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Protective effect of *Undaria pinnatifida* sporophyll extract on iron induced cytotoxicity and oxidative stress in PC12 neuronal cells

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Abstract

The abnormal accumulation of iron in the brain has previously been proposed to be responsible for the neurodegenerative diseases through the oxidative damage to neuronal cells. Therefore, it seems practically meaningful to search for novel active substances protecting neurons against the iron-induced oxidative neurotoxicity *in vitro*. For this purpose, the aqueous extract was prepared from a sporophyll of brown seaweed *Undaria pinnatifida*, called "MEKABU" in Japanese, and the protective effect of MEKABU extract against the iron-induced damage to PC12 cells was examined. The extract prevented the iron-induced oxidative damage, but not the azide-induced hypoxic damage to the cells. Further studies indicated that MEKABU extract showed the radical scavenging and antioxidant activities, which might contribute to its protective effect against the iron-induced cytotoxicity. These observations suggest that MEKABU extract may contain novel substance(s) protecting neuronal cells against the iron-induced toxicity.

Keywords: Brown seaweed, Iron-induced cell death, PC12 cells, Antioxidant; *Undaria pinnatifida*

Introduction

The transition metals, such as iron, copper, and zinc, are generally known as an essential biometals required for maintaining the integrity of physiological functions, and the fundamental roles of these metals in various metabolic processes have been exhaustively studied. Particularly, iron is one of the most principal biometals involved in many critical functions for life, and hence the biochemical iron homeostasis is strictly regulated through

highly sophisticated mechanisms. As the characterization of iron homeostasis has advanced, the dysregulation of iron metabolism is being watched with interest, and the recent studies on the pathogenesis of neurodegenerative diseases have proposed the possibility that the progressive accumulation of iron in the brain may be responsible for neurodegenerative alterations associated with Alzheimer's (AD), Parkinson's (PD) and Huntington's (HD) diseases (Bonda, et al., 2011; Salvador, 2010; Salvador, et al., 2010). Based on these previous findings, the iron-chelating agents have recently been introduced into the clinical treatment of neurodegenerative disorders (Arduino, et al., 2008; Britton, et al., 2002; Weinreb, et al., 2009; Youdim, et al., 2000), and the iron-induced neurotoxicity has also been energetically studied *in vivo* and *in vitro* to find a clue to new drugs as well as novel prophylaxes and treatments for the neurodegenerative disorders.

Recently, the cytotoxic and neurotoxic effects of iron have been investigated, and found that the iron-induced toxicity may be due to the oxidative damage to the cells and tissues (Bostanci, et al., 2010; He, et al., 2007; Liu, et al., 2003; Salazar, et al., 2006; Sohn and Yoon, 1998), and these previous findings have stimulated the ceaseless effort to investigate the protective effect against the iron-induced oxidative damage to different-types of the cells and tissues (Bermejo-Bescos, et al., 2008; Jagetia and Reddy, 2011; Nahdi, et al., 2010; Nunez Figueredo, et al., 2011; Oktar, et al., 2009; Xu, et al., 2010). In particular, it seems scientifically interesting and practically important to carry out the screening of novel substance(s) effective in the protection of the cells and tissues against the iron-induced oxidative stress *in vitro*. Based on this fundamental idea, the aqueous and alcoholic extracts of various agricultural and fishery wastes have been prepared to test their protective effects against the oxidative cell damage as well as their toxic effects on the different-types of the cells in culture.

Brown seaweed *Undaria pinnatifida* is popular as a food stuff, and used for medical care in East Asian countries. However, the sporophyll, called "MEKABU" in Japanese, is considered to have lower utility value as compared with other parts, and usually damped as a fishery waste. Since brown seaweeds have been reported to show anti-cancer and anti-proliferative activities (Furusawa and Furusawa, 1989; Lins, et al., 2009; Maruyama, et al., 2003; Riou, et al., 1996; Yuan and Walsh, 2006), and the effective utilization of MEKABU is considered to be important and necessary for developing new medicinal resources. In our preliminary study, both aqueous and alcoholic extracts were prepared from MEKABU, and their cytotoxic effects were examined using human and rat cancer cells in culture. Consequently, the alcoholic extract, but not the aqueous extract, was shown to be toxic to these cancer cells, thus suggesting that the aqueous extract might be suitable to investigate its protective effect against the oxidative cell damage. In the present study, to assess a possible effectiveness of MEKABU extract in the prevention of neurodegenerative disorders, we therefore examined the protective effect of the aqueous extract against the iron-induced cytotoxicity using PC12 cells as a model of neuronal cells.

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Materials and methods

Chemicals

Neutral red solution, Folin-Ciocalteu's phenol reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Potassium permanganate ($KMnO_4$), ferrous sulfate ($FeSO_4$), sod-

ium azide (NaN_3) and indigo carmine were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other chemicals were commercially available reagent grade.

