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Original Article

Scavenging Effects of Methanolic Extracts of Broad Beans on Free-Radical Species

Mizue OKADA*[1], Yoshinori OKADA*[2], Ryoichi INABA*[1] and Hirotoshi IWATA*[1]

*[1]*Department of Hygiene, Gifu University School of Medicine, Gifu*
*[2]*Laboratory on Ageing, Chukyo Junior College, Mizunami*

Abstract

This report describes the antioxidant characteristics of methanolic extracts from broad beans (*Vicia fava*). The methanolic extracts of broad beans (MEBB) exhibited a marked scavenging effect on superoxide. MEBB also exerted scavenging activities on hydrogen peroxide and 1, 1-diphenyl-2-picrylhydrazyl radical. The radical scavenging activity of MEBB was highest when the scavenging effect of MEBB on superoxide (IC_{50} = 0.15 mg/ml) was examined. These results suggest that MEBB have effective activities both as a radical scavenger and as a hydrogen donor. The chelating activity of MEBB (0.70 mg/ml) on Fe^{2+} and Cu^{2+} was 31.2% and 28.5%, respectively. The antioxidant effect of MEBB on lipid peroxidation might be attributed to their properties of scavenging free-radical species and their chelating activity on metal ions. The antioxidant activity of MEBB against *tert*-butyl hydroperoxide (BHP)-induced oxidative stress in WI-38 cells was assessed. The activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSH-Px) were measured as indices of oxidative stress. WI-38 cells incubated with 0.1 mM BHP for 2 hr exhibited the increase of SOD, catalase and GSH-Px activities over the control. When the cells incubated in MEBB (45-450 μg/ml) for 18 hr were subjected to a BHP challenge test, SOD activity returned to its control value or lower at all levels tested. When catalase activity was determined, a similar trend occurred except in the cells incubated in 112.5 μg/ml MEBB. These results imply that MEBB inhibit oxidative stress in WI-38 cells.

Key words: *Vicia fava*, Broad beans, Methanolic extracts, Free radicals, Oxidative stress

Introduction

All aerobes are at risk of being damaged by activated oxygen species such as superoxide radical, hydrogen peroxide, and hydroxyl radical, since their high reactivity often results in injury including carcinogenesis, inflammation, and aging[1]. In biological systems, superoxide and hydrogen peroxide react together to generate the highly reactive hydroxyl radical that can attack and destroy almost all known biomolecules[2,3]. Free radical damage in biological systems is also attributed to the transition metal-dependent generation of hydroxyl radical, known as 'the metal-catalyzed Haber-Weiss reaction'[4]. Recently, a novel mechanism, independent of transition metal ions, has been proposed for hydroxyl radical formation in biological systems. This mechanism involves the production of peroxynitrite ($ONOO^-$) from the reaction between superoxide and nitric oxide. The peroxynitrite is an unstable intermediate at physiological pH, rapidly decaying by the proton-catalyzed decomposition of peroxynitrous acid. It has been suggested that such a decomposition occurs through homolytic fission, giving rise to hydroxyl radical and nitrogen dioxide. Experimental evidence, however, supports that hydroxyl radical is one of the major damaging species formed in Fenton systems under biologically relevant conditions[5,6].

To survive, all aerobes are equipped with several antioxidants that prevent oxygen cytotoxicity. Several physiologically important enzymes in the body including superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSH-Px) protect the living body against free radicals and oxidative stress. Natural antioxidants are found in various vegetables, fruit, and tea leaves. Plant tissues are rich in a variety of phenolic compounds. Flavonoids and other plant phenolics have been reported to have multiple physiological effects such as antioxidant activity, antiinflammation and antiallergy[7]. Preliminarily, we screened 40 species of edible

