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# Neuroprotective Effect of Rough Aster Butanol Fraction against Oxidative Stress in the Brain of Mice Challenged with Kainic Acid

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The neuroprotective effect of the butanol fraction from the methanol extract of *Aster scaber* Thunb. (rough aster butanol fraction) on oxidative damage in the brain of mice challenged with kainic acid was examined using behavioral signs and biochemical parameters of oxidative stress. The rough aster butanol fraction (0.4−1.0 g/kg) was administered to ICR male mice, 6−8 weeks, through a gavage for 4 days consecutively, and on the third day, kainic acid (50 mg/kg) was ip administered. When compared to the vehicle-treated control, no significant changes in body and brain weight were observed in mice administered the rough aster butanol fraction. Administration of kainic acid only, causing a lethality of ∼54%, resulted in a significant decrease of total glutathione level and an increase of the thiobarbituric acid-reactive substances (TBARS) value in brain tissue. When the rough aster butanol fraction was examined for neuroprotective action, the rough aster butanol fraction (0.4 g/kg) alleviated the lethality (25%) of kainic acid and the behavioral sign of its neurotoxicity. Moreover, administration of the rough aster butanol fraction at a dose of 0.4 g/kg restored the glutathione level in the cytosolic portion of brain homogenate to ∼80% ($p < 0.05$). Also, the rough aster butanol fraction (0.4 g/kg) led to a significant reduction of kainic acid-induced increase of TBARS value. In addition, the glutathione peroxidase activity was restored significantly ($p < 0.05$) in the cytosolic portion of brain homogenate, whereas glutathione reductase activity was not. On the basis of these results, the rough aster butanol fraction is suggested to contain a functional agent to prevent oxidative stress in the brain of mice.

**KEYWORDS: Rough aster butanol fraction; kainic acid; neuroprotection; glutathione; lipid peroxidation**

## INTRODUCTION

Glutamate receptor-mediated excitotoxicity is known to be a causative factor in a variety of neurological disease states (*1*). Kainic acid (KA) has been used as a model agent for the study of neurotoxicity of various excitatory amino acids, because KA-induced excitotoxicity is known to be associated with the excessive release of glutamate that may underlie the pathogenesis of neuronal injury (*2*, *3*). Kainic acid may induce neuronal damage through the excessive production of reactive oxygen species (ROS) and lipid peroxidation (*4*, *5*), triggered by the activation of excitatory amino acid receptor. The brain may be particularly vulnerable to oxidative stress in that it consumes a large amount of the body's oxygen but has a relative paucity of protective systems, such as glutathione peroxidase, glutathione (GSH), and vitamin E (*3*, *6*), which function as major antioxidants against oxidative stress in tissues including the brain and participate nonenzymatically and enzymatically in supporting cellular redox balance and in protecting against ROS-mediated oxidative damage (*6*−*8*). The intracellular level of reduced glutathione is maintained by glutathione reductase, a dimeric cytosolic enzyme that uses NADPH as a cofactor to catalyze the reduction of oxidized glutathione (*9*). Enzymes that clear or prevent ROS formation are superoxide dismutase, catalase, and glutathione peroxidase (*10*). Previous studies showed that the GSH level decreased and lipid peroxidation increased in the brains of rats treated with kainic acid (*4*, *11*). In support of this, recently, the antioxidant action of glutathione, melatonin, or *trans*-resveratrol was suggested to be responsible for the neuroprotective action in KA-induced neurotoxicity (*4*, *5*, *11*). In this respect, vegetable extracts, which contain various antioxidants, are expected to prevent ROS-mediated brain damage. For this purpose, vegetable antioxidants, which can readily penetrate the blood−brain barrier, may have a potential role in preventing excitotoxin-induced neurotoxicity.