Preparation of MEKABU extract

A sporophyll of *Undaria pinnatifida* (Harvey) Suringar, called MEKABU in Japanese, was kindly gifted from Choi Co. Ltd. (Naruto, Japan). Frozen MEKABU was chopped with a food processor, and the chopped MEKABU (about 40 g wet weight) was suspended in 100 ml of distilled water. The suspension was autoclaved at 121^{o}C for 30 min, and then kept in an autoclave chamber for overnight to allow it cool down. The autoclaved suspension was filtered through a Whatman No. 1 filter paper, and the obtained filtrate was then centrifuged at 5,000 x g for 20 min to precipitate the dregs. The supernatant fraction was moreover centrifuged at 12,000 x g for 20 min to clarify the solution, and the extract was sterilized by filtering through a 0.2 μm syringe-top disk filter. The obtained extract was aliquoted, and stored at -21^{o}C until use.

Cell culture

Rat pheochromocytoma PC12 cells were purchased from The American Type Culture Collection (Rockville, MD, USA), and then maintained on a 100-mm culture plate in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% bovine serum, 5% horse serum, 50 units/ml of penicillin, 50 μg/ml of streptomycin and 50 μg/ml of gentamicin sulfate at 37^{o}C in a humidified incubator containing 95% air-5% CO_2 atmosphere. The cells were collected from a subconfluent culture with trypsinization, and seeded onto a 24-well cluster plate or a 60-mm culture dish at an appropriate density for each experiment. The plated cells were cultured for 72 h in the standard growth medium to allow them to restore the surface of their plasma membrane, and maintained for additional 24 h in the serum-free medium containing insulin (5 μg/ml), transferrin (5 μg/ml) and sodium selenite (5 ng/ml) before exposing to test materials.

Determination of cell viability

Cells were plated on a 24-well plastic cluster plate at a density of $5x10^4$ cells/ml/well, and then exposed to MEKABU extract for different time periods. The cell viability was assessed by measuring the amount of neutral red taken up into the cells as described previously (Fautz, et al., 1991; Morita, et al., 1999; Morita and Wong, 2000). Briefly, the cells were washed with saline solution, and incubated in 0.5 ml of DMEM containing neutral red (50 μg/ml) for 2 h at 37^{o}C in a humidified incubator. The cells were rinsed with saline solution, and then extracted with 400 μl of acidified ethanol solution (50% ethanol - 1% acetic acid) for 20 min at room temperature with constant gentle shaking. The amount of neutral red taken up into the cells was determined by measuring the absorbance at 540 nm

Determination of radical scavenging activity

The radical scavenging activity of MEKABU extract was estimated by determining the reduction of DPPH radicals using a HPLC analysis as reported previously (Yamaguch, et

al., 1998). Briefly, the sample solution (100 μl) containing different amounts of the extract was added to 400 μl of 0.1 mM Tris-HCl (pH 7.4), and then mixed with 500 μl of 0.5 mM DPPH ethanol solution. The mixture was shaken vigorously, and then left for 20 min in the dark at room temperature. Then, aliquots of the mixture were analyzed by a reverse-phase HPLC system with a UV-VIS detector. Unchanged DPPH remained in the mixture was separated on a TSK gel Octyl-80Ts column (4.6 x 150 mm) with a mobile phase consisting of methanol : water (70 : 30, v/v) at a flow rate of 1 ml/min, and detected by measuring the absorbance at 517 nm.

Determination of ferric reducing antioxidant potency

The ferric reducing antioxidant potency of the extract was determined as reported previously (Benzie and Strain, 1996; Benzie and Szeto, 1999). Briefly, the mixture was made up by mixing 500 μl of 0.3 M sodium acetate (pH 3.6), 50 μl of 10 mM TPTZ and 50 μl of 20 mM ferric chloride on ice, and the reaction was carried out at 37^{o}C for 30 min after adding the sample solution (50 μl) containing different amounts of the extract to the mixture. The optical density of the reaction mixture was measured at 593 nm.

Determination of phenolic compounds

Total phenolic compounds in the extract were estimated using Folin-Ciocalteu's phenol reagent according to the method described previously (Chen, et al., 2007). The sample solution (100 μl) containing different amounts of the extract was mixed with 400 μl of Folin-Ciocalteu's reagent (diluted with water at 1 : 10), and then mixed with 500 μl of 5% (w/v) sodium carbonate solution. The assay mixture was kept for 20 min at room temperature, and the absorbance was measured at 765 nm. Gallic acid was used as an authentic sample to confirm the reliability of the method. As an alternative way to determine the contents of total polyphenolic compounds, the amounts of tannin contained in the aqueous extract was measured using a classic Lowenthal permanganate titration method (Lowenthal, 1877).

Statistical analysis

Results were presented as the mean ± SEM, and the statistical analyses were performed using a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. A *p*-value < 0.05 was regarded as indicating a statistically significant difference between two groups.