Reprint requests to: Mizue Okada,
Department of Hygiene, Gifu University School of Medicine,
40 Tsukasa-machi, Gifu 500-8076, Japan
TEL: +81(58)265-1241
FAX: +81(58)267-2958

plants (broad beans, broccoli, burdock, carrots, tomatoes, and so on) for superoxide-scavenging activity in an attempt to discover natural antioxidants. The superoxide-scavenging activity of broad beans was higher than all other tested edible plants except burdock (unpublished). Before we studied the scavenging activities, Kim *et al.*[8] had reported that the ethanolic extract of burdock could enhance SOD activity by 28%. However, to date, no reports on the scavenging effect of methanolic extracts of broad beans (MEBB) on free-radical species have been published. Thus, our objective was to explain the mechanism of action of the antioxidant effect by MEBB. Lawlor *et al.*[9] have studied several antioxidant substances using *in vitro* models. Since readjustment of intracellular antioxidant activities is a fundamental cellular response to defense against oxidative stress, we report here the effects of MEBB on three antioxidants including SOD, catalase, and GSH-Px using *in vitro* models.

Materials and Methods

Chemicals

Cytochrome *c*, *tert*-butylated hydroxyanisole (BHA), *N*-2-hydroxyethylpiperazine- *N'*-2-ethanesulfonic acid (HEPES), and 1,1-Diphenyl-2-picrylhydrazyl (DPPH) were obtained from Sigma Ltd. (St. Louis, MO). WI-38 cells (JCRB9017) were obtained from the Health Science Research Resources Bank (Osaka, Japan). Fetal bovine serum (FBS) was purchased from Flow Laboratories (North Ryde, N. S. W., Australia), and trypsin from Difco Laboratories (Detroit, MI, U.S.A.). All other chemicals were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Extractions

Broad beans were purchased from an outdoor market, and then hand-shelled. Broad beans were stored at - 20℃ until use. Frozen broad beans (100 g) were minced into 5-mm fragments and extracts were obtained with methanol (300 ml) overnight at room temperature. The extracts were filtered; the residue was re-extracted under the same conditions. The combined filtrates were evaporated in a vacuum slightly below 40℃ in a rotary evaporator to a final volume of 5 ml. The concentration of the extracts in the solvent was 95.0 ± 2.00 mg/ml (the average of triplicate analyses). MEBB was decolorized with cartridges (Sep-Pak C_{18}, Waters) to avoid interference in spectrometrical analysis.

Determination of total phenolic compounds

The total of the phenolic compounds present in MEBB was estimated using Folin- Denis (F-D) reagent[10]. Briefly, 0.1 ml MEBB solution was combined with 2% Na_2CO_3 (2.0 ml). After 2 min, 0.1 ml of 50% F-D reagent was added, and the mixtures were incubated for 30 min at 20℃. Absorbance was then measured at 750 nm using a Nihonbunko UV-460 spectrophotometer. The concentration of total phenolic compounds present in MEBB was determined by comparing with the absorbance rates of standard chlorogenic acid at different concentrations.

Determination of the effects on superoxide

The ability of MEBB to inhibit the superoxide-dependent reduction of cytochrome *c* was monitored by measuring the rate of increase in absorbance for 3 min at 25℃ at 550 nm. Superoxide was generated enzymatically, by 0.01 μM xanthine oxidase in a 3-ml assay containing 10 μM ferricytochrome *c* and 50 μM xanthine in 50 mM potassium phosphate (pH7.8) included in 0.1mM EDTA at 25℃[11]. The reaction was initiated by adding xanthine oxidase. The concentration of the sample giving 50% inhibition (IC_{50}) was determined from a dose response curve. Catechin and BHA were dissolved in 100% methanol. The concentrations of catechin and BHA were in accordance with Yen and Duh[12]. All tests and analyses were run in triplicate and averaged.

