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Anti-stress Activity of Mulberry Juice in Mice

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Abstract. *The possible anti-stress activity of mulberry juice was investigated in mice. When mice were subjected to water immersion restraint stress at 25˚C for 8 h, the plasma lipid peroxide level, determined by the d-ROMs test performed 12 h thereafter, was almost doubled. After administration of mulberry juice one or two weeks before the stress loading, the lipid peroxidation was completely blocked. Administration of mulberry juice after the stress loading, without pre-administration, was also protective. ESR spectroscopy revealed that mulberry juice scavenged superoxide anion (generated by hypoxanthine and xanthine oxidase reaction), hydroxyl radical (produced by the Fenton reaction) and NO radical (generated by a NO donor) at approximately 50% efficiency of blueberry juice. Mulberry juice produced smaller amounts of radical at neutral to alkaline pH. The cytotoxic and anti-HIV activities of mulberry juice were 18% and >4-fold those of blueberry juice, respectively. These data suggest that the anti-stress activity of mulberry juice in vivo may be derived from its radical scavenging activity.*

In contrast to mulberry leaves, trees and barks, mulberry fruits have been much less investigated. 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 showed the concentration-dependent antioxidant activity (inhibition of copper-induced peroxidation of liposome and the inhibition of 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 with narrow therapeutic range and dramatically decreased the nitric oxide production by the activated macrophages (4). Black mulberry juice inhibited the human cytochrome CYP3A activity in the pooled human liver microsome system (5). These reports support the possibility that mulberry juice contains several components with antioxidant activity.

Although mulberry juice is nutritionally rich, whether it is beneficial to the human body is not clear. Whether the oral intake of mulberry juice induces anti-stress activity in mice was investigated, by monitoring the change in the plasma lipid peroxide concentration of mice forcibly put under water immersion restraint stress. Since anti-stress activity is tightly coupled with antioxidant activity, the radical scavenging activity of mulberry juice was also measured in comparison with that of blueberry juice, using ESR spectroscopy. Limited numbers of reports on the general biological activity of mulberry juice are available. Since some plant polyphenols, especially those with higher molecular weights, showed potent anti-HIV activity (6), the anti-HIV activity of mulberry and blueberry juices were also investigated.

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, Japan), Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Grand Island, NY, USA); fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS, USA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) (Wako Pure Chem. Ind., Ltd., Osaka, Japan); hypoxanthine (HX), xanthine oxidase (XOD),

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Key Words: Mulberry juice, anti-stress activity, radical scavenging activity, anti-HIV activity.

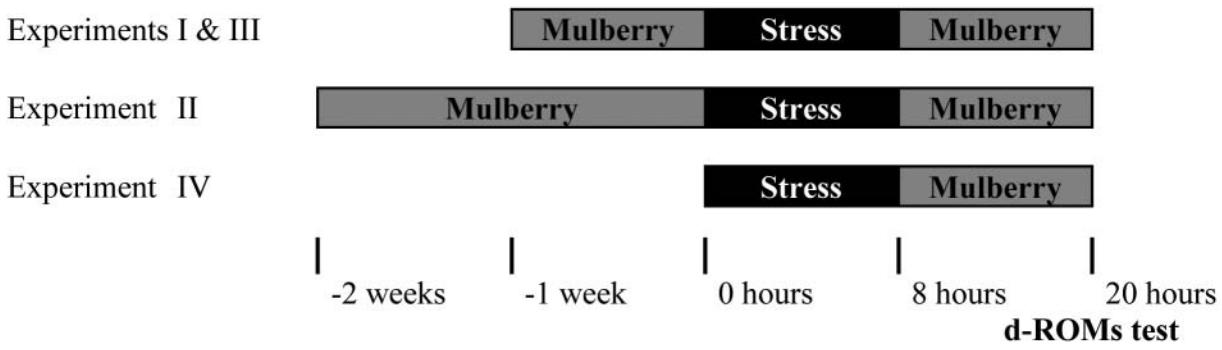

Figure 1. *Design for mouse stress experiments.*

diethylenetriaminepentaacetic 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), 1-hydroxyl-2-oxo-3-(*N*-3-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC-7), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) (Dojin, Kumamoto, Japan); curdlan sulfate (79 kDa) (Ajinomoto Co., Inc., Tokyo, Japan); dextran sulfate (8 kDa) (Kowa, Tokyo, Japan).