Results and Discussion

Brown seaweeds are known to have both pro-oxidant and antioxidant activities, and therefore expected to cause either the cytotoxic or the cytoprotective effect on the cells, depends on the experimental conditions. Then, the aqueous extract was prepared from MEKABU, a sporophyll of *Undaria pinnatifida,* and the protective effect of MEKABU extract against the iron-induced cytotoxicity was examined using PC12 cells. As shown in Figure 1, the exposure to $FeSO_4$ reduced the cell viability in a concentration-dependent man-

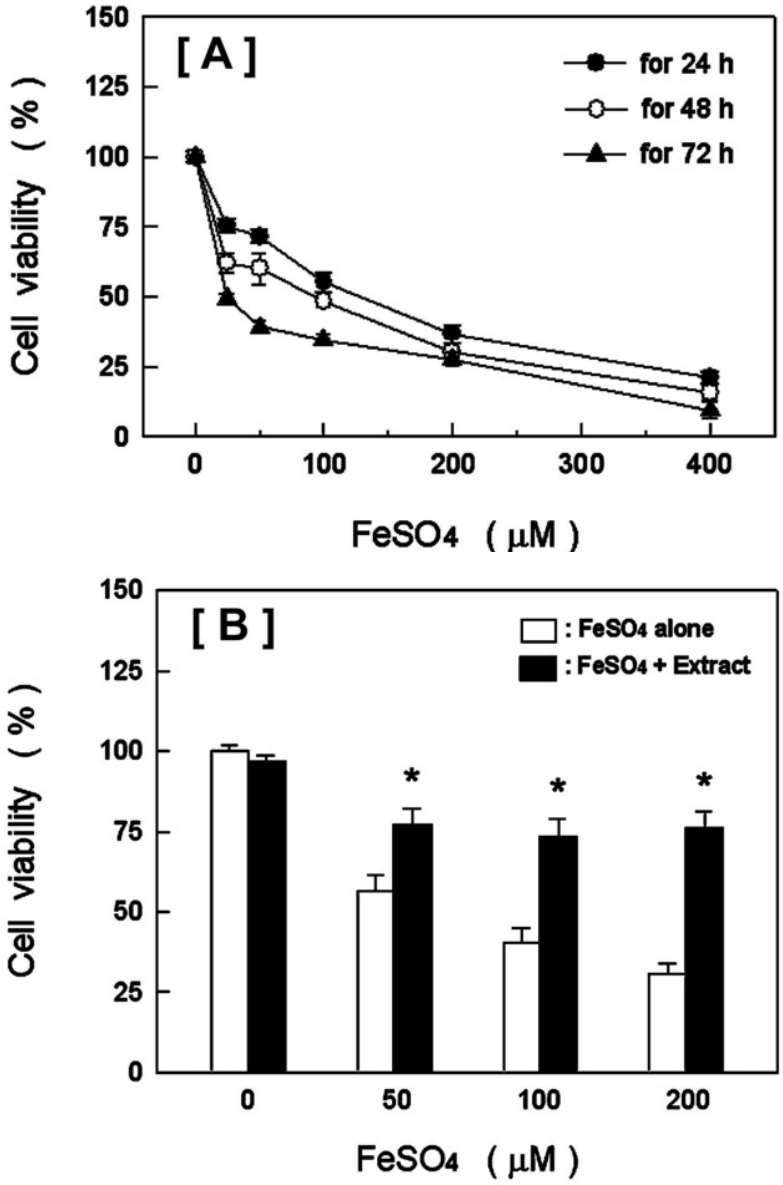


Figure 1. Effect of MEKABU extract on iron-induced reduction of cell viability. PC12 cells were exposed to various concentrations of FeSO4 for different period [A] or exposed to $FeSO_4$ for 24 h in the presence and absence of 100 μl/well of MEKABU extract [B]. The cell viability was determined as described in the text, and the results were expressed as the percent of non-treated cells. Values are the mean ± SEM (*$p < 0.05$ *vs.* $FeSO_4$ alone, n = 6).

ner (Figure 1A), and the iron-induced reduction of the cell viability was recovered by adding 200 μl of the extract prior to the exposure to $FeSO_4$ (Figure 1B). Furthermore, the iron-induced reduction of the cell viability was notably recovered by adding 50 μl of MEKABU extract, and the complete recovery was obtained by adding 100 μl of the extract (Figure 2). These results clearly indicated that the aqueous extract of MEKABU caused the recovery of the iron induced reduction of the cell viability under the experimental conditions used here, thus suggesting that MEKABU extract might be protective against the iron-induced toxic damage to PC12 cells in culture. The preliminary studies have shown that the alcoholic extra-

