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Manda, a Fermented Natural Food, Suppresses Lipid Peroxidation in the Senescent Rat Brain

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The level of lipid peroxidation reflects the degree of free radical-induced oxidative damage in brain tissue of the elderly. We examined the effects of Manda, a product prepared by yeast fermentation of several fruits and black sugar, on lipid peroxidation in the senescent rat brain as model of aging. Senescent rats were provided with a diet containing 50 g/100 g Manda for 8 days, supplemented on day 8 with an intragastric administration of Manda (6.0 g/kg body wt.) twice daily. The hydroxyl radical scavenging activity was generated by the $FeSO_4$-H_2O_2 system and analyzed by electron spin resonance spectrometry. Using this method, the addition of Manda (2.88 mg/ml) to brain homogenates of adult rats (0.06 mg/ml) had an additive inhibitory effect on lipid peroxidation compared with control adult rats not treated with Manda. Incubation of brain homogenates with Manda for 2 h and 3 h, significantly inhibited the increase in lipid peroxides (malondialdehydes and 4-hydroxyalkenals) levels in aged rats due to auto-oxidation. In addition, oral administration of Manda significantly suppressed the age-related increase in lipid peroxidation in the hippocampus and striatum, although such change was not observed in the cerebral cortex. Although Manda contains trace level of α-tocopherol, the level of α-tocopherol in Manda did no correlate with its antioxidant effect. Our results suggest that Manda protects against age-dependent oxidative neuronal damage caused by oxidative stress and that this protective effect may be due, in part, to its scavenging activity against free radicals.

KEY WORDS: Lipid peroxidation; aging; antioxidant; brain; Fischer rats.

INTRODUCTION

Since Harman (1) first proposed the involvement of free radicals in the aging process, there has been extensive discussion of this hypothesis (2–11). Reactive oxygen species (ROS), especially hydroxyl radicals, are probably responsible for cell death and tissue injury (12–14) under unbalanced conditions between oxidant and antioxidant processes such as aging and chronic ischemia. The central nervous system is particularly susceptible to free radical-induced damage since it is rich in polyunsaturated fatty acids. Several types of antioxidants have been used to reduce free radical-induced tissue damage in the body. These antioxidant agents with free radical-scavenging activities in vitro (15,16) decrease lipid peroxidation and attenuate the age-related reduction in membrane fluidity in vivo. In addition to the increased scavenging activity, the activity of antioxidant enzymes is also enhanced by the addition of antioxidants (17,18). In fact, such increase in enzymatic activity may represent one of the mechanisms of action of these anti-oxi-

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dants. Recent studies have also shown that many components of herbal remedies (19), sesame seeds, soybean, yeast (20,21) and miso (fermented soybean paste) (16) contain free radical scavengers such as α-tocopherol, ascorbic acid and β-carotene.

Manda is a natural product produced by yeast fermentation of several fruits and black sugar. The type and composition of the raw material and the method of preparation have been previously reported (22). Furthermore, Manda is a scavenger of the hydroxyl, superoxide and diphenyl-p-picrylhydrazyl (DPPH) radicals in vitro (22), and inhibits the formation of thiobarbituric acid-reactive substances (TBARS) in iron-induced epileptic foci in rats (23).

To elucidate the antioxidant effects of Manda on aging, we examined its scavenging activity in brain tissue of senescent rats in vitro by electron spin resonance (ESR) spectrometry-spin trapping. Furthermore, we also studied the age-dependent changes in lipid peroxides (malondialdehydes, MDA, and 4-hydroxyalkenals, 4-HDA) (24,25) in rat brain, and the effects of long-term administration of Manda.

EXPERIMENTAL PROCEDURE

Reagents. Manda, a brown, sweet and sticky fermented natural food, was provided by Manda Fermentation Co. (Hiroshima, Japan). It is prepared by yeast fermentation of black sugar, fruits, seeds, vegetables and seaweeds (22) (Table I). The analytical data and microorganisms of final products are shown in Tables II and III.