Anti-stress assay. BALB/c mice (Charles River Japan Co. Ltd., Atsugi, Japan) (5-6 mice/group) were pre-administered mulberry juice [*ad. lib.*] for one or two weeks before the stress loading as described below. The mulberry juice was diluted with water to the final concentration of 0, 10, 25, 50 or 100 (v/v)%. Each drinking bottle was replaced with fresh water or juice every morning and night (Experiment I, II, III, Figure 1). One group of mice was omitted for the pre-administration of mulberry juice (Experiment IV in Figure 1). Mice were subjected to water immersion restraint stress at 25˚C for 8 hours and were then administered the same concentrations of mulberry juice for 12 h. The mice were anesthetized with ether and blood was collected from the heart. The 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 by 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.

Cytotoxic activity. Normal human gingival fibroblast, pulp cell and periodontal ligament fibroblast (6-8 population doubling level) prepared from the periodontal tissue (7), and human oral squamous cell carcinoma (HSC-2, HSC-3 and HSC-4) were cultured at 37˚C in DMEM supplemented with 10% heat-inactivated FBS in a humidified 5% CO_2 atmosphere. Near confluent cells were incubated for 24 h with various concentrations of mulberry juice and the relative viable cell number was determined by the MTT method to calculate the 50% cytotoxic concentration (CC_{50}) (7). All data represent the mean values of triplicate measurements.

Assay for anti-human immunodeficiency virus (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 (control) MT-4 cells were incubated for 5 days with various concentrations of test samples and the relative viable cell number was determined by the MTT assay. The 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 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: $SI=CC_{50}/EC_{50}$

Radical scavenging activity. The radical intensity of samples was determined at 25˚C in 0.1 M Tris-HCl (pH 7.0, 8.0), 0.1 M Na_2CO_3/$NaHCO_3$ (pH 9.0, 10.0, 11.0) or 0.1 M KOH (pH 13.5), using ESR spectroscopy (JEOL JES REIX, X-band, 100 kHz modulation frequency) (9). The instrument settings were as follows: 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 the determination of superoxide anion (O_2^-), 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.

Hydroxyl radical (•OH) was 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.

The radical intensity of NO, produced from the reaction mixture of 20 μM carboxy-PTIO and 50 μM NOC-7, was determined in 0.1 M phosphate buffer, pH 7.4 in the presence of 30% DMSO. The microwave power and gain were changed to 5 mW and 250, respectively. When NOC-7 (NO generator) and carboxy-PTIO (spin trapping agent) were mixed, NO was oxidized to NO_2, and carboxy-PTIO was reduced to carboxy-PTI, which produces seven-line signals. The NO radical intensity was defined as the ratio of signal intensity of the first peak of carboxy-PTI (indicated by arrows in Figure 3C) to that of MnO.

Results

Anti-stress activity. When the mice were subjected to stress, the plasma lipid peroxide level was elevated from 97 CARR U to 194 CARR U. However, when mice were pre-administered the straight mulberry juice for 1 week before

Table I. *Effect of pre- or post-treatment of mice with mulberry juice on the stress-induced elevation of plasma lipid peroxide levels.*

		Lipid peroxide in plasma (CARR U) Murberry juice (%)				
Exp	Control	0	10	25	50	100
Pre- and post-treatment						
I (1 wk)	97±13 (100)	194±23 (199)			101±5 (104)	101±10 (104)
II (2 wk)	90±9 (100)	204±17 (225)			92±12 (101)	88±17 (98)
III (1wk)	89±3 (100)	268±39 (302)	299±33 (337)	292±12 (329)		
Post-treatment						
IV (12h)	115±5 (100)	206±8 (180)		236±37 (206)	97±14 (85)	111±17 (97)

Mice were fed *ad. lib.* the indicated concentrations of Mulberry juice for 1 (Experiments I, III) or 2 weeks (Experiment II), submitted to stress in water for 8 h, fed again the same concentrations of mulberry juice for 20 h and then the plasma lipid peroxide level was measured by d-ROMs test as described in Figure 1. Other groups of mice were first submitted to stress in water for 8 h (omitting the pretreatment with mulberry juice), and then fed *ad. lib.* the indicated concentrations of Mulberry juice for 12 h (Experiment IV). Each value represents mean±S.D. from 5 mice.

the stress loading, the plasma lipid peroxide level was elevated only 4% (Experiment I, Table I). Similar results were also obtained when mice were administered mulberry juice for 2 weeks before the stress loading (Experiment II, Table I). Dilution of mulberry juice by 2-fold with water also showed the inhibitory effect (Experiment I, II, Table I). However, further dilution of mulberry juice (10 and 25% of the original concentration) resulted in the complete loss of the inhibitory effect (Experiment III, Table I). The inhibitory effect of mulberry juice was reproduced, even if the mulberry juice was administered only after the stress loading (Experiment IV, Table I).

