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Anti-stress, Anti-HIV and Vitamin C-synergized Radical Scavenging Activity of Mulberry Juice Fractions

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Abstract. *Anti-stress and anti-HIV activity of mulberry juice were separated by centrifugation. The anti-stress activity was enriched in the supernatant fraction whereas the anti-HIV activity in the precipitate fraction. Oral administration of the supernatant fraction significantly reduced the elevated plasma level of lipid peroxide in mice loaded with water immersion restraint stress. The kinetic study revealed that the anti-stress activity was maintained for 4 hours after cessation of the administration of mulberry juice. The lignin fraction in the precipitate fraction scavenged superoxide and hydroxyl radicals more efficiently than other fractions, in a synergistic fashion with sodium ascorbate. Anti-HIV activity of mulberry juice was concentrated in the lignin fraction, whereas blueberry juice, which has no precipitating fibrous materials, did not show anti-HIV activity. The present study suggests the functionality of mulberry juice as an alternative medicine.*

Mulberry fruit juice is rich in anthocyanins (1) and minerals such as iron and magnesium. Cyanidin-3-*O*-β-D-glucopyranoside isolated from mulberry fruits inhibited the cerebral ischemic damage caused by oxygen glucose deprivation in PC12 cells (2). The anthocyanin-rich fruits inhibited the copper-induced peroxidation of liposome and the co-oxidation of linoleic acid and β-carotene (3). Morin, a flavonoid present in mulberry and herbs, significantly reduced the tissue level of cyclosporin, a potent immunosuppressive agent and dramatically decreased nitric oxide production by the activated macrophages (4). Black mulberry juice inhibited human cytochrome CYP3A activity in a pooled human liver microsome system (5). These reports support the possibility that mulberry juice contains several antioxidants.

The anti-stress activity of straight mulberry juice against mice was recently described (6). When mice were subjected to water immersion restraint stress at 25˚C for 8 h, the plasma lipid peroxide level, determined by d-ROMs test performed 12 h thereafter, was almost doubled. Administration of mulberry juice completely blocked the lipid peroxidation. ESR spectroscopy showed that mulberry juice scavenged superoxide, hydroxyl and nitric oxide radicals at approximately 50% efficiency of blueberry juice, suggesting that the anti-stress activity of mulberry juice *in vivo* is derived from its radical scavenging activity (6). We also found that anti-HIV activity of mulberry juice was more than 4-fold than that of blueberry juice. The apparent difference between these two juices is that the former has insoluble fibers, while the latter does not. This suggests that the anti-HIV activity of mulberry juice is derived from some substances in the precipitate fraction, possibly lignified materials (7). To test this possibility, we fractionated the mulberry juice by the method used for lignin preparation

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and investigated which fractions of mulberry juice are responsible for the anti-HIV activity, as well as anti-stress and antioxidant activity.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Mulberry juice, blueberry juice (Nakamura Chiro Association, Shibuya-ku, Tokyo), dimethyl sulfoxide (DMSO) (Wako Pure Chem. Ind. Ltd., Osaka, Japan); hypoxanthine (HX), xanthine oxidase (XOD), diethylenetriamine-pentaacetic acid (DETAPAC), 3'-azido-2', 3'-dideoxythymidine (AZT), dideoxycytidine (ddC) (Sigma Chem. Co., St. Louis, MO, USA); 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO); curdlan sufate (79 kD, Ajinomoto Co. Inc., Tokyo), dextran sulfate (8 kD, Kowa, Tokyo, Japan).

Fractionation of mulberry juice. Mulberry juice was separated into the following two fractions by centrifugation at 5,000 rpm at 4˚C for 30 min, using Method I (Figure 1). The supernatant fraction (sup) was passed successively through Millipore filters with different pore sizes (1.0 and 0.45 μm) to remove insoluble materials. The precipitate fraction (ppt) was washed three times with distilled water, and finally reconstituted by re-suspension in 50 mL of distilled water (Method I, Figure 1).