Animal Experiments. Male adult (10 wk old) and senescent (30 month) Fischer rats (Charles River Japan Inc., Kanagawa, Japan) were housed in an environment of a constant temperature (25 ± 2°C) and humidity (50 ± 5%). Experimental rats were provided with a standard diet (MF; Oriental Yeast Co., Japan) and water ad libitum before the start of this experiment. The animals were divided into four groups: control diet-treated adult rats (CA), Manda-treated adult rats (MA), control diet-treated senescent rats (CS) and Manda-treated senescent rats (MS). Control standard diet containing 50 g/100 g mannan (mannan, also known as konnyaku paste is a general food in Japan of no energy value made from konnyaku flour), diet containing 50 g/100 g Manda and water were provided freely for 8 days. For equal volume of standard diet with diet containing Manda, mannan is contained in the control diet. On day 8, Manda (6.0 g/kg body wt) was administered twice daily directly into the stomach of Manda-treated groups using a canula, while the same volume of saline was administered to the control groups. All rats were sacrificed by decapitation 15 h after the last administration. The cerebral cortex, hippocampus and striatum were dissected carefully and immersed in liquid nitrogen immediately. The brain tissues were kept at −80°C for 24 h until lipid peroxides were measured. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of our institution.

Effects of Manda on Hydroxyl Radical-Generating System In Vitro. The following compounds were mixed together in a test tube and served as the control vehicle (all data represent final concentration): 75 μl of 0.34 mM $FeSO_4$, 75 μl of 0.34 mM H_2O_2 and 50 μl of 22 mM sodium phosphate buffer (pH 7.4). To this mixture, we added 0.06, 0.12 or 0.23 mg/ml tissue homogenate of the cerebral cortex, 2.88 mg/ml Manda dissolved in 0.1 M sodium phosphate buffer or 34 ng/ml α-tocopherol (VE). Using a stock solution (20 mg/ml VE in ethanol), 34 ng/ml VE solution was diluted in 0.1 M sodium phosphate buffer, then added 20 μl of 8.4 mM 5,5'-dimethyl-1-pyrroline-1-oxide (DMPO). Ethanol used for dissolving VE had no effects on the results of this experiment. The mixture was then transferred to an ESR spectrometry cell and the DMPO-OH spin adduct formed was quantified 40 s after the addition of H_2O_2 using an ESR spectrometer (JES-FE1X,JEOL Co., Tokyo). The signal intensity was evaluated using the peak amplitude of the second signal of the DMPO-OH spin adduct. Mn^{2+} was used as an internal standard. Values were expressed as percentage of the control peak amplitude (vehicle only).

Measurement of Lipid Peroxides in Brain Tissues. MDA and 4-HDA as lipid peroxides were measured using the LPO-586 kit (Bonneuil Sur Marde, France) (24,25). These compounds are formed to a large extent by peroxidation of unsaturated fatty acids and are widely used as an index of lipid peroxidation (26). Furthermore, the kit contains a chromogenic reagent that reacts with MDA and 4-HDA at 45°C, yielding a stable chromophore with a maximum absorbance at a wavelength of 586 nm. Dissected brain tissue sections of each treated group were homogenized in 10 volumes of 20 mM Tris-HCl buffer (pH 7.4) and measured directly for MDA and 4-HDA (Figs. 3–5). To examine auto-oxidation in rat brain homogenates (*see* Figs. 2 and 5), 100 mg wet weight (wt)/ml homogenate of the cerebral cortex of the adult and senescent rats were incubated at 37°C for 0.5 to 3 h prior to the measurement of lipid peroxides. Manda and VE were prepared with 20 mM Tris-HCl buffer. VE solution was diluted to 20 mg/ml ethanol stock solution by this buffer. The levels of MDA and 4-HDA were measured in all prepared samples as follows. Immediately after centrifugation of brain homogenate at 2,500 × g for 10 min at 45°C, the pellet was resuspended in 2 or 3 volumes of 20 mM Tris-HCl buffer and 200 μl was used for the assay. In the next step, 650 μl of freshly prepared chromogenic reagent, in 11.4 mM of acetonitrile, were added to each test tube and then mixed with 200 μl of the sample. After the addition of 150 μl of 10.4 M methanesulfonic acid, the reaction mixture was throughly mixed and then incubated for 40 min at 4°C. The test tube was placed on ice after incubation to stop the reaction prior to measurement of absorbance at 586 nm.