Determination of the effects on hydrogen peroxide

The ability of MEBB to scavenge hydrogen peroxide was detected spectrophotometrically[13]. A solution (2 mM) of hydrogen peroxide was prepared in phosphate-buffered saline (PBS), pH 7.4, at 20℃. The hydrogen peroxide concentration[14] was determined spectrophotometrically at 230 nm using a molar extinction coefficient for hydrogen peroxide of 81 M^{-1} cm^{-1}. MEBB was added to the hydrogen peroxide solution at 20℃ Absorbance of hydrogen peroxide at 230 nm was determined 10 min later in a spectrophotometer against a blank solution containing a MEBB sample in PBS without hydrogen peroxide. The concentrations of catechin and BHA were in accordance with Yen and Duh[12]. The concentration of the sample giving 50% inhibition (IC_{50}) was determined from a dose response curve. All tests and analyses were run in triplicate and averaged.

Determination of the effects on DPPH radical

Two milliliters of MEBB sample were added to a solution (1 ml) of DPPH radical (final concentration of DPPH was 2.0×10^{-4} M). The mixture was shaken vigorously and left to stand for 30 min. The absorbance rate of the resulting solution was then measured at 517 nm with a spectrophotometer[15]. The concentrations of catechin and BHA were in accordance with Yen and Duh[12]. The concentration of the sample giving 50% inhibition (IC_{50}) was determined from a dose response curve. All tests and analyses were run in triplicate and averaged.

Measurements of chelating activity on metal ions

The chelating activity of MEBB on Fe^{2+} and Cu^{2+} was measured according to the method of Shimada *et al.*[16]. Two milliliters of MEBB solution were added to 2 ml of 10 mM hexamine buffer containing 10 mM KCl (pH 5.0) and 3 mM $FeSO_4$ or 3 mM $CuSO_4$, and then 0.2 ml of 1 mM tetramethyl murexide was added. Absorbance rate at 480 nm was measured with a spectrophotometer at 20℃.

Antioxidant activity

Bovine liver was homogenized in 19 volumes of 50 mM phosphate buffer (pH 7.4), and 0.5 ml of the homogenate was added to a mixture that consisted of 25 μl of 1.0% aqueous solution of BHP, 0.5 ml of a sample solution, and 2.225 ml of H_2O. This reaction mixture was incubated at 37℃ for 15 min and the content of thiobarbituric acid reactive substances (TBARS) in the solution was determined by the method of Yoshino *et al.*[17] (= TBARS 1). Trichloroacetic acid (12.5%, 0.2 ml) was added to an aliquot of the reaction mixture. Thiobarbituric acid (TBA) reagent (0.4 ml of 0.67% TBA and 1mM EDTA) was added. The mixture was heated at 95℃ for 20 min in a water bath. After cooling with tap water, 3 ml of 1-butanol were added, and the mixture was shaken vigorously for 30 sec. After centrifugation at 3,000 rpm for 10 min, the 1-butanol layer was removed. Absorbance at 532 nm was measured with a spectrophotometer at room

temperature. As a control, the homogenate was peroxidized by BHP without the antioxidants (= TBARS 2). The reactions without BHP were carried out for each test substance as a blank test (= TBARS 3 and 4). The antioxidant potential of the sample was calculated by the following equation.

Antioxidant activity (%) = (1- (TBARS 1- TBARS 3) / (TBARS 2 - TBARS 4)) × 100

The concentration of the sample giving 50% inhibition (IC_{50}) was determined from a dose response curve. The concentrations of catechin and BHA were in accordance with Yoshino *et al.*[17].

Human lung fibroblasts WI-38

WI-38 cells were cultured by methods previously described[18]. Briefly the cells were cultured in an atmosphere of air containing 5% CO_2 at 37℃ in Eagle's minimum essential medium (MEM) supplemented with 10% FBS, 28 mM HEPES and 1.5 μg/ml Fungizone (hereafter, MFHF medium). After four days of culture, the cells were harvested by trypsin treatment, resuspended in MFHF medium and seeded in 60 mm tissue culture dishes (1×10^5 cells/dish). After four days of secondary culture, the MFHF medium was removed and replaced by a fresh medium. The cells were then incubated in the MFHF medium with or without MEBB for the predetermined times.

