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Lotus root (*Nelumbo nucifera* rhizome) extract causes protective effect against iron-induced toxic damage to C6 glioma cells

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Abstract

Glial cells are suggested to contribute to retaining the integrity of brain function through the protection of neuronal cells against toxic insults, and the protection of glial cells is proposed to be beneficial for the prevention of neurodegenerative diseases. Then, to search for substance(s) protecting glial cells against the cytotoxic insults, the aqueous extract was prepared from a lotus root (a rhizome of *Nelumbo nucifera*), and the cytoprotective effect of the extract was examined. The chemical analyses showed that the extract contained polyphenolic compounds, which might confer the radical scavenging and antioxidant activities on the extract, thereby being expected to protect the cells. Actually, the extract prevented the iron-induced oxidative damage, but not the azide-induced hypoxic damage to the cells. Therefore, lotus root extract is considered to contain novel substance(s) protecting glial cells against the iron-induced oxidative insults.

Keywords: Lotus root extract, Iron-induced cytotoxicity, C6 glioma cells, Polyphenols, Radical scavenging activity, Antioxidant potency

Introduction

As a potential cause of the neurodegenerative diseases, the abnormal accumulation of iron in the brain tissue, thereby resulting in the oxidative damage to neuronal cells, has been proposed, and the highly sophisticated mechanism regulating the biochemical iron homeostasis in the brain has been extensively investigated. Therefore, the dysregulation of iron metabolism is being watched with keen interest, and the recent studies on the pathogenesis of

neurodegenerative diseases have also suggested that the progressive accumulation of iron within the brain may be responsible for neurodegenerative alterations associated with Alzheimer's, Parkinson's and Huntington's diseases (Bonda, et al., 2011; Salvador, 2010; Salvador, et al., 2010). Based on these previous findings, the iron-chelating agents have 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 facilitate the development of new drugs as well as novel prophylaxes and treatments for the neurodegenerative diseases.

Previous studies have shown that the iron-induced cytotoxicity 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 observations 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). Particularly, it seems scientifically interesting and practically important to prove into novel substance(s) effective in the protection of the cells and tissues against the iron-induced oxidative stress *in vitro*. Then, we have prepared the extracts from various agricultural and fishery products, and investigated their protective effects against the toxic damage to different-types of the cells in culture. Practically, the alcoholic extract prepared from a sporophyll of brown seaweed *Undaria pinnatifida* has been shown to cause the cytotoxic effect on human colorectal cancer cells *in vitro* (Nishibori, et al., 2012). In contrast, the aqueous extract of brown seaweed sporophyll has been reported to prevent the oxidative damage to PC12 cells (Nishibori, et al., 2012). On the other hand, the aqueous extract of lotus root has been shown to cause the oxidative damage to human colorectal cancer cells probably through its pro-oxidant activity, which may be due to polyphenolic compounds in the extract (Arimochi, et al., 2011). However, because of containing polyphenolic compounds, it still remains to be investigated whether the extract may be able to show the cytoprotective activity probably through its antioxidant potency, depending upon the cells and other conditions used for the experiments. In the present study, to assess a cytoprotective effect of lotus root extract against neurodegenerative insults in the brain, the direct effect of the aqueous extract prepared from lotus root on the iron-induced cytotoxicity was examined using C6 glioma as a model system.

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). Ferrous sulfate ($FeSO_4$), sodium azide (NaN_3) and calf thymus DNA were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other chemicals were commercially available reagent grade.