Statistical Analyses. Data were expressed as mean ± SD. Differences between groups were statistically analyzed by using one-way ANOVA or Mann-Whitney *U*-test (27) (*see* Figs. 1 and 4) and by two-way ANOVA followed by *post-hoc* Fischer's PLSD multiple comparison test (27) (*see* Figs. 2 and 3) using the StatView software (Abacus Concepts, Inc., Berkeley, CA). A p value less than 5% denoted the presence of statistically significant difference.

RESULTS

Average Daily Food and Water Intake. The average daily food intake in the adult-control diet, adult-Manda, senescent-control diet and senescent-Manda groups was $68.5 \pm 13.6^{a*}$, 85.7 ± 29.8^{bc}, 27.0 ± 4.7^{ab} and 51.3 ± 13.3^{c} g/kg body wt, respectively, where * denotes $p < 0.01$ for comparison of data with the same letters. The water intake in each group was $100.9 \pm 31.4^{d**}$, 63.8 ± 15.6, 57.9 ± 15.9^{d} and 73.7 ± 19.3 ml·kg body

Table I. Composition of Manda

Composition		g/100 g
Fruits	apple, persimmon, banana, pineapple, akebi, silvervine, fig, wild vine, etc.	26.1
Citrus	orange, chinese citron, navel orange, *Citrus* Hassaku, true citron, etc.	14.0
Roots	burdock, carrot, garlic, lotus root, etc.	5.3
Cereals	brown rice, glutinous rice, wheat, rice, etc.	8.1
Pulse	soy bean, black sesame, white sesame, etc.	5.2
Marine algae	kombu, laver, etc.	5.3
Sugar	black sugar, etc.	33.4
Others	honey, starch, etc.	2.6
	Total	100.0

Table II. Essential Nutrients Present in Manda

Nutrient	Content (g/100 g)
Water*	33.8
Protein	2.1
Lipid	0.1
Carbohydrate	62.2
Ash	1.8
Total	100.0

Analyzed by the Institute of Food Hygiene, Japan Food Hygiene Association.
*Lost on drying (Vacuum at 70°C)

Table III. Vitamins and Inorganic Elements Present in Manda

Element	Concentration
Vitamins	
A (as β-carotene) (mg/100 g)	0.10
B_1 (mg/100 g)	0.02
B_2 (mg/100 g)	0.07
B_6 (mg/100 g)	0.36
C (ascorbic acid) (mg/100 g)	0.00
E (α-tocopherol) (mg/100 g)	1.20
Niacin (mg/100 g)	1.54
Minerals	
Calcium (mg/100 g)	33.8
Phosphorus (mg/100 g)	43.0
Iron (mg/100 g)	2.40
Sodium (mg/100 g)	57.0
Potassium (mg/100 g)	670
Magnesium (mg/100 g)	70
Copper (ppm)	1.0
Arsenic (ppm)	<0.2
Lead (ppm)	<0.5
Cadmium (ppm)	<0.1
Mercury (ppm)	<0.05

Analyzed by the Institute of Food Hygiene, Japan Food Hygiene Association.

$wt^{-1}\cdot day^{-1}$, respectively, where ** denotes $p < 0.05$ for comparison of data with the same letters.

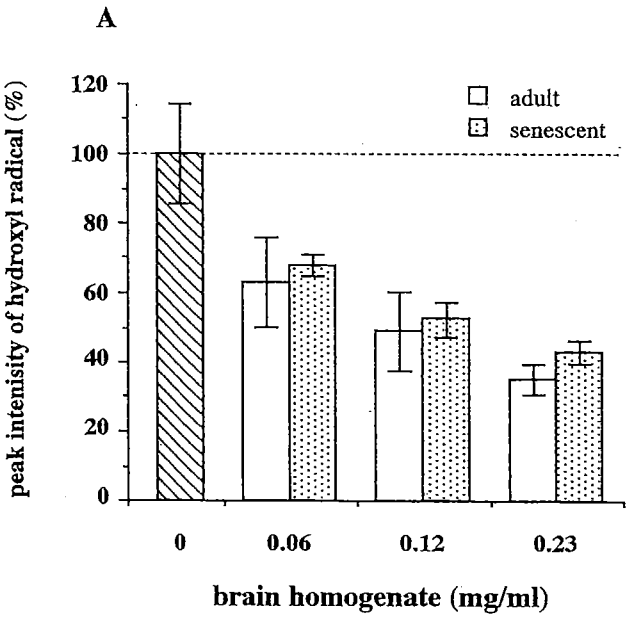
Effects of Manda and Brain Homogenate on Hydroxyl Radical Generation In Vitro. Brain homogenate dose-dependently scavenged hydroxyl radicals generated by the Fenton reaction system ($FeCl_2$-H_2O_2) as shown in Fig. 1A. The scavenging activity of adult brain homogenate was not significantly different from that of senescent rats at homogenate concentration of 0.06, 0.12 mg/ml and 0.23 mg/ml (Fig. 1A).