Mulberry juice was also separated into six fractions using Method II (Figure 1). Mulberry juice (720 mL) was centrifuged at 8,000 xg at 4˚C for 10 min to fractionate into the supernatant (Fr. I) (yield 57 g) and precipitate. The precipitate was extracted at 90˚C for 2 hours with 100 mL of H_2O, and the hot water extract was successively mixed with 1 and then 5 volumes of ethanol to precipitate Fr. II (yield 0.11 g) and Fr. III (yield 0.28 g), respectively. The reside after hot water extraction was extracted for 2 hours at room temperature with 100 mL of 1% NaOH, and insoluble materials were removed by centrifugation at 8,000xg at 4˚C for 10 min. The pH of NaOH extract was adjusted to 5.0 by drop-wise addition of acetic acid to precipitate Fr. IV (yield 1.19 g). The supernatant obtained was successively mixed with 1 and then 5 volumes of ethanol to precipitate Fr. V (yield 0.11 g) and Fr. VI (yield 0.022 g), respectively (Method II, Figure 1). All fractions were extensively dialyzed against distilled water and lyophilized.

Determination of dietary fiber. Values of the soluble and insoluble dietary fibers were determined by the standard method (8) with slight modifications. The content of the soluble dietary fiber was determined to be 0.52%(w/w), and that of the insoluble dietary fiber, which contains lignin was 1.88%(w/w). The content of the total dietary fiber was 2.40%(w/w).

Anti-stress assay. BALB/c mice (Japan Charls River) (5-6 mice/group) were subjected to water immersion restraint stress at 25˚C for 12 h ("stress loading period"), and then they were administered *ad. lib.* water, mulberry juice (or its supernatant, or pellet fraction) for a total of 12 h ("stress expression period"), as shown in Figure 2. Mice were then anesthetized with ether and the blood was collected from the heart. Plasma was obtained as a supernatant after centrifugation at 3,000 rpm for 15 min at 25˚C and the plasma lipid peroxide level was determined using d-ROMs test (DIACRON s.r.l., Via Zircone, Italy) and expressed as CARR U. I CARR U=0.08 mg H_2O_2/dL (6).

Assay for anti-human immunodeficiency virus (HIV) activity. Human T-cell leukemia virus I (HTLV-I)-bearing CD4-positive human T cell line, MT-4 cells, were infected with HIV-1_{IIIB} at a multiplicity of infection (m.o.i.) of 0.01. 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 (9). All data represent the mean values of triplicate measurements. The anti-HIV activity was evaluated using a selectivity index (SI), which was calculated with the following equation:

$$SI = CC_{50}/EC_{50}$$

Radical scavenging activity. The radical intensity was determined at 25˚C in 0.1 M Tris-HCl (pH 8.0) or 0.1 M Na_2CO_3/$NaHCO_3$ (pH 10.0, 11.0), using ESR spectroscopy (JEOL JES REIX, X-band, 100 kHz modulation frequency) (6). Instrument settings: center field, 336.0±5.0 mT; microwave power, 8 mW; modulation amplitude, 0.1 mT; gain: 500; time constant: 0.1 sec; scanning time: 2 min.

For determination of superoxide anion (in the form of DMPO-OOH), produced by 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 DMSO) 40 μL, H_2O 30 μL, XOD (0.5 U/mL in PB) 30 μL), the time constant and scanning time were changed to 0.03 sec and 2 min, respectively.

For the determination of 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.