Radical production and scavenging activity. ESR spectroscopy revealed that mulberry juice did not produce radical at pH 7.0-11.0, whereas blueberry juice produced detectable radical at pH 8.0-13.5 (Figure 2).

Mulberry juice (stored for 2 months in refrigerator) (IC_{50}=0.18%), mulberry juice (freshly opened) (IC_{50}=0.16%), blueberry juice (freshly opened) (IC_{50}=0.06%) and sodium ascorbate (IC_{50}=15.6 μM) dose-dependently scavenged the O_2^- produced by HX-XOD reaction (Figure 3A). The O_2^- scavenging activity of straight mulberry (stored, fresh) and blueberry juice (fresh) was equivalent to 8.7, 9.8, and 26 mM sodium ascorbate, respectively.

Table II. *Anti-HIV activity of mulberry and blueberry juices.*

	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
Dextran sulfate	389 μg/mL	1.19 μg/mL	327
Curdlan sulfate	>1000 μg/mL	0.139 μg/mL	>7179
AZT	51 μM	0.015 μM	3346
ddC	399 μM	0.662 μM	603

Mulberry juice (stored for 2 months in refrigerator) (IC_{50}=1.63%), mulberry juice (freshly opened) (IC_{50}=1.71%), blueberry juice (freshly opened) (IC_{50}=1.03%) and sodium ascorbate (IC_{50}=56 μM) also scavenged OH•, produced by the Fenton reaction (Figure 3B) in a dose-dependent manner. The OH• scavenging activity of mulberry (stored, fresh) and blueberry juice (fresh) was equivalent to 3.4, 3.3 and 5.4 mM sodium ascorbate, respectively.

Mulberry and blueberry juices (freshly opened) showed comparable NO scavenging activity (IC_{50}=0.96 and 0.81%, respectively) (Figure 3C). These data suggested that storage of mulberry juice in a refrigerator for 2 months did not significantly reduce the radical scavenging activity and that the radical scavenging activity of mulberry juice was about 35-62% that of blueberry juice.

Cytotoxic activity. Mulberry juice showed very weak cytotoxic activity against both human normal cells (gingival fibroblast, pulp cell, periodontal ligament fibroblast) and human oral squamous cell carcinoma (HSC-2, HSC-3, HSC-4) (CC_{50}>10%). However, mulberry juice showed higher cytotoxic activity against a human T-cell leukemia MT-4 cell line (CC_{50}=2.24%), compared to the other cells, but this cytotoxicity was about 18% of that of blueberry juice (CC_{50}=0.40%) (Table II).

Anti-HIV activity. In contrast to the positive control (dextran sulfate (SI=327), curdlan sulfate (>7179), AZT (SI=3346), ddC (SI=603)), mulberry juice showed a much lower, anti-HIV activity (SI=3.7), whereas blueberry juice was inactive (SI<1.0). (Table II).

Discussion

The results of the present study demonstrated that mulberry juice showed anti-stress activity against mice, inhibiting the elevation of plasma lipid peroxide levels induced by stress. The anti-stress activity was expressed even if mulberry juice was administered only after the stress, without pre-