*Aster scaber* Thunb. (rough aster) is widespread and cultivated as a culinary vegetable in Korea. Aster species have been used

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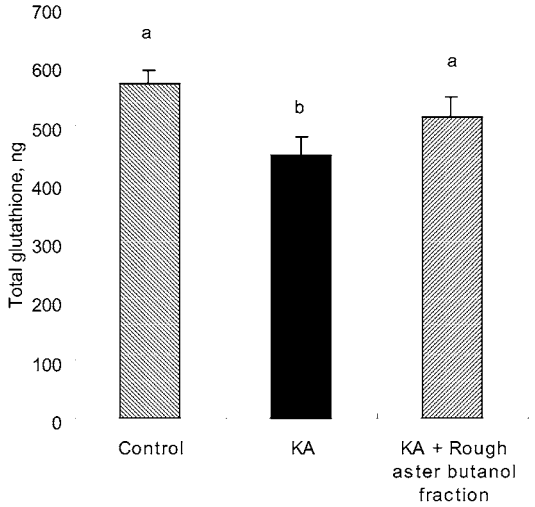


**Figure 1.** Total glutathione level in the brain tissue of mice administered kainic acid (KA) and rough aster butanol fraction. Brain tissues from mice administered KA and the rough aster butanol fraction (0.4 g/kg) were homogenized with 6% metaphosphoric acid, and the supernatant, after centrifugation, was used for the determination of GSH. Values with the same letter are not significantly different ($p < 0.05$). Values are means ± SE of three determinations from four to eight mice.

in traditional Chinese medicine to treat bruises, snakebite, headache, and dizziness (*12*). In our previous studies, rough aster ethanol extract at a high dose appeared to recover the NMDA-induced decline in total glutathione level of brain tissue (*13*). Very recently (*14*), it was shown that the ethyl acetate fraction of methanol extract from rough aster contained antioxidants, such as caffeoylquinic acid, which demonstrated a neuroprotective action in PC12 cells. Nevertheless, there is no attempt to test the neuroprotective effect of the solvent fraction of the methanol extract from rough aster on KA-induced neurotoxicity in vivo. In the course of screening vegetable extracts, we found that the methanol extract from rough aster showed a significant antioxidant action.

The objective of our study is to examine the anticonvulsant effect and the neuroprotective effect of the butanol fraction of the methanol extract from rough aster on the oxidative stress in the brains of mice treated with kainic acid. The oxidative stress was evaluated by measuring the level of total glutathione, the extent of lipid peroxidation, and the activity of glutathione-related enzymes in brain tissue.

## MATERIALS AND METHODS

**Materials.** Fresh rough aster (*A. scaber* Thunb.) was purchased from a local market in Taejon, Korea. Kainic acid, glutathione reductase (type III from baker's yeast), cumene peroxide, reduced glutathione (GSH), oxidized glutathione (GSSG), tetramethoxypropane, 5,5′-dithio-2-nitrobenzoic acid (DTNB), xanthine, NADP, NADPH, thiobarbituric acid (TBA), and bovine serum albumin were products of Sigma Chemical Co. (St. Louis, MO).

**Preparation of Vegetable Extracts.** Washed and chopped fresh rough aster was dried in a convection oven (at 80 °C) for 24 h. The dried leaves of rough aster were extracted three times with methanol in the dark (15 °C). The methanol extract, after concentration at 40 °C by rotary evaporator (RE111, Büchi, Switzerland), was suspended in distilled water, and the suspension was partitioned three times with hexane, chloroform, ethyl acetate, and *n*-butanol, successively (**Figure 1**). Each fraction was evaporated at low temperature under reduced pressure and then used for in vitro or in vivo antioxidant experiment.

**Prevention of Lipid Peroxidation in Vitro.** $Fe^{2+}$-mediated lipid peroxidation in brain homogenate was induced with $Fe^{2+}$ and ascorbic acid in vitro. Brain homogenate was incubated with 0.2 mM $Fe^{2+}$ and 25 mM ascorbic acid in the presence or absence of vegetable extract, and the mixture was placed in a shaking water bath at 37 °C. After 30 min, equal volumes of 15% trichloroacetic acid (TCA) and 0.75% thiobarbituric acid (TBA) were added to the mixture. The reaction mixtures were heated in boiled water for 15 min, kept in ice for 5 min, and then centrifuged for 10 min at 3000 rpm to separate corpuscolate particles. The absorbance (*A*) of the supernatant was measured using a spectrophotometer at 533 nm. Calibration was performed using a malondialdehyde standard prepared by hydrolysis of 1,1,3,3-tetraethoxypropane (*15*). $IC_{50}$ values for inhibition of lipid peroxidation were derived by interpolation of a log concentration versus inhibition plot using eight concentrations of the extract, spanning the 50% inhibition point. All experiments were run in triplicate. The antioxidant activity was expressed as percent decrease of thiobarbituric acid-reactive substances (TBARS) relative to the control using the following equation:

$$\text{antioxidant activity (\%)} = [1 - (A_3 - A_4)/(A_1 - A_2)] \times 100$$

$A_1$ A(brain homogenate + ascorbate/$Fe^{2+}$)
$A_2$: A(brain homogenate)
$A_3$: A(brain homogenate + ascorbate/$Fe^{2+}$ + sample)
$A_4$: A(brain homogenate + sample)

**Animal Experiments.** Male ICR mice (22.5 ± 2.5 g), 6−8 weeks of age, were housed in polycarbonate cages (five per cage) and fed unrestricted amounts of water and pelleted commercial diet (SamYang Co.). The temperature and relative humidity were 23 ± 3 °C and 60 ± 10%, respectively, and 12-h light/12-h dark cycles were maintained. All animal experiments were conducted in compliance with the *Guide for Care and Use of Laboratory Animals* of the National Institutes of Health Guidelines (*16*). The mice were assigned randomly to treatment groups and were weighed individually every day. Mice were administered orally the suspended butanol fraction of the methanol extract from rough aster (0.4 or 1 g/kg per day) using an esophagus needle for 4 days consecutively before and 1 day after KA injection. KA was dissolved in saline, and the solution (50 mg/kg) was injected intraperitoneally into the mice. The brains of mice were quickly excised after fasting for 1 day, frozen in liquid nitrogen, and stored at −80 °C until used (within 48 h). Control mice were administered the same volume of saline without rough aster butanol fraction. Behavioral changes after KA administration were monitored according to the method for mice described by Kondo et al. (*17*). In addition, the onset time (minutes) of the first behavioral change and the mortality were examined.

**Measurement of Lipid Peroxidation in Vitro.** Brain tissue, rinsed with 0.15 M KCl solution containing 2 mM EDTA, was homogenized in 9 volumes of 10 mM phosphate buffer (pH 7.4) using a tissue homogenizer with a Teflon pestle. To the brain homogenate (1.0 mL) was added 1.0 mL of 8.1% SDS, 2 mL of 20% acetic acid, and 1 mL of 0.75% TBA. The mixture was boiled and placed on ice after 30 min of boiling. The mixture was centrifuged (14000 rpm, 10 min), and then the absorbance of the supernatant was measured at 533 nm as previously described (*18*).

**Determination of Total GSH.** Brain tissue (∼0.2 g wet wt) was pulverized in a cooled ceramic percussion mortar with 6% metaphosphoric acid, and the mixture was centrifuged (27000*g*, 20 min) at 4 °C. Total GSH was determined enzymatically according to a published procedure (*19*, *20*) with modifications. To 0.05 mL of supernatant was added 0.39 mL of 100 mM phosphate buffer (pH 7.4) containing 5 mM EDTA, 0.025 mL of 10 mM DTNB, and 0.08 mL of 5 mM NADPH. After 3 min of equilibration at 25 °C, the reaction was started by adding 2 units of GSH reductase. The formation of 2-nitro-5-thiobenzoic acid was continuously recorded at 412 nm with a UV−vis spectrophotometer. The total amount of GSH in the samples was determined from a standard curve obtained by plotting the known amount of GSH versus the rate of change of absorbance at 412 nm.