In adult and senescent rats, a mixture of 0.06 mg/ml brain homogenate and 2.88 mg/ml Manda solution reduced the level of hydroxyl radicals, i.e., there was a significantly higher scavenging activity than 0.06 mg/ml brain homogenate alone or 2.88 mg/ml Manda solution alone (Fig. 1B). Although brain homogenate and Manda had superoxide radical scavenging activity, a combination of the two did not produce an additive effect on the scavenging activity of this particular radical (data not shown).

Inhibitory Effect of Manda on Auto-Oxidation of Rat Brain Homogenate. Changes in the concentration of lipid peroxides in cerebral cortex homogenates were measured in brain tissue homogenates of the adult and senescent rats. Treatment with Manda significantly inhibited the increase in lipid peroxides concentration observed during auto-oxidation at 2 h and 3 h of incubation of brain homogenates of the senescent rats (Fig. 2). In the adult rat brain, however, treatment with Manda failed to influence the level of lipid peroxides after incubation (Fig. 2).

In Vivo Effects of Manda on Age-Dependent Changes in Lipid Peroxides in Different Rat Brain Regions. The level of lipid peroxide (MDA and 4-HDA) was measured in the cerebral cortex, hippocampus and striatum in homogenates of the adult and senescent rat brains. The level of lipid peroxides in the cerebral cortex of Manda-treated adult animals was similar to that of senescence rats (Fig. 3A). In contrast, its level in the hippocampus and striatum in the control diet-treated senescent group was significantly higher than in adult control rats (Figs. 3B and C). Furthermore, in both the hippocampus and striatum, the level of lipid peroxides in Manda-treated senescent rats was significantly lower than in control diet-treated senescent group.

Effects of Manda, the VE Implicit in Manda and Brain Homogenate on Hydroxyl Radical Generation In Vitro. The amount of VE in 2.88 mg/ml Manda was 34 ng/ml (see Table III), but this amount of VE had no effect on hydroxyl radical generation (Fig. 4). Although the combination of brain homogenate and VE showed an additive effect on the scavenging activity, this effect



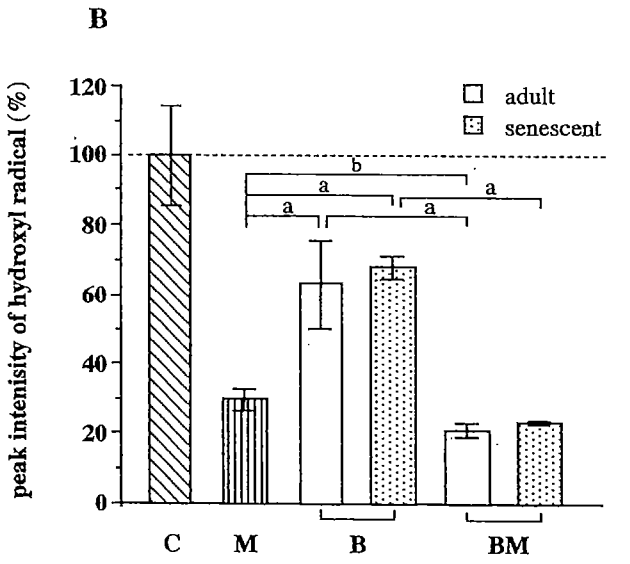


Fig. 1. Scavenging effects of brain homogenates of control group and/or Manda against hydroxyl radicals generated by the Fenton reaction and trapped with 5,5'-dimethyl-1-pyrroline-1-oxide. (A) Dose-dependent scavenging effect of brain homogenate alone; (B) Scavenging effects of 0.06 mg/ml of brain homogenate in the absence or presence of Manda solution (2.88 mg/ml) on generated hydroxyl radicals. Data are mean ± SD (n = 5 to 6). Bars with same letters are significantly different (a; $p < 0.01$, b; $p < 0.05$). Abbreviations: *C*, control (vehicle only); *M*, Manda; *B*, brain homogenate; BM, brain homogenate + Manda.

was significantly lower than the scavenging effect of a combination of brain homogenate and Manda (Fig. 4).