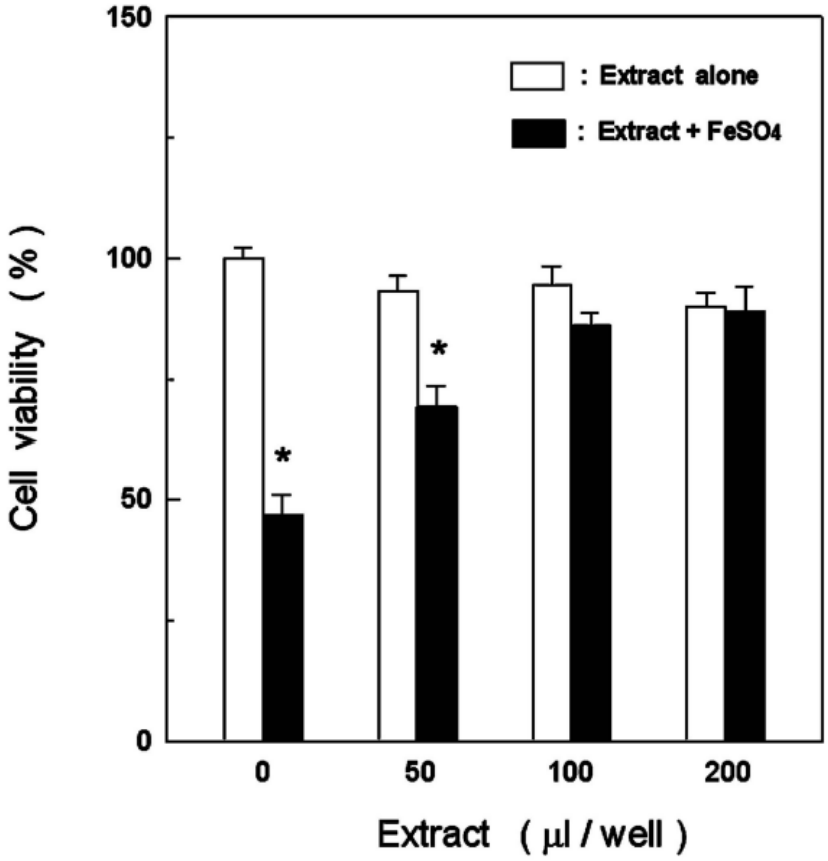
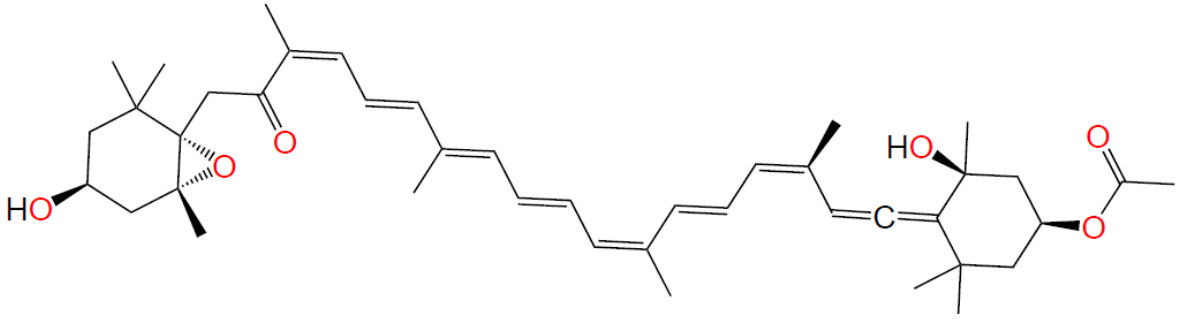


Figure. 2. Inhibitory effect of MEKABU extract on iron-induced reduction of cell viability. PC12 cells were exposed to 200 µM of $FeSO_4$ for 24 h in the presence of different concentrations of MEKABU extract, and the cell viability was determined as described in the text. Results were expressed as the percent of non-treated cells. Values are the mean ± SEM (*$p < 0.05$ *vs.* Extract alone, n = 6).

Ct of MEKABU causes the toxic effect on PC12 cells, C6 glioma cells and human colorectal canc-er cells, thus suggesting that the cytotoxic effect of the alcoholic extract may be attributed to fucoxanthin, a powerful antioxidant contained in brown seaweeds and easily extracted with alcohol (Chemical structure was illustrated below). However, it seems unlikely that fucoxanthin may be responsible for the cytotoxic effect of the alcoholic extract observed in these studies, because the concentration of fucoxanthin estimated within the culture is not high enough to cause the cell death. On the other hand, MEKABU pigments including fucox-



Chemical structure of fucoxanthin.