**Assay of GSH Peroxidase and Reductase.** Brain tissue was homogenized in 9 volumes of 20 mM phosphate buffer containing 0.1 M KCl, 1 mM EDTA, and 0.5% Triton X-100. The homogenate was centrifuged (15000*g*, 15 min), and the supernatant was recentrifuged (105000*g*, 30 min). The last supernatant was retained for enzymatic

**Scheme 1.** Systematic Solvent Fractionation of Rough Aster Extract

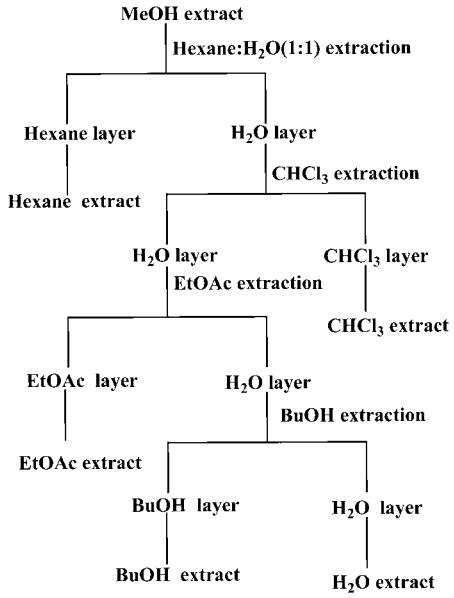


**Table 1.** $IC_{50}$ Values of Solvent Fractions of Rough Aster Extract[a]

| fraction | $IC_{50}$ (mg/mL) | fraction | $IC_{50}$ (mg/mL) |
|---|---|---|---|
| ethyl acetate | 0.142 | water | 0.034 |
| chloroform | 0.142 | ascorbic acid | 0.790 |
| butanol | 0.028 | | |

[a] Lipid peroxidation assay was carried out in the presence of each solvent fraction at different concentrations as described under Materials and Methods.

assays. The assay of GSH peroxidase activity was carried out as described previously (*21*). A mixture containing 0.1 M phosphate buffer (pH 7.0), 3 mM EDTA, 1 mM GSH, 0.1 mM NADPH, 2 units of GSH reductase, and 0.05 mL of supernatant was incubated for 3 min, and 0.01 mL of 10 mM cumene hydroperoxide was then added to the reaction mixture. GSH reductase activity was measured in a mixture containing 0.1 M phosphate buffer (pH 7.0), 0.5 mM EDTA, 1 mM GSSG, 0.1 mM NADPH, and 0.05 mL of the supernatant (*4*).

**Protein Determination.** Protein was determined according to the method of Bradford (*22*) using bovine serum albumin as a standard.

**Statistical Analyses.** All statistical analyses were performed using a SAS program (*23*). Duncan's multiple-range test was used to determine a significant difference among treatment groups after initial demonstration of a treatment-related effect by analysis of variance. All data are presented as mean ± standard error (SE). Statistical assessments were performed using ANOVA followed by post-hoc Duncan's multiple-range test. Statistical significance refers to results where $p < 0.05$ was obtained.

## RESULTS

**In Vitro Screening of a Potent Antioxidant Fraction of Rough Aster Extract.** To select a fraction of rough aster extract expressing a potent antioxidant action, each fraction (**Scheme 1**) was examined for the prevention of lipid peroxidation of brain membrane. As represented in **Table 1**, the $IC_{50}$ values of the solvent fraction of the methanol extract from rough aster ranged from 0.028 to 0.142 mg/mL. The greatest protection against lipid peroxidation was shown by the butanol fraction, with an $IC_{50}$ value of 0.028 mg/mL. Therefore, the butanol fraction was employed in the further study to evaluate the neuroprotective action in vitro.

**Behavioral Change and Mortality.** First, the neurotoxic effect of kainic acid on the behavior of mice was investigated.

**Table 2.** Effect of Rough Aster Butanol Fraction on Kainic Acid-Induced Neurobehavioral Changes and Death in Mice[a]

| treatment | death (%) | onset time of first sign (min) |
|---|---|---|
| control | 0 | |
| KA (50 mg/kg) | 54 | 10.7 ± 1.5a |
| KA (50 mg/kg) + rough aster butanol fraction (400 mg/kg) | 25 | 16.4 ± 1.3b |

[a] Values are means ± SE of determinations from four to eight mice. Any two means in the same row with different letters represent a significant difference at $p < 0.05$.