Dimethyl sulfoxide (DMSO) was used to dilute MEBB and was added alone to the cells in 0 μg/ml MEBB and the control. Ten μl DMSO (0.02% final concentrations) was added to the control sample.

Cells homogenate and measurements of antioxidant enzyme activity

WI-38 cells were incubated for 18 hr at 37℃ in 5% CO_2 in MFHF medium supplemented with MEBB (45-450 μg/ml) or DMSO. Following the removal of the medium from dishes, cells were washed with Hanks' balanced salt solution (HBSS) and then exposed either to BHP (0.1 mM)[19] for MEBB (0-450 μg/ml) or to HBSS for control. After 2 hr of incubation, BHP or HBSS was rapidly aspirated to prepare the cell homogenates for enzymatic analysis. The cells were washed with HBSS and then removed from the dishes by scraping, placed on ice and subsequently homogenized with 5 ml of 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA at 4℃. The homogenates were centrifuged at 1,000 ×g for 10 min and the supernatant fractions were centrifuged at 10,000 ×g for 20 min at 4℃. The supernatant fractions were used for determination of SOD, catalase and GSH-Px activities.

Superoxide dismutase activity

SOD activity was measured by the method of Crapo *et al.*[20] using xanthine- xanthine oxidase (X-XOD). One unit of activity is the amount of enzyme needed to cause half-maximal inhibition of cytochrome *c* reduction.

Catalase assay

Catalase activity was determined by direct measurement of the decrease of light absorption at 250 nm for 4 min at 25℃ which was caused by the decomposition of hydrogen peroxide by catalase[21]. Three milliliters of assay mixture containing 60 mM hydrogen peroxide and 50 mM phosphate buffer, pH 7.0 was placed in a 1.0-cm cuvette. The reaction was initiated by adding an enzyme source.

Glutathione peroxidase assay

The rate of production of oxidized glutathione in the peroxidase reaction catalyzed by GSH-Px was measured in the presence of glutathione reductase by following the rate of NADPH oxidation. The decrease was monitored for 3 min in absorbance at 340 nm at 30℃. The assay mixture was modified from Tappel[22]. The reaction mixture (3.0 ml) contained 133 μM NADPH, 1 mM glutathione reduced form, 1 mM NaN_2, 1 mM EDTA, 2 units of glutathione reductase, 70 μM of BHP, a source of GSH-Px, and 100 mM Tris- HCl, pH 7.0. The reaction was started by adding BHP solution.

Statistical analysis

Data are expressed as mean values with standard deviations. Mean values were assessed for significance by Student's *t*-test. *p*-Values < 0.05 were considered significant.

Results

Measurement of total phenolic compounds

The content of the total phenolic compounds in MEBB was 2.13 mg/g of broad beans. The content of the total phenolic compounds in the MEBB was also 0.70 mg/ml of methanol extracted solution. The following experiments used this concentration of MEBB (0.70 mg/ml). As the MEBB may contain different phenolic compounds, MEBB cannot be expressed as a molar concentration. Therefore, it is difficult to compare the MEBB with other compounds.

The visible spectrum of MEBB was recorded using Nihonbunko UV-460. At a concentration of 0.70 mg/ml, no detectable light absorption was observed in the range of 400 to 650 nm, showing that MEBB does not contain prosthetic groups such as heme and flavin. Light absorbance intensity of 0.001 could have been detected easily (data not shown).

Scavenging of superoxide by MEBB

The ability of MEBB to scavenge superoxide generated by X-XOD is shown in Table 1. The addition of MEBB (0.70 mg/ml) to the X-XOD system significantly ($p < 0.01$) diminished production of superoxide. SOD (8 Units) inhibited 94% of superoxide. Catechin and BHA at concentrations of 2.5 and 75 μM, respectively, did not inhibit superoxide. The IC_{50} of MEBB was 0.15 mg/ml. Catechin and BHA also showed concentrations of 7.0×10^{-6} and 2.0×10^{-4} M, respectively. Similar results were obtained by non-enzymatic reduction of alloxan according to the method of Miwa *et al.* (data not shown)[23].