Antimicrobial activity. Streptococcus mutans, *Streptococcus gordonii* and *Actinomyces viscosus* were grown anaerobically at 37˚C in brain heart infusion broth (Difco Laboratories, Detroit, MI, USA). *Capnocytophaga ochracea*, *Fusobacterium nucleatum*, *Prevotella intermedia*, *Porphyromonas gingivalis* and *Porphyromonas melaninogenica* were cultivated anaerobically at 37˚C in heart infusion broth (Difco) supplemented with hemin (5 μg/mL) and menadione (50 μg/mL). *Candida albicans*, *Escherichia coli* and *Staphylococcus aureus* were grown aerobically at 37˚C in nutrient broth (Eiken Chemical Co., Ltd., Tokyo, Japan). Each bacterium was cultivated for 2 days in the respective medium with or without 10 mg/mL of mulberry juice Fr. I. Antimicrobial activity was determined by growth in each medium containing 10 mg/mL of Fr. I.

Results

Anti-stress activity. The plasma lipid peroxide concentration of untreated control mice was 96 CARR U. When mice were given water immersion restraint stress for 12 hours, and then returned to and maintained under normal unstressed condition for 12 h allowing the mice drink water, the level of plasma lipid peroxide was elevated to 217 CARR U (Figure 2). The first 12 h is referred to as the "stress loading period" and the following 12 hours as the "stress expression period". However, if mice were administered mulberry juice instead of water for the stress expression period, the elevation of