**Table 3.** Change of Body Weight and Brain Weight of Mice Administered Kainic Acid and Rough Aster Butanol Fraction[a]

| treatment | initial body wt (g) | final body wt (g) | Δ body wt (g) | brain wt (g) |
|---|---|---|---|---|
| control | 31.48 ± 1.09NS | 32.06 ± 1.06NS | 0.58 ± 0.080NS | 0.355 ± 0.0059NS |
| KA (50 mg/kg) | 31.39 ± 0.49 | 32.34 ± 0.58 | 0.95 ± 0.385 | 0.346 ± 0.0038 |
| KA (50 mg/kg)+ rough aster butanol fraction (400 mg/kg) | 31.3 6± 0.91 | 32.40 ± 1.09 | 1.03 ± 0.308 | 0.352 ± 0.0034 |

[a] Values are means ± SE of determinations from four to eight mice. NS = not significant at $p < 0.05$.

When KA only was administered (50 mg/kg) ip to male mice, the typical sustained seizure was evident 10 min after ip administration with a tremor persisting for 20−30 min, in contrast to the vehicle-treated group, which showed no seizure activity. In the following experiment, we examined the neuroprotective effect of each fraction of methanol extract from rough aster on the neurotoxicity of KA. For this purpose, the butanol fraction was chosen, because it was most effective among all of the solvent fractions of rough aster methanol extract screened in preventing the lipid peroxidation of brain membrane in vitro experiment (**Table 1**). Therefore, the butanol fraction of the methanol extract from rough aster (rough aster butanol fraction) was administered by gavage (0.4 g/kg) to mice before the exposure to kainic acid. Although the characteristic behavioral toxicity of KA was not altered by the administration (0.4 g/kg) of the butanol fraction, the onset time of neurobehavioral change was significantly ($p < 0.05$) delayed in the butanol fraction/KA-treated mice as compared to the vehicle/KA-treated mice (**Table 2**). Moreover, mortality in the butanol fraction/KA-treated mice group decreased to 25%, in contrast to 54% in the vehicle/KA-treated group. However, the increase of the dose to 1 g/kg failed to further ameliorate the mortality. Separately, when the effect of the rough aster butanol fraction on body weight and brain weight was examined (**Table 3**), it was found that no significant change in body weight or brain weight was observed with the rough aster butanol fraction. Thus, mice survived the 5 day gavage administration of the rough aster butanol fraction.

**Protective Effect of Rough Aster Butanol Fraction on Glutathione Level in Brain of Kainic Acid-Treated Mice.** To see whether oxidative stress was involved in the neurotoxicity of KA, we examined the change in the amount of total glutathione, a biochemical marker of oxidative stress, in the brain of mice administered kainic acid (50 mg/kg). As shown in **Figure 1** the administration of KA at 50 mg/kg reduced the level of total glutathione in the cytosol of mouse brain to ~73% of control level ($p < 0.05$). Meanwhile, administration of the rough aster butanol fraction showed a significant restoration of glutathione level (**Figure 2**); the rough aster butanol fraction at

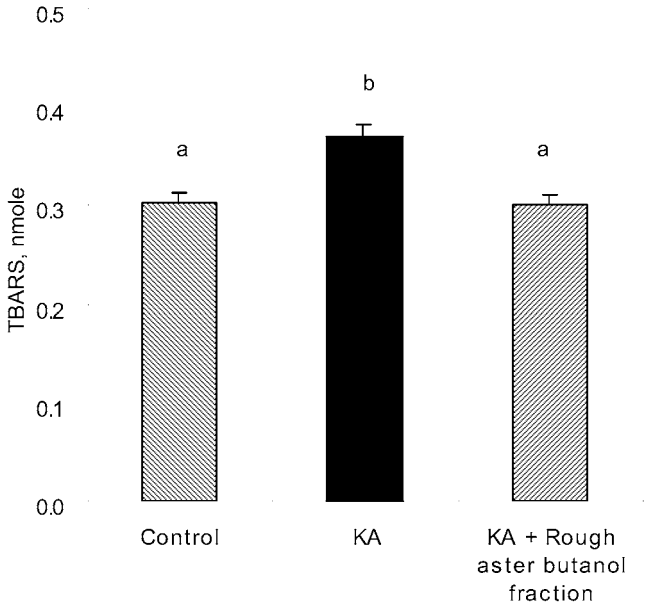


**Figure 2.** Thiobarbituric acid-reactive substances (TBARS) in brain tissue of mice administered kainic acid (KA) and rough aster butanol fraction. The whole homogenate of brain tissue was used for the determination of TBARS value. Values with the same letter are not significantly different ($p < 0.05$). Values are means ± SE of three determinations from four to eight mice.