Table 1. Superoxide scavenging activities of investigated compounds.

Compound	Concentration	Rate of increase in absorbance at 550 nm (OD/min)	Inhibition (%)
Control	0	0.017 ± 0.003	0
MEBB	0.70 (mg/ml)	0.003 ± 0.001*	94.0 *
BHA	75.0 (μM)	0.017 ± 0.003	0
Catechin	2.50 (μM)	0.015 ± 0.003	6.0
SOD	8.0 (Units)	0.003 ± 0.001*	94.0*

The ability of MEBB to inhibit the superoxide-dependent reduction of cytochrome *c* was monitored by measuring the rate of increase for 3 min at 25℃ in absorbance at 550 nm. The values given were measured as described in Materials and Methods.

Each value is the mean ± standard deviation of triplicate analyses.

* $p < 0.01$, compared with controls.

Table 2. Scavenging effects of investigated compounds on hydrogen peroxide.

Compound	Concentration	Absorbance of H_2O_2 at 230nm	Inhibition (%)
Control	0	3.55 ± 0.05	0
MEBB	0.70 (mg/ml)	0.460 ± 0.05*	87.1 *
BHA	43.0 (μM)	0.629 ± 0.02*	82.3 *
Catechin	14.0 (μM)	3.02 ± 0.06	15.0
Catalase	96.0 (Units)	0.610 ± 0.08*	82.8*

The values given were measured as described in Materials and Methods.
Each value is the mean ± standard deviation of triplicate analyses.
* $p < 0.05$, compared with controls.

Table 3. Scavenging effects of investigated compounds on DPPH radical.

Compound	Concentration	Absorbance of DPPH at 517nm	Inhibition (%)
Control	0	3.24 ± 0.03	0
MEBB	0.70 (mg/ml)	0.324 ± 0.04*	90.0 *
BHA	240 (μM)	0.214 ± 0.05*	93.4 *
Catechin	8.00 (μM)	0.760 ± 0.02*	76.6*

The values given were measured as described in Materials and Methods.
Each value is the mean ± standard deviation of triplicate analyses.
* $p < 0.05$, compared with controls.

Table 4. Chelating effects of different compounds on Fe^{2+} and Cu^{2+}.

Compound	Concentration	Absorbance at 480nm Fe^{2+}	Cu^{2+}
Control	0	0.944 ± 0.002	1.211 ± 0.004
MEBB	0.7 (mg/ml)	0.650 ± 0.004	0.878 ± 0.004
	0.07 (mg/ml)	0.900 ± 0.003	1.089 ± 0.003
	0.007 (mg/ml)	0.915 ± 0.005	1.137 ± 0.006
EDTA	0.5 (M)	0.379 ± 0.004	0.388 ± 0.002
Citric acid	0.5 (M)	0.568 ± 0.003	0.611 ± 0.003

The values given were measured as described in Materials and Methods.
Each value is the mean ± standard deviation of triplicate analyses.

Table 5. Antioxidant activity of investigated compounds.

Compound	Concentration	Antioxidant activity (%)
MEBB	0.70 (mg/ml)	61.5 ± 1.30
BHA	240 (μM)	91.3 ± 1.50
Catechin	8.00 (μM)	81.0 ± 2.50

The activities given were measured as described in Materials and Methods.
Each value is the mean ± standard deviation of triplicate analyses.
The homogenate of bovine liver was peroxidized by BHP both with and without the antioxidants (=TBARS 1 and 2). The reactions without BHP were carried out for each test substance as a blank test (=TBARS 3 and 4). The antioxidant potential of the sample was calculated by the following equation.
Antioxidant activity (%) = (1- (TBARS 1- TBARS 3) / (TBARS 2 - TBARS 4)) ×100

Scavenging of hydrogen peroxide by MEBB

The scavenging activity of MEBB on hydrogen peroxide is shown in Table 2. MEBB scavenged 87.1% of hydrogen peroxide after 10 min of incubation. With MEBB (0.70 mg/ml), the concentration of hydrogen peroxide was diminished from 2 to 0.258 mM, representing a hydrogen peroxide reduction to 2.49 μmol of H_2O_2/mg of MEBB. BHA also scavenged 82.3% of hydrogen peroxide. The IC_{50} of MEBB was 0.40 mg/ml. BHA and catechin also showed concentrations of 2.0×10^{-5} and > 2.0×10^{-5} M, respectively.