In Vitro Effect of Manda and the Implicit Level of VE in Manda on Auto-Oxidation of Rat Brain Homogenate. We also measured changes in lipid peroxides in cerebral cortex homogenates using brain tissue homogenates of adult rats with Manda and the implicit level of VE in Manda. Although treatment with Manda inhibited the rise in lipid peroxides concentration observed during auto-oxidation at 1–3 h of incubation of brain homogenates of adult rats, the use of 10-times higher concentrations of VE in Manda did not show the inhibitory effect observed by Manda (Fig. 5).

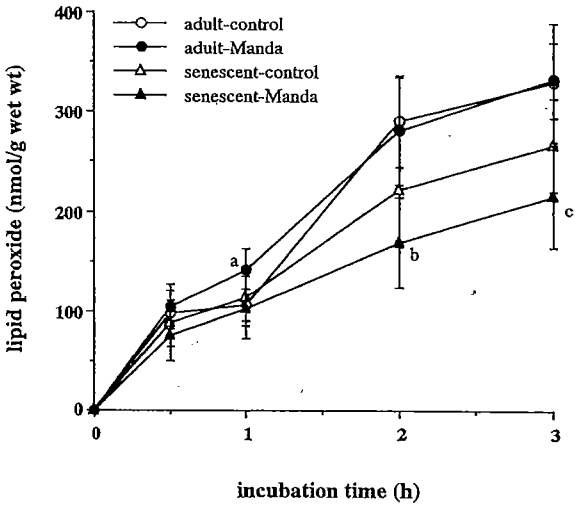


Fig. 2. Effects of Manda on serial changes in lipid peroxides in homogenates of cerebral cortex of adult and senescent rats. 100 mg/ml of brain homogenates were incubated for 0.5, 1, 2 and 3 h at 37°C. Each data point represents the mean ± SD of 6 to 12 samples. Letters represent data points significantly different from that of adult control at 1 h (a; $p < 0.05$), and that of senescent control at 2 h (b; $p < 0.01$) and 3 h (c; $p < 0.05$).

DISCUSSION

The lipids of neuronal cell membrane are rich in polyunsaturated fatty acids, and high concentrations of iron are also present in the brain (28). Hydroxyl radicals are produced from (ROS) by electron transfer. ROS produced by the mitochondria and not scavenged by superoxide dismutase (SOD) may react with the membrane lipids, resulting in the accumulation of lipid peroxides, causing a chain reaction that can eventually lead to cell death or damage. Brain homogenates scavenge superoxide and hydroxyl radicals in vitro in a dose dependent manner (29,30). The present results also showed that the brain homogenate of adult and senescent rats effectively protected against damage by free radicals in vitro (Fig. 1A).