0.4 g/kg restored the GSH level to ~80.3% of the control group ($p < 0.05$).

**Effect of Rough Aster Butanol Fraction on Lipid Peroxidation in the Brain of Kainic Acid-Treated Mice.** Subsequently, the effect of the rough aster butanol fraction on lipid peroxidation in the brains of mice treated with KA was investigated. **Figure 2** shows that the TBARS value was increased to 122% of the control value in the homogenate of whole brain of mice treated with KA. Meanwhile, treatment with the rough aster butanol fraction (0.4 g/kg) diminished significantly the level of TBARS value to a control level ($p < 0.05$), indicating that the butanol fraction may be effective in preventing lipid peroxidation.

**Protective Effect of Rough Aster Butanol Fraction on GSH Peroxidase or GSH Reductase Activities.** In an independent study to elucidate the mechanism responsible for the restoration of GSH level by the rough aster butanol fraction (0.4 g/kg), the change in glutathione-related enzymes such as GSH peroxidase and GSH reductase in the rough aster butanol fraction/KA-treated group was examined. **Figure 3A** indicates that treatment with the rough aster butanol fraction appeared to restore the loss of the GSH peroxidase activity, which was caused by KA. However, there was no significant change of GSH reductase activity among the vehicle-treated group, KA-treated group, and rough aster butanol fraction/KA-treated group (**Figure 3B**), suggesting that under the experimental conditions used, the neurotoxicity of KA may not be sufficient to alter the level of GSH reductase activity.

## DISCUSSION

Some part of kainic acid-triggered excitotoxicity in the brain involves the production of reactive oxygen radicals, which are known to cause the reduction of the GSH level and the increase of lipid peroxidation and protein oxidation (*24*). Consistent with this, the present study confirms that total GSH level, TBARS value, and glutathione peroxidase activity are indicators of KA-induced oxidative imbalance in the brain tissue of mice. The brain has endogenous antioxidants such as glutathione and melatonin. A primary protective compound is glutathione, which acts both as a substrate in the glutathione peroxidase-mediated destruction of hydroperoxides and as a scavenger of ROS (*7*). In addition, melatonin also offers an endogenous protective defense against the deleterious actions of the reactive hydroxyl radicals, which cannot be detoxified enzymatically (*24*). Nevertheless, the ability of these antioxidants to combat oxidative stress in brain tissue is limited (*6*). Probably in support of the above, extraneous administration of GSH was reported to protect against KA-induced neuropathological changes in rat brain (*5*), suggesting the value of a pharmacological strategy directed toward the regulation of endogenous GSH levels. Therefore, maintenance of the GSH level appears to be important for the effective prevention of KA-induced oxidative stress. For this purpose, the use of natural antioxidants, membrane-permeable, would be a potential approach, because they have been employed to potentiate intracellular antioxidant capacity.

In our study, administration of the rough aster butanol fraction to mice treated with KA exhibited a GSH-sparing effect as well as a preventive action against lipid peroxidation in mouse brain. Therefore, it is supposed that the rough aster butanol fraction may contain antioxidants expressing a neuroprotective activity against the oxidative stress induced by kainic acid. This might