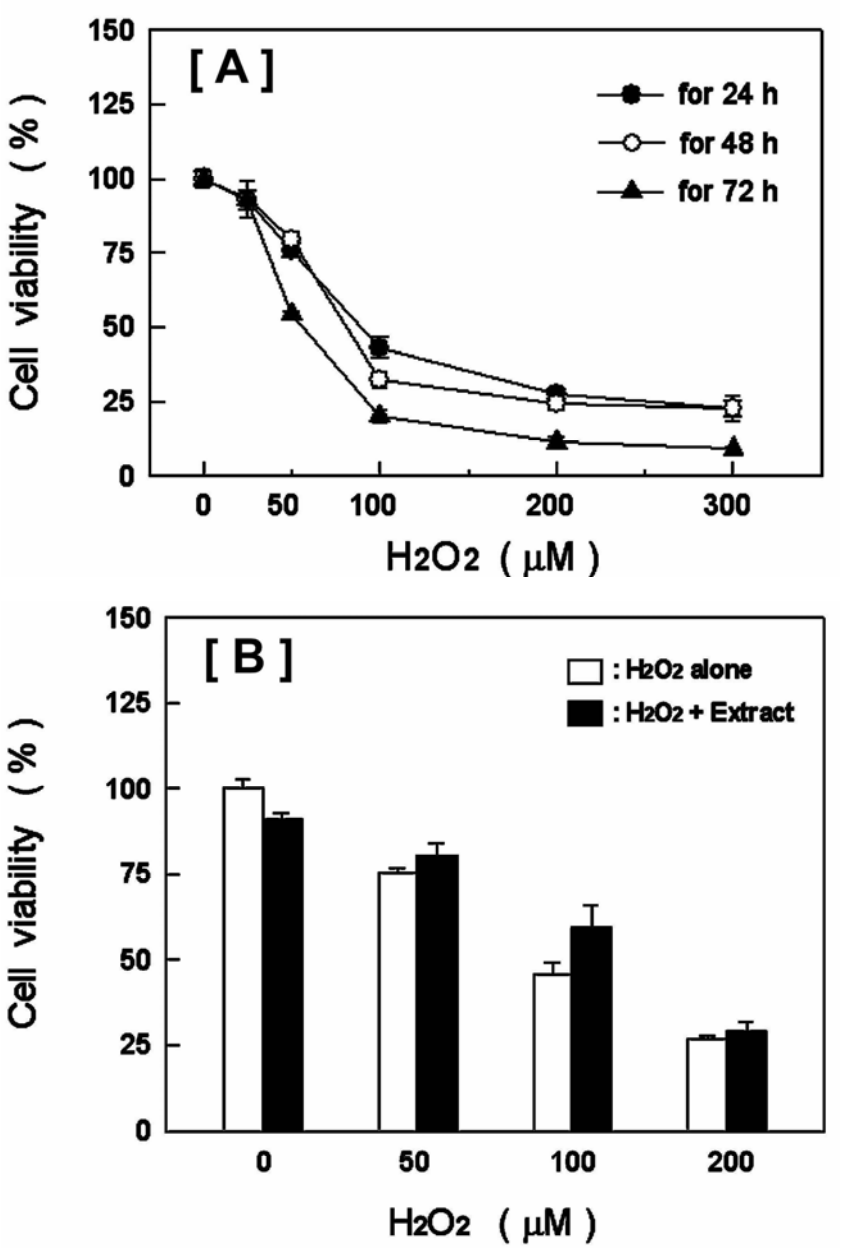


Figure 3. Effect of MEKABU extract on peroxide-induced reduction of cell viability. PC12 cells were exposed to various concentrations of H_2O_2 for different period [A] or exposed to H_2O_2 for 24 h in the presence and absence of 100 μl/well of MEKABU extract [B]. The cell viability was determined as described in the text, and the results were expressed as the percent of non-treated cells. Values are the mean ± SEM (n = 6).

anthin have been shown to be hardly extracted by the method of the aqueous extraction used in the present study. Therefore, it seems reasonable to consider that MEKABU may contain novel active substances, which may be easily extractable with hot water and protective against the iron-induced damage to PC12 cells in culture. The iron-induced cytotoxicity is generally considered to be based on the oxidative damage to the cells. Then, the effect of MEKABU extract on the peroxide-induced cell dam-age was also examined to confirm the possibility that the protective effect of MEKABU extract against the iron-induced cytotoxic-city might be simply due attributed to its antioxidant activity. As shown in Figure 3, the exp-