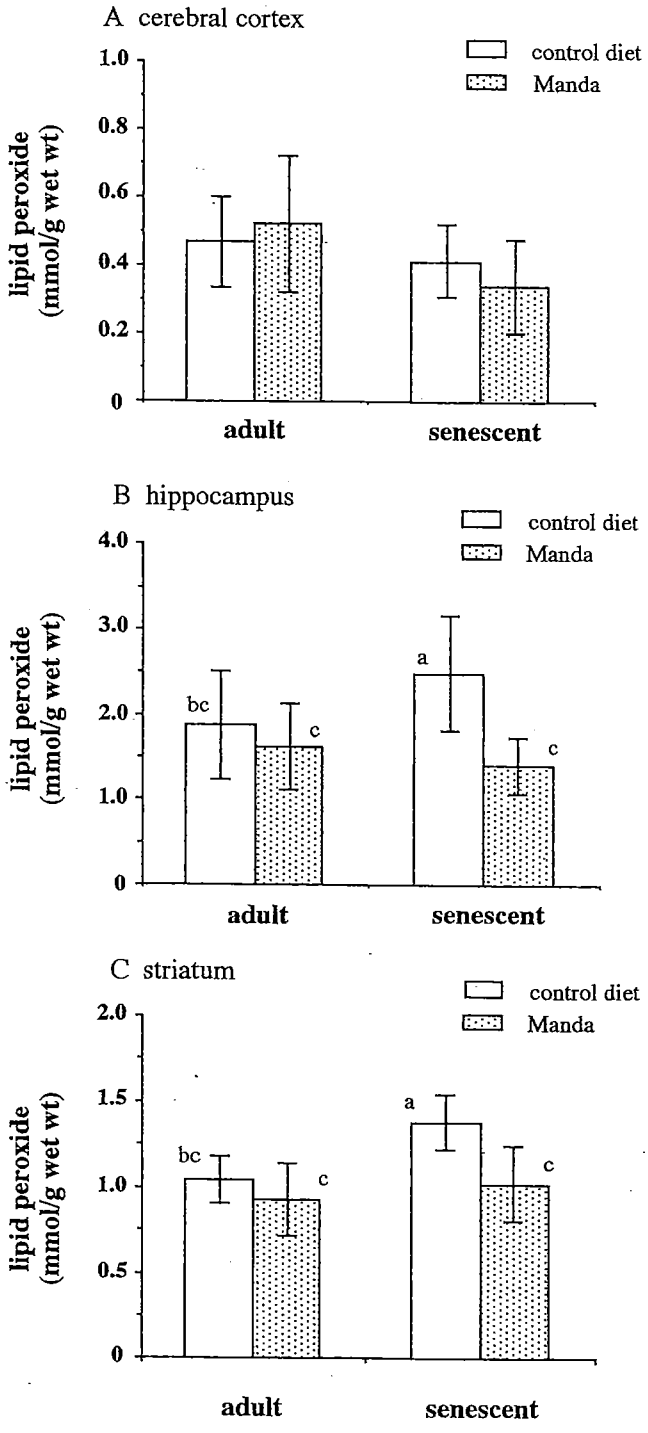


Fig. 3. Effects of long-term administration of Manda on age-related changes in lipid peroxides in three regions of the brain in the adult and senescent rats (A) cerebral cortex; (B) hippocampus; (C) striatum. Data are mean ± SD of 6 to 12 samples. Bars with the different letters are significantly different, $p < 0.01$.

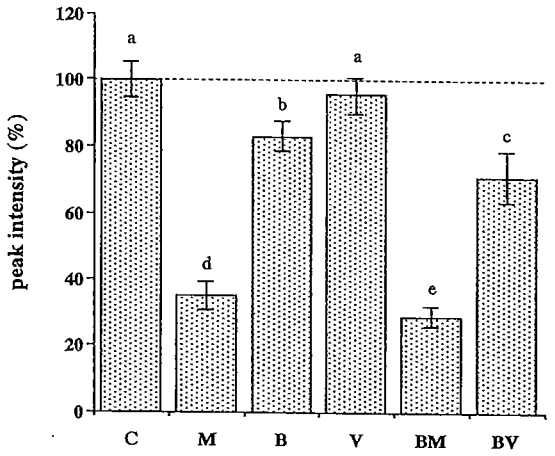


Fig. 4. Scavenging effects of brain homogenate (0.06 mg/ml) of control group and/or Manda (2.88 mg/ml), α-tocopherol (34 ng/ml) against hydroxyl radicals generated by the Fenton reaction and trapped with 5,5'-dimethyl-1-pyrroline-1-oxide. Data are mean ± SD of 5 to 7 samples. Bars with the different letters are significantly different (a; $p < 0.05$, b, c, d and e; $p < 0.01$). Abbreviations: as in Fig. 1; V, α-tocopherol; BV, brain homogenate + α-tocopherol.

Manda scavenges superoxide, hydroxyl and DPPH radicals in vitro (22) and inhibits lipid peroxides (TBARS) in epileptic foci in rats (23), findings similar to those reported for other fermented foods (16). In the present study, Manda scavenged hydroxyl radicals and acted in an additive manner to increase the scavenging activity of adult and senescent brain homogenates (Fig. 1B).

The level of lipid peroxides increases with time in brain homogenates due to auto-oxidation (31). Therefore, to examine the effect of Manda administration on auto-oxidation in vivo, we measured first the serial changes in lipid peroxides in homogenates of the cerebral cortex of adult and senescent rats. The results shown in Fig. 2 suggest that daily treatment with Manda increased the enzymatic and nonenzymatic antioxidative effects.