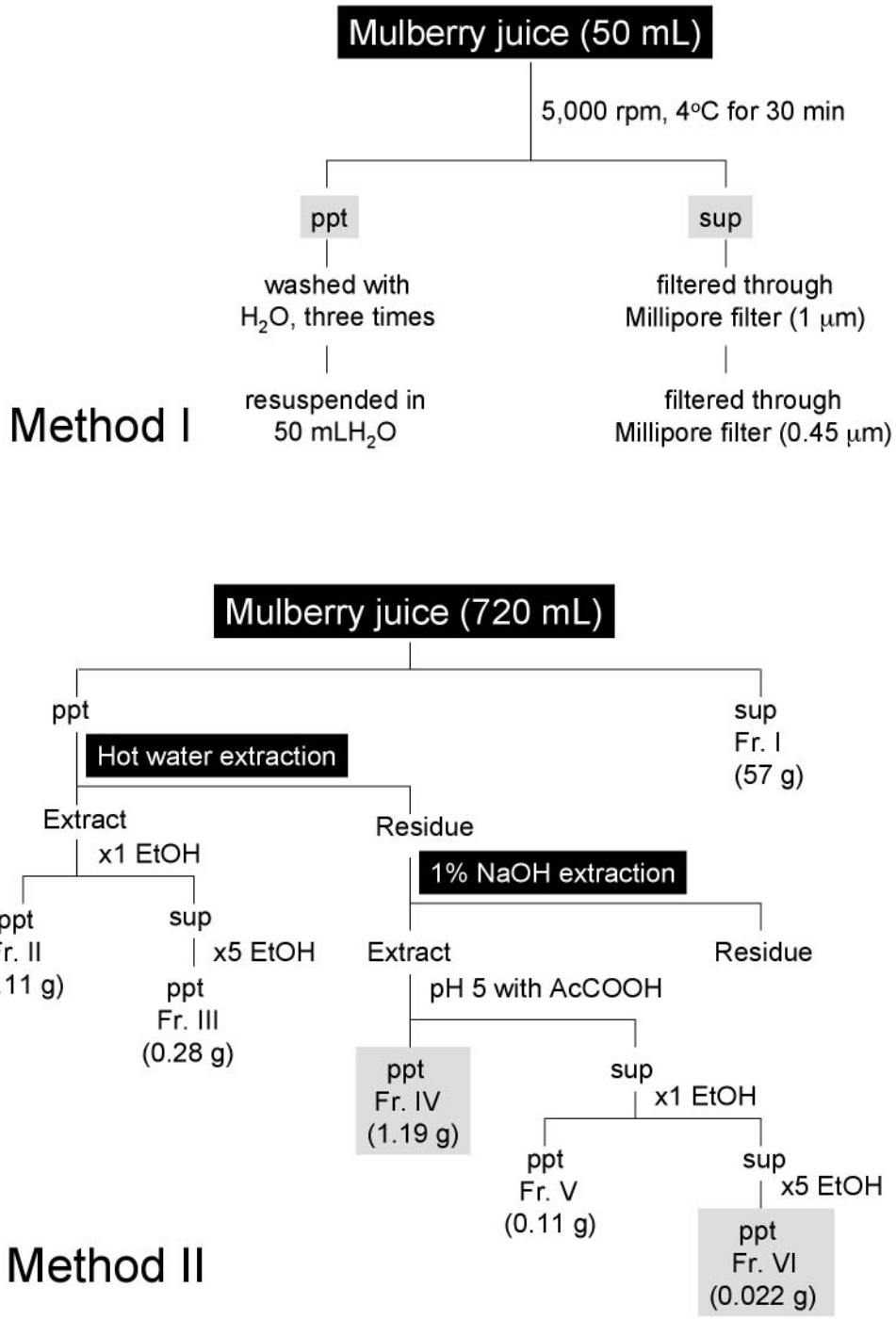

Figure 1. *Fractionation of mulberry juice.*

plasma lipid peroxide level was prevented (93 CARR U) (Group I) (Exp. I, Figure 2), confirming our previous report of the anti-stress activity of mulberry juice (6).

The mulberry juice was separated into two fractions by centrifugation: supernatant (containing approximately 22w/w% of total fiber) and precipitate (containing approximately 78w/w% of total fiber) to investigate which fractions are responsible for the anti-stress activity. The administration of the supernatant fraction produced a similar magnitude of prevention effect (94 CARR U) (Group II), whereas the precipitate fraction was slightly less active (135 CARR U) (Group III) (Figure 2).