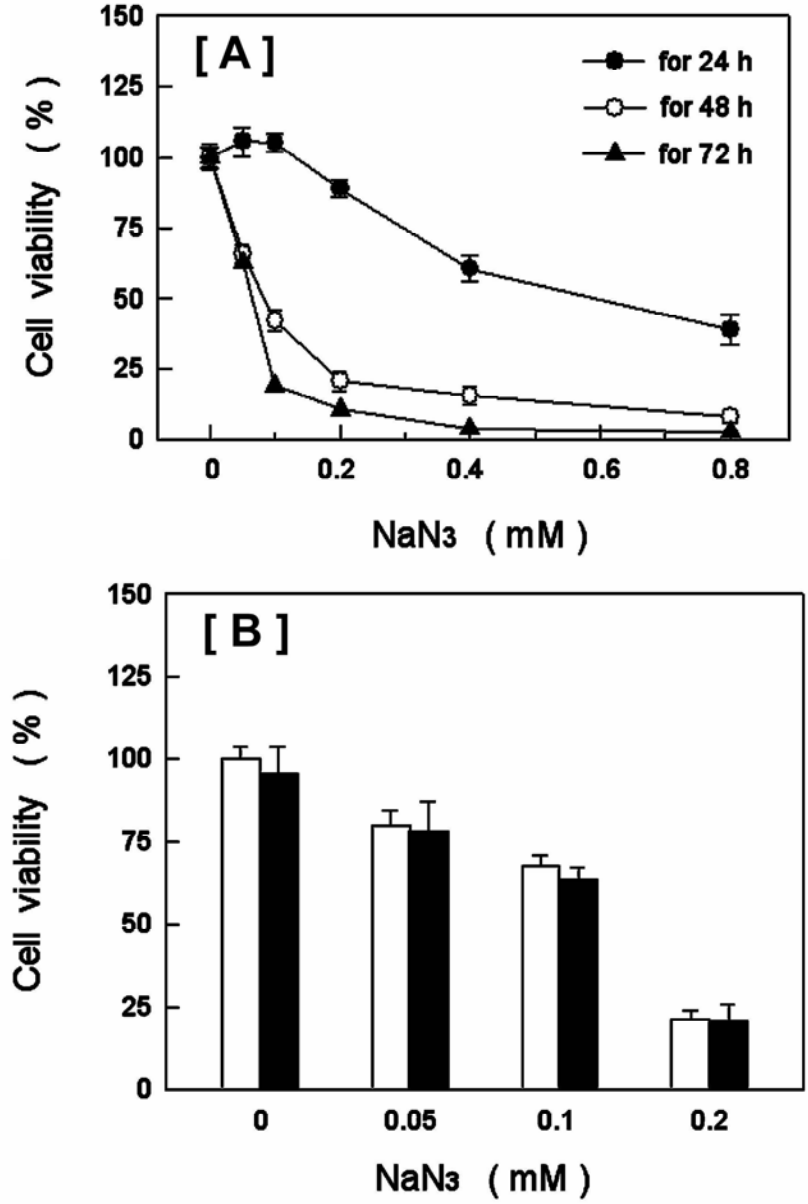


Figure 4. Effect of MEKABU extract on hypoxia-induced reduction of cell viability. PC12 cells were exposed to various concentrations of NaN_3 for different period [A] or exposed to NaN_3 for 24 h in the presence and absence of 100 µl/well of MEKABU extract [B]. The cell viability was determined as described in the text, and the results were expressed as the percent of non-treated cells. Values are the mean ± SEM (n = 6).

osure to H_2O_2 caused the concentration-dependent reduction of the cell viability, which was quite similar to the iron-induced reduction (Figure 3A). In contrast to the iron-induced toxicity, the peroxide-induced cytotoxicity was not prevented by adding 200 µl of MEKABU extract (Figure 3B). In addition, the effect of NaN_3 a chemical hypoxia-inducing agent, on the cell viability was examined, and the exposure to NaN_3, reduced the cell viability in a concentration- and time-dependent manner (Figure 4A). Similar to the peroxide-induced cell damage, MEKABU extract failed to prevent the chemical hypoxia-induced toxic damage to the cells under the same experimental conditions (Figure 4B).