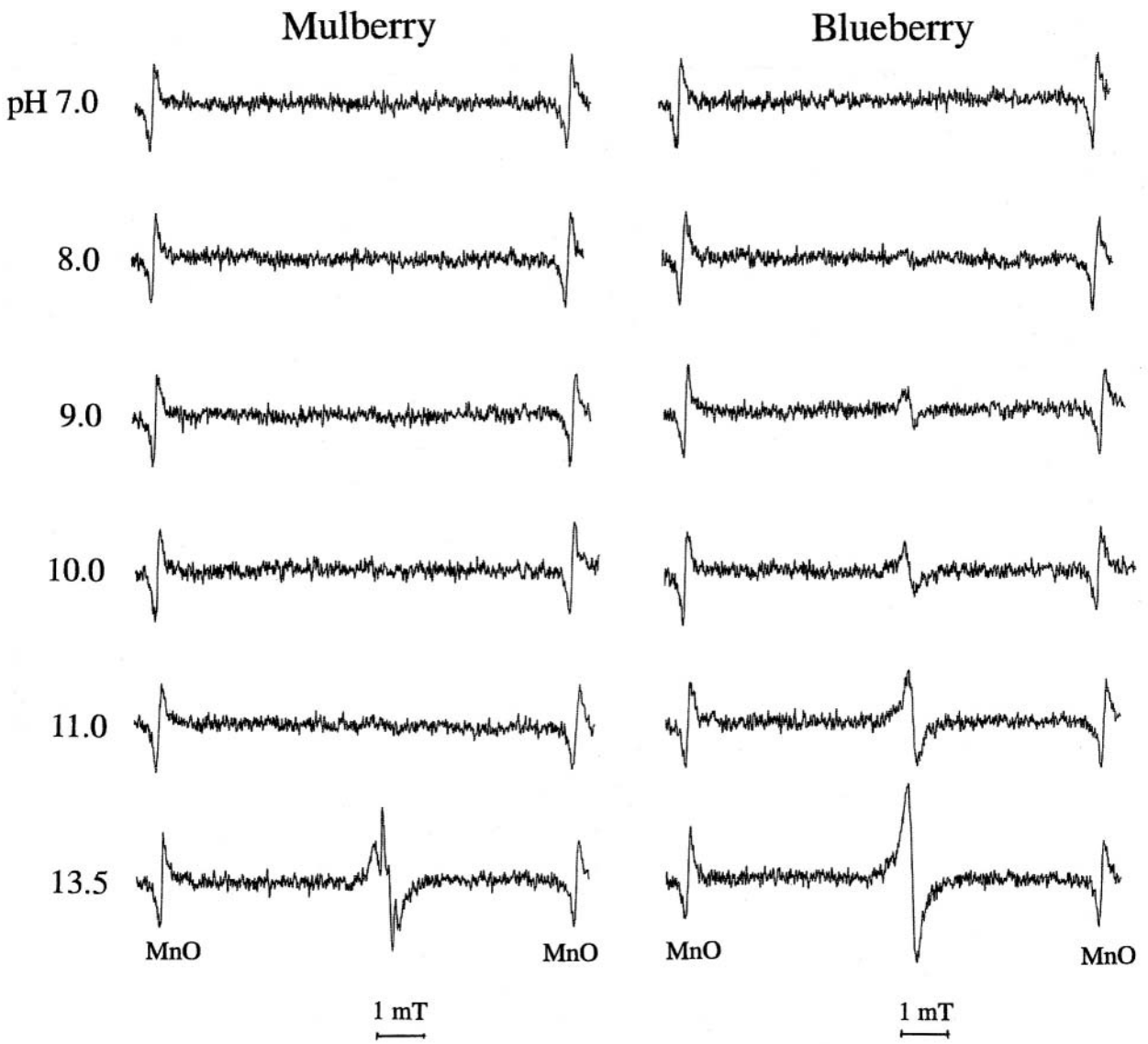

Figure 2. *ESR spectra of mulberry and blueberry juices in 0.1 M Tris-HCl (pH 7.0, 8.0), 0.1 M $NaHCO_3/Na_2CO_3$ (pH 9.0, 10.0, 11.0) or 0.1 M KOH (pH 13.5).*

administration. It is generally recognized that stress could produce several types of free radicals, such as O_2^- and •OH, and that these radicals attack any organic molecule, generating compounds, called reactive oxygen metabolites, including hydroperoxides (10-12). From these reports, the present results may be interpreted that mulberry juice scavenges free radicals produced by the stress and results in inhibition of hydroperoxide formation. This interpretation may be supported by the present observations showing that mulberry juice exerted scavenging activities against O_2^-, •OH and NO. The dry powder of mulberry leaf was reported to have antioxidative activity in streptozotocin-diabetic rats (13). The 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging activity of oxyresveratrol in mulberry wood (14) as well as the O_2^- and DPPH scavenging activity and hepatoprotective activities of 5,7-dihydroxycoumarin 7-methyl ether, cudraflavone B and oxyresveratrol from *Morus alba* were also reported (15). These observations further strengthen our speculation that mulberry juice could inhibit hydroperoxide formation through the free radical scavenging activity of the juice. We found that the radical scavenging activity of mulberry juice was slightly lower than that of blueberry juice. However, the mulberry juice had a much lower cytotoxicity and radical generating activity than blueberry juice, confirming the positive relationship between radical generation/scavenging activity and cytotoxicity (16-18).