We also examined the effects of Manda administration on age-dependent changes in MDA and 4-HDA, representing the end-products of lipid peroxidation. In the hippocampus and striatum of the senescent rat brain, tissue homogenates of Manda-treated rats significantly inhibited the age-related rise in lipid peroxide (Fig. 3), suggesting that administration of Manda in vivo increases the antioxidative activity of the brain tissue.

As shown Table III, Manda contains various vitamins and minerals. Although, Manda is free from ascorbic acid, it is possible that VE which is one of the components of final production, might influence the antioxidant effects of Manda demonstrated in Figs. 1–3.

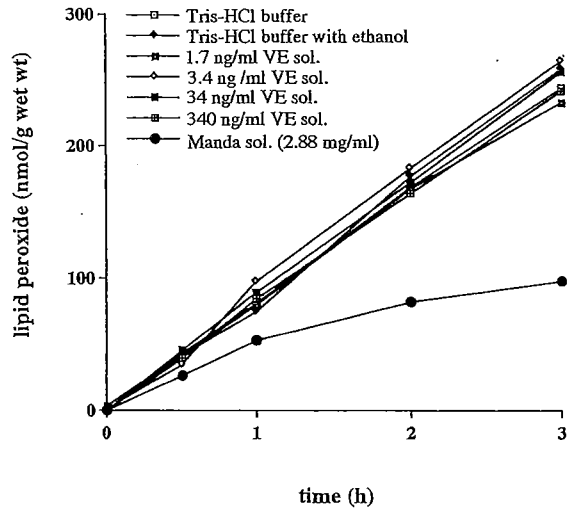


Fig. 5. Effects of Manda and implicit α-tocopherol in Manda on serial changes in lipid peroxides in homogenates of rat cerebral cortex. Manda (2.88 mg/ml) and each concentration of α-tocopherol (VE) including implicit VE component (34 mg/ml) in Manda were incubated with brain homogenate (0.06 mg/ml) for 0.5, 1, 2 and 3 h at 37°C.

Therefore, to confirm the separate effect of VE in vitro, we investigated the influence of 2.88 mg/ml of Manda which contained 34 ng/ml of VE. The equivalent trace level of VE in Manda had no effect on the scavenging action of hydroxyl radical (Fig. 4). The additive effect of brain homogenate and VE is significantly lower than the combination of brain homogenate and Manda.

Furthermore, we compared the inhibitory effects of Manda and VE on auto-oxidation of rat brain homogenate in vitro. However, the equivalent trace level of VE and the use of 10 times higher concentrations of VE showed no inhibitory effect (Fig. 5). These results suggest that other yet unknown components of Manda, apart from VE may be responsible for the anti-oxidant effects of Manda. It is suggested that these components may have a direct radical scavenger activity and/or support the enzymatic or nonenzymatic antioxidant action.

In this regard, we found that Manda increased the mitochondrial SOD activity in the striatum of senescence-accelerated mouse (SAMP8) brain (unpublished data). The increased SOD activity and inhibition of lipid peroxidation by the administration of this agent in this study indicate that Manda may enhance the endogenous and compensatory reactions against cell aging caused by oxygen-derived free radicals such as superoxide, hydroxyl radicals, H_2O_2, and peroxides.

Antioxidative mechanisms may play an important role in protecting the brain against aging. As such, they may be related to catalase, glutathione peroxidase, α-tocopherol, ubiquinone, glutathione and ascorbic acid. The complex interaction among these intrinsic cellular antioxidative systems and nutrition may lead to imbalances in the oxidation-reduction status of the brain tissue, increasing its susceptibility to oxidative stress. Therefore, the effects of this fermented antioxidant on lipid peroxidation in brain tissue may be based not only on the multiple effects of the dietary components themselves, but also on their interaction with the cellular antioxidative systems.

In conclusion, we showed that Manda exerts a scavenging activity on hydroxyl radicals and that the administration of Manda ameliorates the age-dependent increases in lipid peroxidation in the brain. Therefore, this agent may be beneficial as an antioxidant to protect the neurons in the senescent brain against excessive amounts of ROS.

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