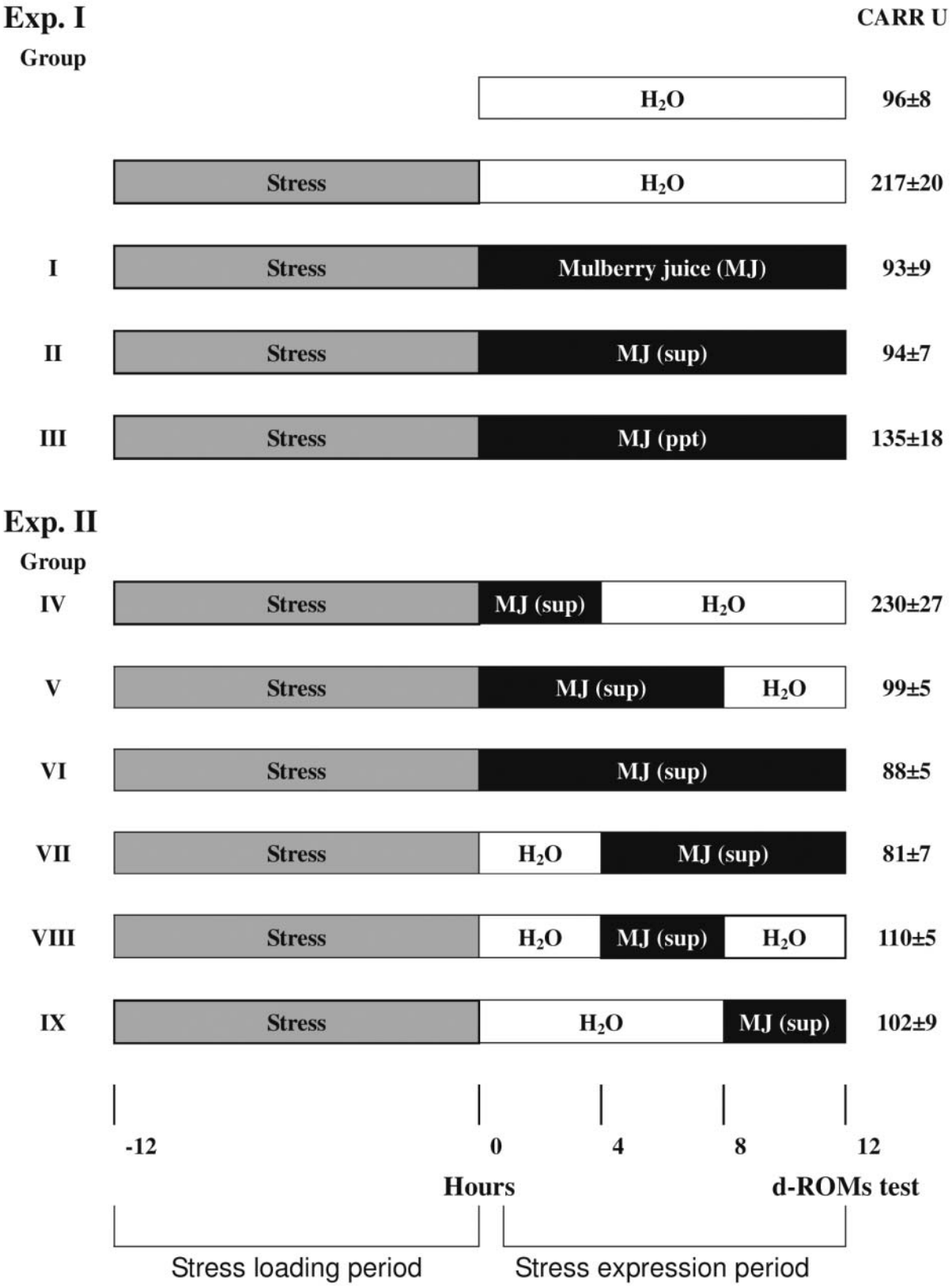

Figure 2. *Design for mouse stress experiments.*

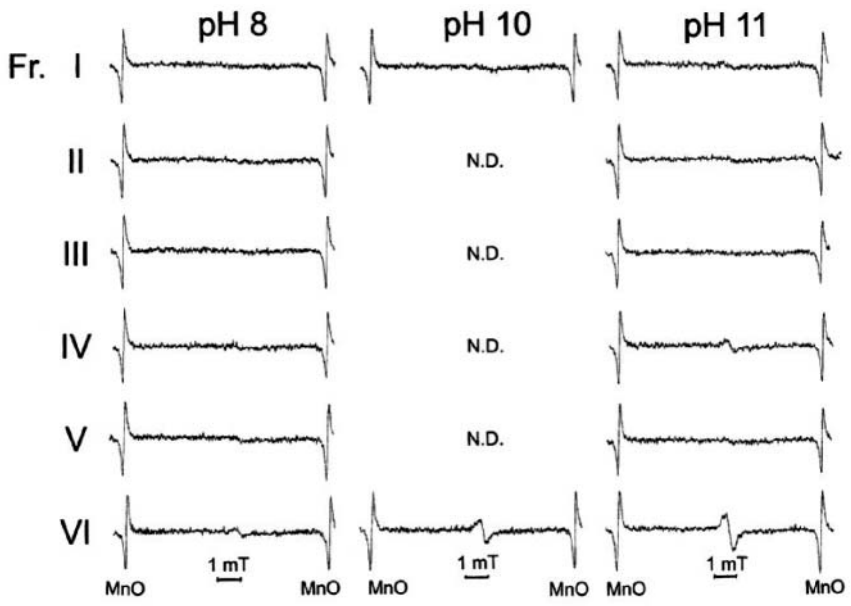

Figure 3. *ESR spectra of mulberry juice fractions (2 mg/mL) in 0.1 M Tris-HCl (pH 8.0) or 0.1 M $NaHCO_3/Na_2CO_3$ (pH 10.0, 11.0).*