Preparation of lotus root extracts

Lotus root (a rhizome of *Nelumbo nucifera*) was kindly donated by the Naruto Agricultural Cooperative in Tokushima (Naruto, Japan). Lotus root was cleaned by washing

with water, and sectioned into two different parts, the edible and joint parts. Then, each of them was sliced in approximately 1 cm thickness, and the slices were dehydrated by exposing them to wind stream for several weeks before grinding them into coarse powder. To prepare the aqueous extract from either the edible or joint parts, the powder was soaked in distilled water (4 g /100 ml), and kept it at 4^{o}C for 1 h. The suspension was filtered through a Whatman No. 1 filter paper, and the filtrate was centrifuged at 6,000 x g for 20 min to remove the insoluble materials. Then, the supernatant fraction was further centrifuged at 12,000 x g for 20 min to clarify the solution, and the obtained extract was aliquoted and stored at -20^{o}C until use. For the cell culture experiments, the aqueous extracts were sterilized by filtering through a 0.2 μm syringe-top filter, and the obtained filtrates were aliquoted and stored at -20^{o}C until use. The amounts of lotus root extracts were expressed as the weight of lotus root in the chemical reaction (mg/assay) and in the cell culture (mg/well).

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 furthermore 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.

Determination of radical scavenging activity

The radical scavenging activity of the 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 ml 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.

Cell culture

C6 glioma 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 mg/ml of streptomycin and 50 mg/ml of gentamicin sulfate at 37°C in a humidified incubator containing 95% air-5% CO_2 atmosphere. The cells were collected from a subconfluent culture with trypsinization, and then 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 5 x 10^4 cells/ml/well, and then exposed to the aqueous 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°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 then determined by measuring the absorbance at 540 nm.

Determination of DNA protecting activity

The DNA protecting activities of the extracts were determined as reported previously (Lee, et al., 2002). Briefly, the calf thymus DNA was incubated with or without the lotus root extracts in a Fenton's reaction mixture at 37°C for 30 min. and the hydroxyl radical-induced degradation of the DNA was analyzed by subjecting the mixture to a 1% agarose-gel electrophoresis, which was running at 50 volts for approximately 2 h. Then, the image of the agarose-gel was captured under UV-light exposure.

Statistical analysis

Results were presented as the mean $\pm$ SEM, and the statistical analyses of the differences 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

The aqueous extract prepared from the joint part of lotus root has previously been suggested to show the pro-oxidant and the antioxidant activities, both of which may be due to polyphenolic compounds contained in the extract (Arimochi, et al., 2011), and therefore expected to cause either the protective or the toxic effect on the cells, depending upon the experimental conditions. Then, the properties of the aqueous extracts were first analyzed more precisely before applying them to the cell culture system. As shown in Fig. 1, the edible

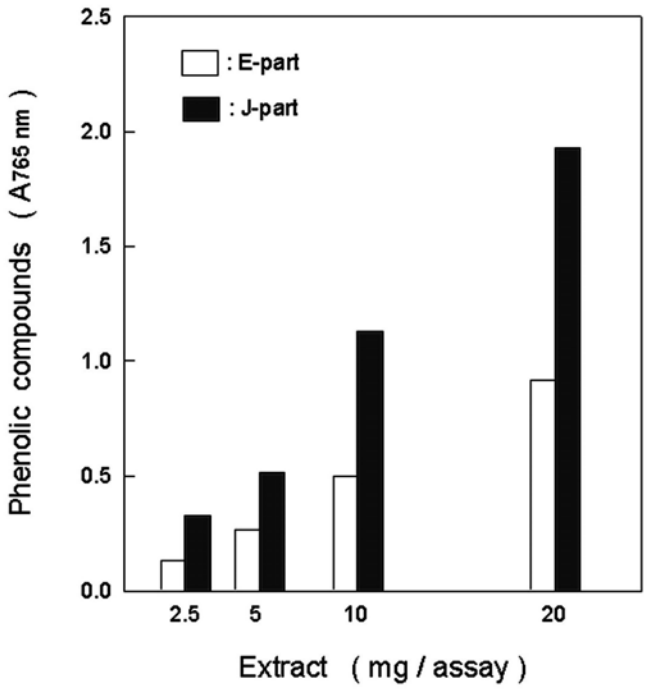

Figure 1. Phenolic compound contents in extracts prepared from edible and joint parts of lotus root. The mixture containing Folin-Ciocalteu's phenol reagent and different amounts of either edible part (E-part) or joint part (J-part) extract was furthermore mixed with sodium carbonate solution, kept for 20 min at room temperature, and the absorbance at 765 nm was measured as described in the text. Values are the mean ± SEM (n = 6).