Measurement of DPPH radical-scavenging activity

The scavenging activity of MEBB on the DPPH radical is shown in Table 3. MEBB (0.70 mg/ml) exhibited about 90% scavenging activity. Catechin and BHA at concentrations of 8 and 240 μM, respectively, exhibited marked scavenging activity (> 75%). The IC_{50} of MEBB was 0.35 mg/ml. Catechin and BHA were also observed at concentrations of 4.8×10^{-6} and 1.3×10^{-4} M, respectively.

Measurement of chelating activity on metal ions

The chelating activity of MEBB on Fe^{2+} and Cu^{2+} was measured according to the method of Shimada *et al.*[16]. The ability of MEBB to form complexes with metal ions is shown in Table 4. The chelating activity of MEBB (0.70 mg/ml) on Fe^{2+} and Cu^{2+} was 31.2% and 28.5%, respectively. EDTA and citric acid also showed a marked chelating effect.

Comparison of antioxidant activities of MEBB and several known antioxidants

Table 5 shows the antioxidant activity of MEBB and several known antioxidants. All samples had a protective effect on the BHP-induced peroxidation of liver homogenate in the concentration of samples tested. The antioxidant activities of MEBB, BHA, and catechin were about 61.5, 91.3, and 81.0%, respectively. The IC_{50} of MEBB was 0.63 mg/ml. Catechin and BHA were also at concentrations of 5.0×10^{-6} and 1.3×10^{-4} M, respectively.

Effect of BHP on SOD, catalase and GSH-Px

BHP affected the three antioxidant enzymes (Fig.. 1a-1c). WI-38 cells incubated with 0.1 mM BHP for 2 hr exhibited significantly increased SOD, catalase and GSH-Px activities ($p < 0.05$) compared with the control.

Protective effects of MEBB against oxidative injury in WI-38 cells

The antioxidant potential of MEBB was evaluated using the following experimental approach. The approach assessed the ability of cells incubated in MEBB to combat a BHP induced oxidative challenge. When the cells incubated in MEBB (45-450 μg/ml) for 18 hr were subjected to a BHP challenge, SOD activity returned to its control value or lower at all levels tested (Fig. 1a). When catalase activity was determined, a similar trend occurred except in the cells incubated in 112.5 μg/ml MEBB (Fig. 1b). BHP increased GSH-Px activity in WI-38 cells, and WI-38 cells incubated in MFHF medium supplemented with all levels of MEBB tested (45-450 μg/ml) also increased GSH-Px activity (Fig. 1c).

Discussion

Several recent studies[12,24-25] have reported that specific polyphenols scavenge superoxide and hydroxyl radicals, and inhibit lipid peroxidation. Phenolic compounds can also contribute to antioxidant activity. The content of total phenolic compounds in MEBB was 0.70 mg/ml. MEBB contained a high content of phenolic compounds, which may contribute to the antioxidant activity of MEBB. The specific mode of inhibition of lipid oxidation by the phenolic compounds is not clear; they may act by chelating Cu^{2+} via the *ortho*-dihydroxy phenolic structure or by acting as hydrogen-donating radical scavengers. The *ortho*-dihydroxy substitution in the B ring is important for stabilizing