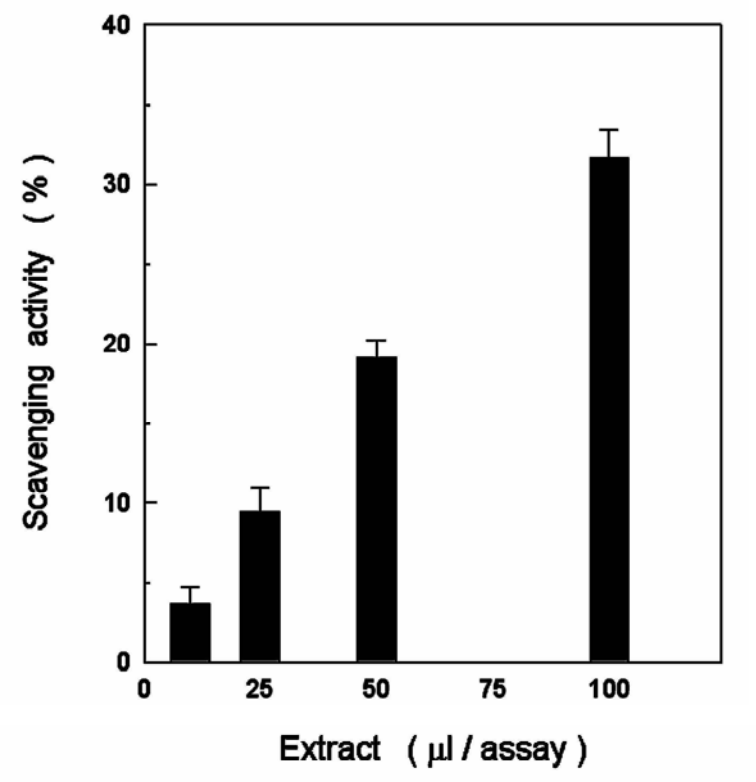


Figure 5. Free radical scavenging activity of MEKABU extract. The mixture containing DPPH and different amounts of MEKABU extract in Tris-HCl buffer (pH 7.4) was vigorously shaken, and left for 20 min at room temperature in the dark. DPPH in the mixture was separated, and then determined as described in the text. Results were expressed as the percent of control. Values are the mean ± SEM (n = 6).

Previously, both iron- and peroxide-induced cytotoxicities have been shown to be oxidative and dependent upon the generation of reactive oxygen species (ROS), but the hypoxia-induced cytotoxicity may be independent of the ROS generation within the cells (Chen, et al., 2006; Chen, et al., 2002; Ko, et al., 2004; Liu, et al., 2003). However, the results presented here suggested that MEKABU extract might cause the protective effect against the iron-induced ROS-dependent cell death, but failed to protect the cells against the hypoxia-induced ROS-independent cytotoxicity. Therefore, it seems possible to consider that MEKABU extract may cause the protective effect against the iron-induced cytotoxicity by scavenging ROS within the cells. However, the extract failed to protect the cells against the peroxide-induced damage, which was also oxidative and dependent on the ROS generation in a similar manner to the iron-induced cell damage. These results indicated that the effect of MEKABU extract on the peroxide-induced cytotoxicity was inconsistent with that on the iron-induced cytotoxicity. Therefore, it seems absolutely necessary to further investigate the critical question of whether MEKABU extract may be selective about its protective effect against the iron-induced damage to PC12 cells.

Based on the results described above, it seemed possible to assume that the protective effect of MEKABU extract against the iron-induced cell damage might be attributed, even at

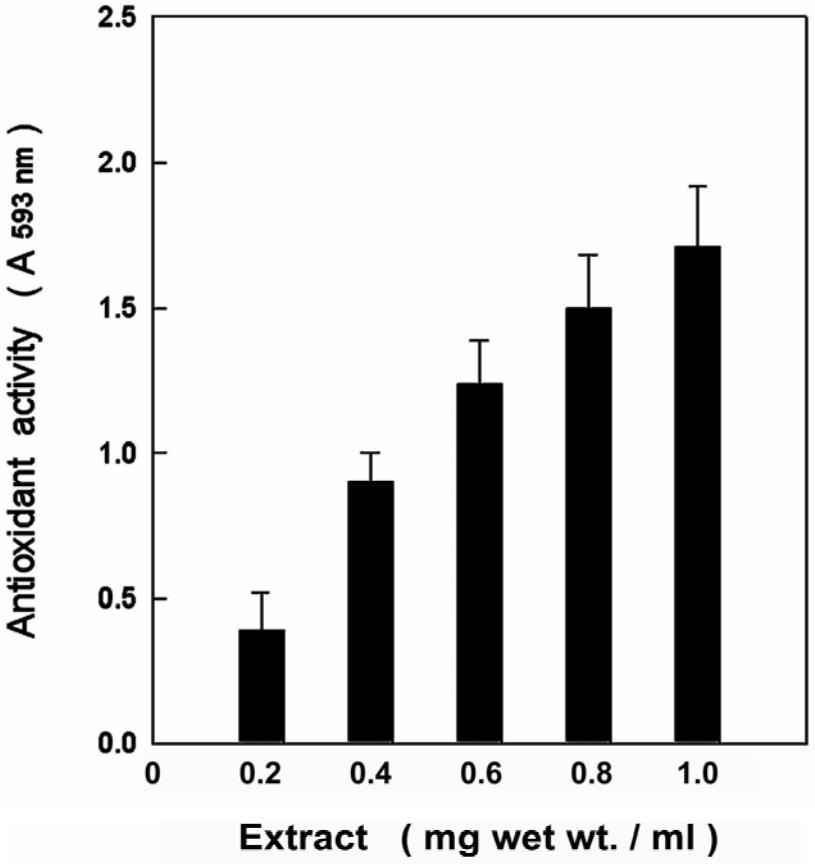


Figure 6. Antioxidant activity of MEKABU extract. The mixture containing sodium acetate buffer (pH 3.6), TPTZ, ferric chloride and various amounts of MEKABU extract was incubated at 37°C for 30 min, and the absorbance at 593 nm was then determined as describe in the text. Values are the mean ± SEM (n = 6).