and joint part extracts contained polyphenolic compounds, and the contents of polyphenolic compounds in the joint part extract was markedly higher than those in the edible part extract, consistent with the previous findings (Arimochi, et al., 2011). Therefore, it seemed highly probable that the aqueous extracts of lotus root might be able to show the radical scavenging and antioxidant activities *in vitro.* Actually, the extracts showed the radical scavenging activities, and the activity in the joint part extract was significantly higher as compared to that of the edible part extract (Fig. 2). In contrast, both the edible and joint part extracts had the antioxidant potencies, but there was no significant difference in the antioxidant potency observed between these two extracts (Fig. 3). These findings are considered to suggest that polyphenolic compounds contained in the extracts may contribute primarily to the radical scavenging activities, but not to the antioxidant potencies observed in the extracts under these experimental conditions. However, the antioxidant potency is uncertain and relatively determined according to the redox potentials of the oxidative materials in the assay system, and therefore it seems impossible to completely exclude the contribution of polyphenolic compounds in these extracts to their antioxidant potencies obtained in the present study.

The iron-induced cytotoxicity is generally known to be caused by the oxidative damage to the cells, and the aqueous extracts of lotus root are therefore considered to cause the protective effect against the iron-induced damage to the cells through the radical scavenging and/or the antioxidant activities of polyphenolic compounds contained in the extracts. Then, to assess their cytoprotective effects, the effects of lotus root extracts on the

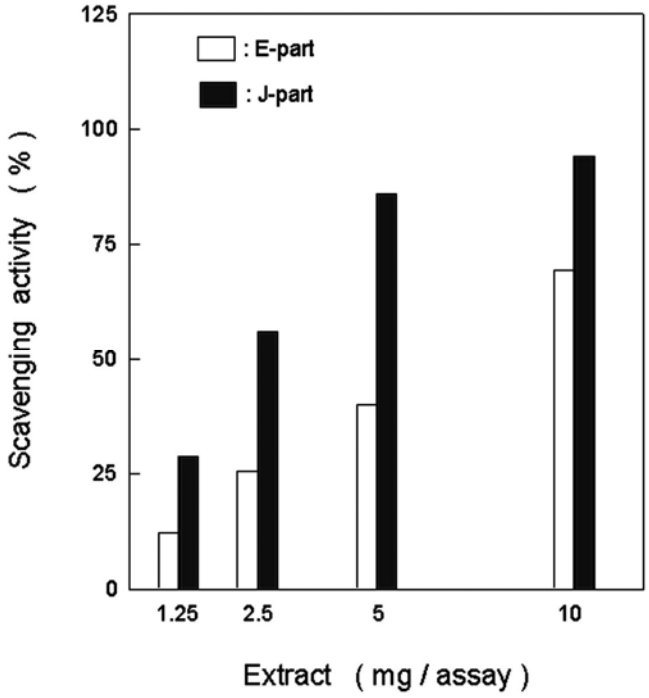

Figure 2. Radical scavenging activities of extracts prepared from edible and joint parts of lotus root. The mixture containing DPPH and different amounts of either edible part (E-part) or joint part (J-part) extract in Tris-HCl buffer (pH 7.4) was vigorously shaken, and left for 20 min at room temperature in the dark. DPPH in the mixture was separated, and determined as described in the text. Results were expressed as the percent of control. Values are the mean ± SEM (n = 6).