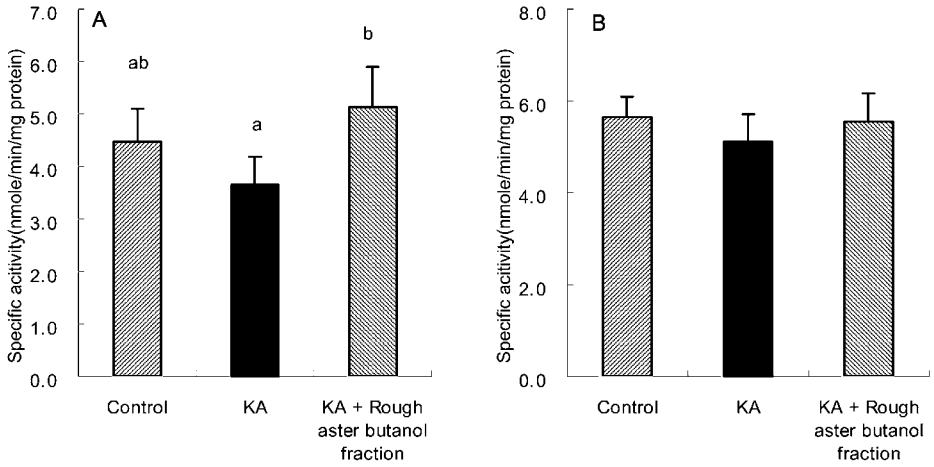


**Figure 3.** (A) Glutathione peroxidase activity and (B) glutathione reductase activity in the brain tissue of mice administered kainic acid (KA) and rough aster butanol fraction. Values are mean ± SE of three determinations from four to eight mice. Values with the same letter are not significantly different ($p < 0.05$).

be supported by the present result that the rough aster butanol fraction effectively prevented the lipid peroxidation of brain membrane in in vitro screening. The capability of the rough aster extract to ameliorate behavioral signs of KA neurotoxicity and to reduce mortality might be related to its prevention of oxidative stress. From these, it is assumed that the primary neuroprotective effect of the rough aster butanol fraction in vitro may be at least partly due to its antioxidant activity. Although the compounds responsible for the neuroprotective activity in the rough aster butanol fraction were not identified, it is supposed that the neuroprotective components would be polyphenol antioxidants as suggested from the previous report that the methanol extract of rough aster contains antioxidants such as polyphenols (*25*) or glycosides (*26*). Recent data (*27*) indicate that some flavonoids such as patuletin, isolated from *Inula britannica*, prevented a decrease in the activities of antioxidant enzymes in catalase, GSH peroxidase, and GSH reductase in glutamate-injured cortical cell cultures, and the loss of GSH induced by glutamate excitotoxicity was also counteracted by those flavonoids. Very recently (*14*), it was reported that caffeinylquinic acids, antioxidants, present in the ethyl acetate fraction of the rough aster extract exhibited a neuroprotective effect in the PC12 cells exposed to kainic acid. However, it is not known whether the same ethyl acetate fraction of the methanol extract from rough aster has a neuroprotective action in animal experiment. On the other hand, our data show that the neuroprotective effect is exhibited by the rough aster butanol fraction, suggesting that the neuroprotective action might be attributed to antioxidants other than caffeinylquinic acids. These results might suggest that antioxidants should pass through the blood−brain membrane to be effective neuroprotective agents (*28*). Alternatively, it is also possible to think that the neuroprotective action of the rough aster butanol fraction might be due to other activities such as the restoration of the imbalance of glutamate/GABA content or the inhibition of the voltage-gated $Ca^{2+}$ channel. In addition, it can antagonize the binding of KA to glutamate receptors, as proposed for the possible mechanism of the neuroprotection by ginsenosides (*29*).

In conclusion, the present data are the first to demonstrate the neuroprotective effect of the rough aster extract in an animal experiment. A key finding of the present study is that the rough aster butanol fraction, an edible vegetable, has a neuroprotective effect against kainic acid excitotoxicity through antioxidant action. Although further investigation is necessary to identify the effective neuroprotective compounds present in the butanol extract, our data suggest that the rough aster butanol fraction may contain central nervous system-selective antioxidants, blood−brain barrier-permeable. Natural products that attenuate the glutamate-induced neurotoxicity might offer a useful choice in the prevention of neurodegenerative disorders caused by oxidative stress.

## ABBREVIATIONS USED

Rough aster butanol fraction, butanol fraction of methanol extract from *Aster scaber* Thunb.; GSH, reduced glutathione; KA, kainic acid; TBARS, thiobarbituric acid-reactive substances; DTNB, 5,5′-dithiobisnitrobenzoate.

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