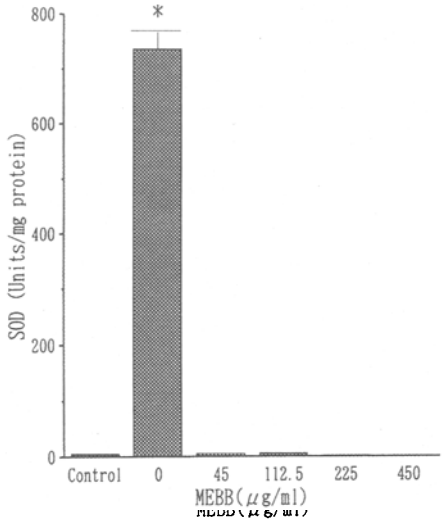

Fig. 1a The effect of MEBB on BHP-induced oxidative stress of WI-38 cells. SOD activities in WI-38 cells. WI-38 cells were incubated with MEBB or DMSO for 18 hr, washed with HBSS, and then exposed either to 0.1 mM BHP (0-450 μg/ml MEBB) or to HBSS (Control) for 2 hr. Data are expressed as the means ± standard deviation of triplicate analyses. N=4 for all treatments. One unit is defined as the amount of SOD required to inhibit the maximum rate of cytochrome *c* reduction by 50%. *$p<0.05$ compared with control.

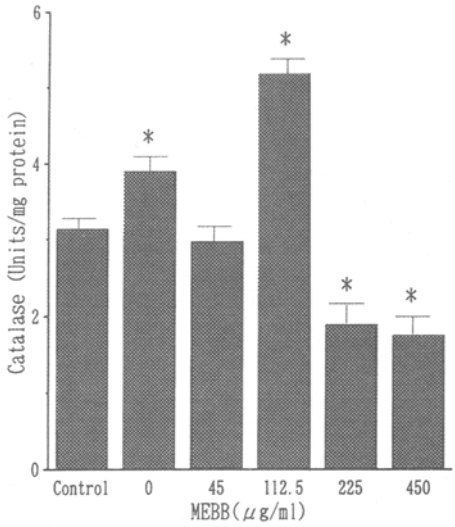

Fig. 1b The effect of MEBB on BHP-induced oxidative stress of WI-38 cells. Catalase activities in WI-38 cells. WI-38 cells were incubated with MEBB or DMSO for 18 hr, washed with HBSS, and then exposed either to 0.1 mM BHP (0-450 μg/ml MEBB) or to HBSS (Control) for 2 hr. Data are expressed as the means ± standard deviation of triplicate analyses. N=4 for all treatments. One unit is defined as 1 μmole hydrogen peroxide removed per minute. *$p<0.05$ compared with control.

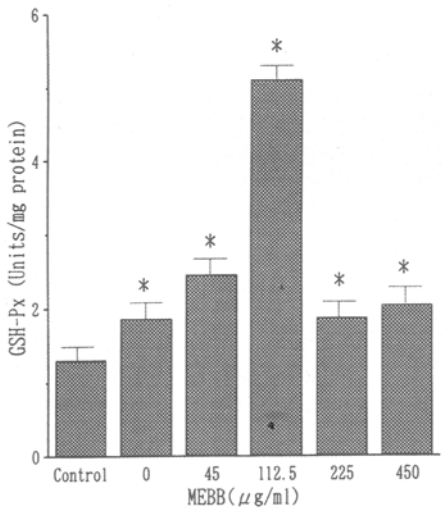

Fig. 1c The effect of MEBB on BHP-induced oxidative stress of WI-38 cells. GSH-Px activities in WI-38 cells. WI-38 cells were incubated with MEBB or DMSO for 18 hr, washed with HBSS, and then exposed either to 0.1 mM BHP (0-450 μg/ml MEBB) or to HBSS (Control) for 2 hr. Data are expressed as the means ± standard deviation of triplicate analyses. N=4 for all treatments. One unit is defined as the oxidation of 1 nmole of NADPH per minute. *$p<0.05$ compared with control.

the free radicals and for chelating metal ions. Therefore, an investigation of MEBB ability to scavenge free radicals or to chelate metal ions would be helpful in understanding the mechanism of antioxidant function of MEBB.