The optimal administration of mulberry juice was further pursued. Administration of mulberry juice for 4 h, followed by administration of water for 8 h during the stress expression period did not prevent the elevation of plasma lipid peroxide level (230 CARR U) (Group IV). Administration of Mulberry juice for 8 hours, followed by water for 4 h, showed a potent, but slightly lower anti-stress activity (99 CARR U) (Group V), as compared with administration during the entire 12 h period (88 CARR U) (Group VI). These data suggest the importance of mulberry juice administration for the last 8 h of the stress expression period. This was confirmed by the experiment described in Group VII (81 CARR U). To narrow the range of the critical time point, this 8 h administration period was split into two parts. As expected from the data of Group IV-VI, both the administration of mulberry juice for the first 4 h (Group VIII), and that for the last 4 h (Group IX), were equally effective (110, 102 CARR U). This indicates that the anti-stress activity of mulberry juice was maintained for at least 4 h after cessation of administration.

Synergistic radical scavenging activity of the lignin fraction and vitamin C. ESR spectroscopy showed that the lignin fractions (IV, VI) produced radical(s) under alkaline conditions and that the radical intensity increased with an increase in pH (Figure 3). On the other hand, the supernatant fraction (Fr. I) and other fractions produced little or no detectable amounts of radicals.

Lignin fractions (IV, VI) showed the greatest superoxide (measured as DMPO-OOH) scavenging activity which was enhanced synergistically with sodium ascorbate (Table I). Lignin fractions (IV, VI) also showed the greatest hydroxyl radical (DMPO-OH) scavenging activity which was synergistically enhanced by sodium ascorbate (Table II). The relatively higher hydroxyl radical scavenging activity of Fr. I (sup) may be due to the higher iron content of this fraction.

Table I. *Synergistic superoxide scavenging activity of mulberry lignin fractions and vitamin C (VC).*

Fraction	DMPO-OOH radical intensity (% of control)		
	1000 μg/mL	250 μg/mL	125 μg/mL+ 1.25 μM VC
I (sup)	29.8	57.5	72.6<75.9 [(57.5+94.3)/2
II	33.5	57.5	71.7<75.9 [(57.5+94.3)/2
III	51.2	75.5	82.1<84.9 [(75.5+94.3)/2
IV (lignin fr.)	18.6	38.7	48.1<<66.5 [(38.7+94.3)/2] synergism
V	32.6	57.5	70.8<75.9 [(57.5+94.3)/2
VI (lignin fr.)	5.6	27.4	40.6<<60.9 [(27.4+94.3)/2] synergism
2.5 μM VC		94.3	

VC: Vitamin C.

Anti-HIV activity. Lignin fractions (IV, VI) showed the greatest anti-HIV activity among 6 fractions of mulberry juice, although their anti-HIV activity was much lower than that of popular anti-HIV agents (dextran sulfate, curdlan sulfate, AZT, ddC) (Table III). On the other hand, the supernatant fraction (Fr. I) was inactive.

Table II. *Synergistic hydroxyl radical scavenging activity of mulberry lignin fractions and vitamin C (VC).*

Fraction	DMPO-OH radical intensity (% of control)	
	1000 μg/mL	500 μg/mL+ 6.25 μM VC
I (sup) (contains Fe)	63.4	39.8<<66.5 [(63.4+69.6)/2] synergism
II	78.6	50.8<74.1 [(78.6+69.6)/2]
III	95.1	60.8<82.4 [(95.1+69.6)/2]
IV (lignin fraction)	71.5	45.0<<70.6 [(71.5+69.6)/2] synergism
V	69.2	56.3<69.4 [(69.2 + 69.6)/2]
VI (lignin fraction)	49.3	35.3<<59.5 [(49.3+69.6)/2] synergism
12.5 μM VC	69.6	

VC: Vitamin C.