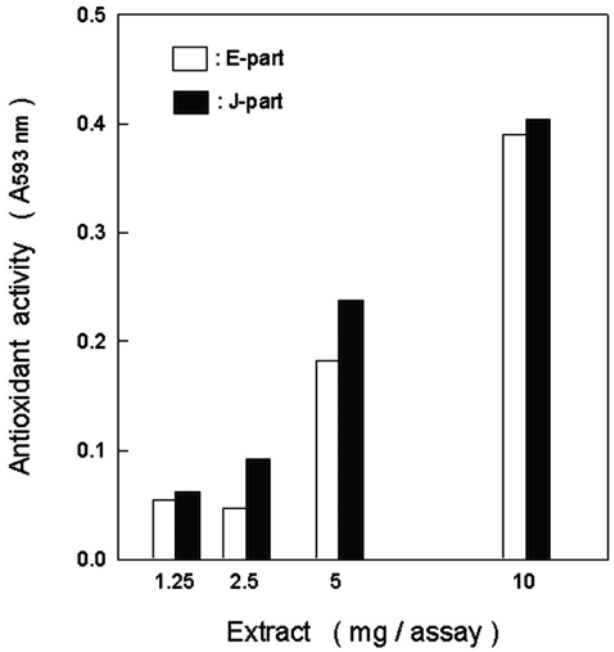

Figure 3. Antioxidant potencies of extracts prepared from edible and joint parts of lotus root. The mixture containing sodium acetate buffer (pH 3.6), TPTZ, ferric chloride and different amounts of either edible part (E-part) or joint part (J-part) extract was incubated at 37°C for 30 min, and the absorbance at 593 nm was determined as describe in the text. Values are the mean ± SEM (n = 6).

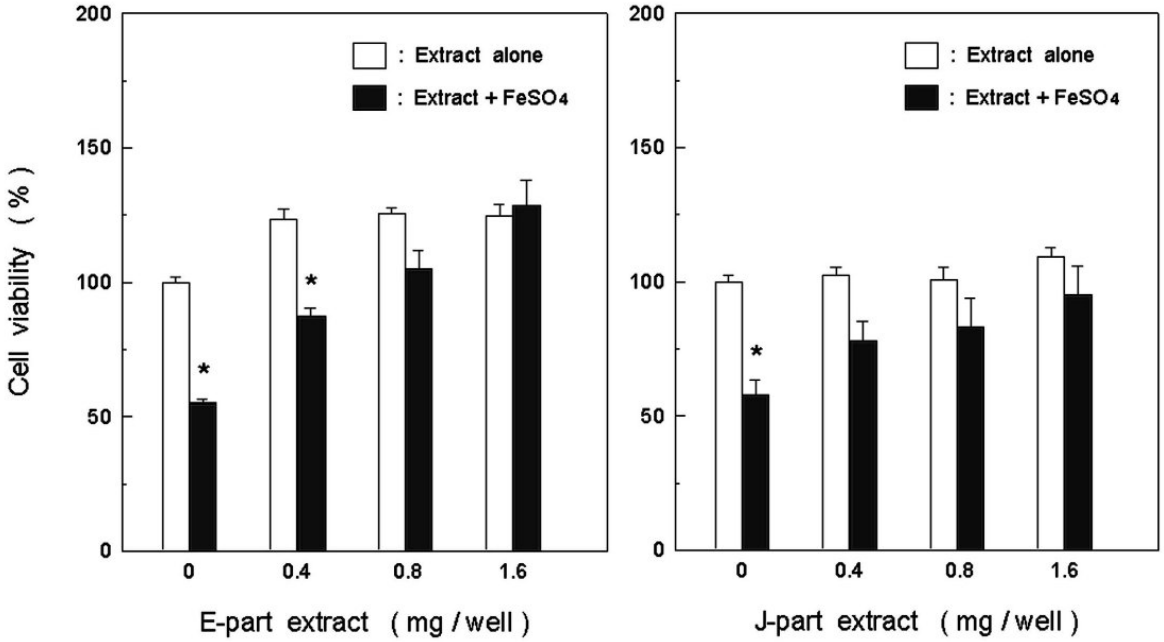

Figure 4. Effects of extracts prepared from edible and joint parts of lotus root on iron-induced cell death. The glioma cells were cultured with or without 200 μM $FeSO_4$ for 24 h in the presence of different amounts of either edible part (E-part) or joint part (J-part) extract. The cell viability was determined as described in the text, and the results were expressed as percent of the non-treated culture. Values are the mean ± SEM (*$p < 0.05$ *vs.* without $FeSO_4$, n = 6).

iron-induced cell damage were examined using C6 glioma cells. As shown in Fig 4, the exposure to $FeSO_4$ (200 mM) caused approximately 40 - 50% reduction of the cell viability, and the iron-induced reduction was completely abolished by adding both the edible and joint part extracts. On the other hand, the exposure to NaN_3 (400 mM) caused the reduction of the cell viability in a similar extent, but both the edible and the joint part extracts failed to cause any significant recovery of the cell viability under the experimental conditions used here (Fig. 5). Thus, the aqueous extracts prepared from the edible and the joint parts of lotus root were clearly shown to protect the glioma cells against the iron-induced, but not the azide-induced cytotoxicity.