Superoxide decomposes to form stronger oxidative species such as hydroxyl radical and hydrogen peroxide that initiate the peroxidation of lipids[26]. Because they serve as precursors of singlet oxygen and hydroxyl radicals, superoxide and hydrogen peroxide indirectly initiate lipid oxidation[27,28]. The antioxidant activity of MEBB is thought to relate to its scavenging effects on superoxide.

Hydrogen peroxide has only a weak activity to initiate lipid peroxidation[25], but it can react with reduced metal ions via the Fenton reaction generating hydroxyl radicals[4]. Therefore, the ability of MEBB to scavenge hydrogen peroxide is thought to contribute to inhibition of the peroxidation of lipids.

MEBB (0.70 mg/ml) exhibited about 90% scavenging activity on DPPH (Table 3). Therefore, MEBB mixed with DPPH decolorized DPPH due to hydrogen-donating abilities[15]. MEBB had high hydrogen-donating activities. This result shows that the ingredients of MEBB are free radical scavengers and primary antioxidants that react with free radicals such as the hydroperoxy radical.

By using the method of Shimada *et al.*[16], we were able to show that MEBB was capable of chelating metal ions (Table 4). MEBB contains phenolics in a structure such as catechol or resorcinol, which implies that the keto group at position 4 and the hydroxy group at position 5 could afford some degree of chelation[9]. This leads to the conclusion that *in vivo* MEBB may be a potent chelator in competing binding ligands with endogenous metal ions. In the presence of transition metals such as iron or copper, superoxide and hydrogen peroxide can form highly reactive hydroxyl radicals through the metal-catalyzed Haber-Weiss or Fenton reactions[4]. Hydroxyl radicals can readily extract a hydrogen atom from an unsaturated fatty acyl group, thus initiating the process of lipid peroxidation consisting of a set of chain reaction. Therefore, the ability of MEBB to chelate metal ions may contribute to inhibition of the peroxidation of lipids.

In this study, the antioxidative activities of MEBB were determined using *in vitro* peroxidation of bovine liver homogenate induced by BHP, which is a system closer to biological conditions than that in which oils and fatty acids are used as the

oxidizable substrate[17].

Phenolic compounds have excellent antioxidant properties in some *in vitro* and *in vivo* model systems[5]. Lawlor and O'Brien[9] have shown that astaxanthin acts as an antioxidant in chicken embryo fibroblasts treated with paraquat. Rong *et al.*[19] also studied the action of *Ginkgo biloba* extract as an antioxidant using the models of oxidative stress induced by BHP. Therefore, by using *in vitro* model systems, we assessed the antioxidant capability of MEBB against BHP-induced oxidative stress in WI-38 cells. During the period when oxidative stress was induced by BHP, the cells enhanced free radical scavengers such as SOD, catalase, and GSH-Px (Fig. 1a-1c). The increases of these enzyme activities may be due to their leakage by mitochondria or peroxisome injury. Incorporation of BHP into the MEBB (45-450 μg/ml) treated cells, resulted in a reduction of SOD activity (Fig. 1a). In the case of catalase activity, a similar trend occurred except in the cells incubated in 112.5 μg/ml MEBB (Fig. 1b). MEBB may inhibit the injury of mitochondria. The reason for the increase of catalase activity in 112.5 μg/ml MEBB is still unknown. Further studies on lipid peroxidation and cell viability are indicated. GSH-Px activity increased beyond the control level (Fig. 1c) due to BHP being a substrate for GSH-Px. These results imply that MEBB exhibited free radical scavenging activities in our model.

In conclusion, we have shown that the ingredients of MEBB have strong hydrogen-donating abilities and are good scavengers of active oxygen species, including superoxide and hydrogen peroxide. Moreover, through the use of oxidative stress models, the ingredients of MEBB may decrease oxidative stress in WI-38 cells. These properties seem to be important in explaining how

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