Table III. *Anti-HIV activity of mulberry juice.*

	CC_{50}	EC_{50}	$SI=CC_{50}/EC_{50}$
Mulberry juice	2.24%	0.60%	3.7
Blueberry juice	0.40%	>0.63%	<1.0
Mulberyy juice fraction			
I (sup)	>1000 μg/mL	>1000 μg/mL	><1.0
II	>1000 μg/mL	368 μg/mL	>2.7
III	>1000 μg/mL	>1000 μg/mL	><1.0
IV (lignin fraction)	488 μg/mL	67 μg/mL	7.2
V	517 μg/mL	>1000 μg/mL	<1.0
VI (lignin fraction)	452 μg/mL	65 μg/mL	7.0
Positive control			
Dextran sulfate	389 μg/mL	1.19 μg/mL	327.0
Curdlan sulfate	>1000 μg/mL	0.139 μg/mL	>7179.0
AZT	51 μM	0.015 μM	3346.0
ddC	399 μM	0.662 μM	603.0

Each value represents the mean of three determinations.

Discussion

The present study demonstrates that anti-stress activity and anti-HIV activity were found in two distinct fractions. The anti-stress activity was recovered mainly from the supernatant fraction, whereas the anti-HIV activity was recovered from the precipitate fraction containing most of the fiber.

We found that 4 h administration of mulberry juice is sufficient to induce anti-stress activity in mice, but it is recommended that the mulberry juice is administered at the most stressful time. Under such conditions, the anti-stress effect can be maintained even 4 h after cessation of administration (Figure 2). This interpretation is supported by our preliminary study which showed that one administration (200 mL) of mulberry juice to two healthy male volunteers without stress did not significantly change the visual acuity evaluated 4 h later by distant visual acuity, near visual acuity, refraction, intraocular pressure, critical flicker fusion frequency and night vision (data not shown). Thus, the anti-stress activity of mulberry juice in humans should be evaluated under more stressful or fatigue conditions.

We confirmed our previous finding that mulberry juice effectively scavenged superoxide and hydroxyl radicals. This radical scavenging activity may inhibit the production of reactive oxygen species as a result of stress (10-12). The present study demonstrates for the first time that the lignin fraction in the precipitate showed higher radical scavenging activity than other fractions and that the radical scavenging activity of lignin fractions was synergistically enhanced with sodium ascorbate. We previously reported that a lignin carbohydrate complex from pine cone extract enhanced the cytotoxicity (13) and radical scavenging activity (7, 14) of vitamin C. These data suggest that lignin carbohydrate complexes of any plant origin acts synergistically with vitamin C. It was unexpected that the precipitate fraction that contains lignin showed lower anti-stress activity (Figure 2). This may be due to the dilution effect of lignin, present at a much lower concentration (only 2% (w/w)) in the mulberry juice, as compared with other ingredients (98%) in the supernatant.

We found that lignin fractions (Fr. VI and IV) in the precipitate of mulberry juice showed the greatest anti-HIV activity, consistent with our previous finding (7). The lack of anti-HIV activity in blueberry juice may be explained by the absence of insoluble fiber composed of lignin carbohydrate complex. Flavonoids from the root bark of *Morus alba* L showed anti-herpes simplex activity (15). The antiviral spectrum of mulberry juice should be investigated.

We found that the supernatant of mulberry juice at 10 mg/mL inhibited the growth of *Porphyromonas gingivalis* and *Prevotella melaninogenica*, although it was ineffective against *Streptococcus mutans*, *Streptococcus gordonii*, *Actinomyces viscosus*, *Prevotella intermedia*, *Fusobacterium nucleatum*, *Capnocytophaga ochracea*, *Candida albicans*, *Escherichia coli* and *Staphylococcus aureus*. Antimicrobial activity of prenylated flavonoids isolated from *Morus alba* L. and *Morus mongolica* Schneider, has also been reported (16). Further analysis of antibacterial activity against *Porphyromonas gingivalis* and *Prevotella melaninogenica* are necessary for the effective application of mulberry juice in the oral environment. The present study suggests the functionality of mulberry juice as an alternate medicine.

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