It was reported that prenylated flavonoids isolated from *Morus alba* L showed antimicrobial activity against four

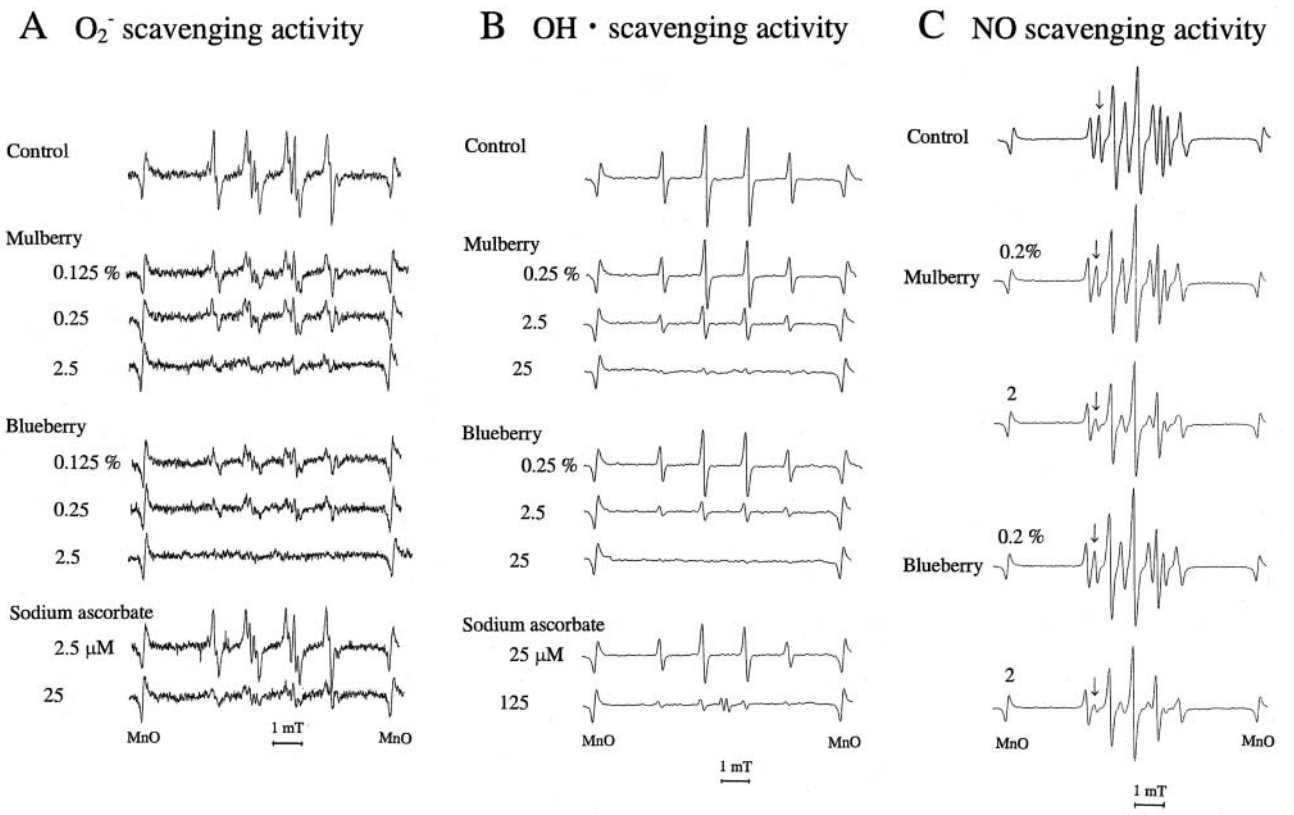

Figure 3. *ESR spectra of O_2^- (DMPO-OOH adduct produced in HX-XOD reaction) (A), hydroxyl radical (DMPO-OH adduct produced in Fenton reaction) (B) and NO (produced by NOC-7 with carboxy-PTIO) (C) in the presence of the indicated concentrations of mulberry juice, blueberry juice or sodium ascorbate.*

bacterial and two fungal microorganisms (19) and antiviral activity against the herpes simplex type 1 virus (20).

The present study demonstrated various biological activities of mulberry juice. Further studies are required to identify the active substances in the mulberry juice.

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