least partly, to its antioxidant activity, and hence the bioactivity of MEKABU extract was then characterized. Firstly, the free radical scavenging activity of MEKABU extract was examined by measuring the scavenging of DPPH radicals, and the extract was shown to have a powerful scavenging activity (Figure 5). Secondarily, the antioxidant activity of MEKABU extract was assessed by measuring the ferric reducing potency, and the extract was shown to have a considerably strong antioxidant activity (Figure 6). Lastly, the contents of total polyphenolic compounds in MEKABU extract was measured by a Folin-Ciocalteu's reagent assay, and the extract was shown to contain considerably low concentrations of polyphenolic compounds, which was calculated approximately 0.41 - 0.48 mM gallic acid equivalent (Figure 7). According to the results presented in Figure 2, the iron-induced reduction of the cell viability is completely restored by adding 50 μl of the extract to the culture, in which the final concentration of polyphenols is estimated at approximately 22.3 μM gallic acid equivalent. Moreover, the concentration of polyphenols in MEKABU extract was measured by a Lowenthal permanganate titration method, which was considered to directly reflect the reducing activity of the extract, and the polyphenol concentration in the extract was hardly detectable by this classical assay method (data not shown). These results suggest that the antioxidant activity of MEKABU extract may be responsible for its protective effect against the iron-induced damage to the cells. However, the estimation of polyphenol concentration in

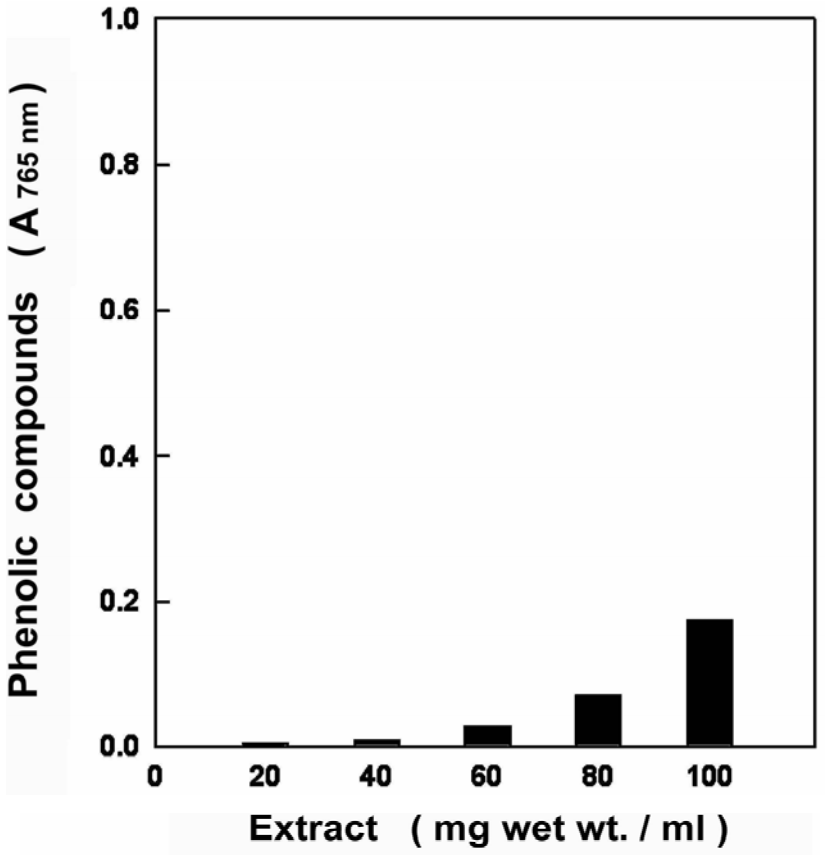


Figure 7. Phenolic compound concentration in MEKABU extract. The mixture of Folin-Ciocalteu's phenol reagent and different amounts of MEKABU extract were mixed with sodium carbonate solution, kept for 20 min at room temperature and the absorbance at 765 nm was then measured as described in the text. Values are the mean ± SEM (n = 6).

the culture medium provides evidence for suggesting that polyphenolic compounds contained in MEKABU extract seem unlikely to contribute as a principal component to its antioxidant activity observed here. Therefore, it is still necessary to determine potential active substance-e(s) responsible for the protective effect of MEKABU extract against oxidative cell damage, and the aqueous extract of MEKABU is now subjected to a HPLC analysis to identify water-extractable active component(s).

In conclusion, MEKABU extract is clearly shown to prevent the iron-induced oxidative damage to PC12 cells, thus suggesting that MEKABU extract may be able to protect neuronal cells against the iron-induced cytotoxicity. Thus, the aqueous extract of MEKABU is suggested to contain potential neuroprotective substance(s) which can effectively protect neurons against the oxidative damage brought by the abnormal accumulation of iron in the brain, thereby being possibly beneficial for preventing neurodegenerative diseases or at least reducing the risks of these disorders.

Acknowledgments

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Conflict of interest

There is no conflict of interest associated with the authors of this paper, and the sponsors supporting the research did not cause any inappropriate influence on this work.

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