetin	<0.1	7-Hydroxycoumarin	<0.1

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