In previous studies, both iron-induced and peroxide-induced cytotoxic-ities have been shown to be oxidative and dependent on the generation of reactive oxygen species (ROS), but the hypoxia-induced cytotoxicity has been shown to 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). Based on these findings, the results presented here suggested that the lotus root extracts 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 speculate that the extracts may protect the cells against the iron-induced cytotoxicity by scavenging ROS within the cells. However, the DNA protection study clearly indicated that these extracts failed to prevent the radical-induced DNA degradation (Fig. 6), thus suggesting that the protective effects of these two extracts might be connected with the prevention of ROS generation rather than the scavenging of radicals within the cells.

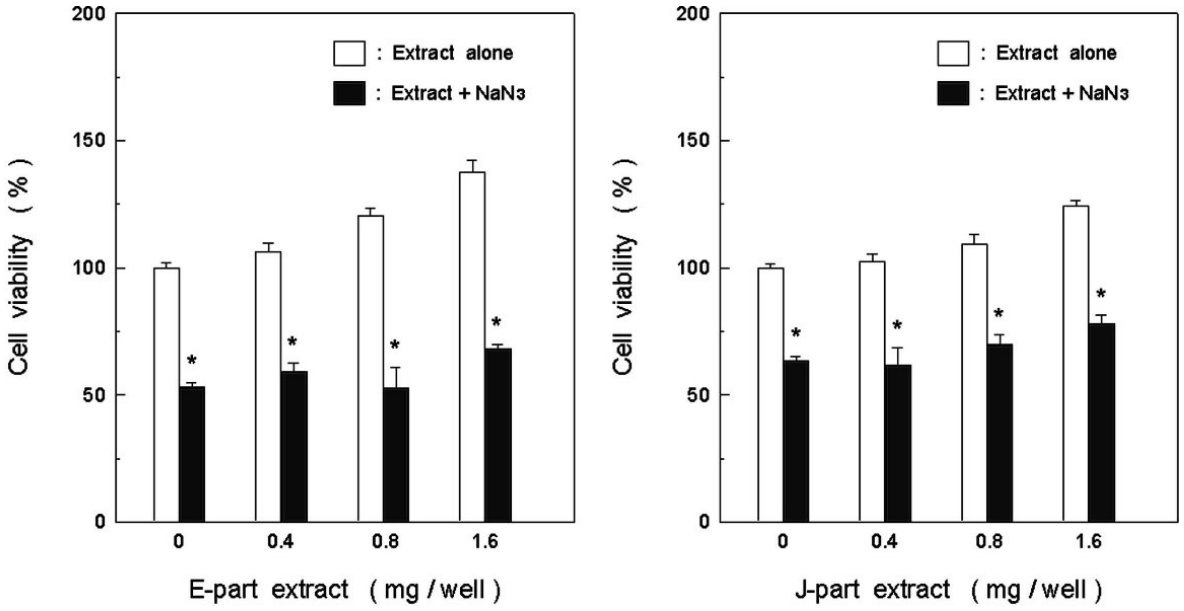

Figure 5. Effects of extracts prepared from edible and joint parts of lotus root on azide-induced cell death. The glioma cells were cultured with or without 400 mM NaN_3 for 24 h in the presence of different amounts of either edible part (E-part) or joint part (J-part) extract. The cell viability was determined as described in the text, and the results were expressed as percent of the non-treated culture. Values are the mean ± SEM (*$p < 0.05$ *vs.* without NaN_3, n = 6).

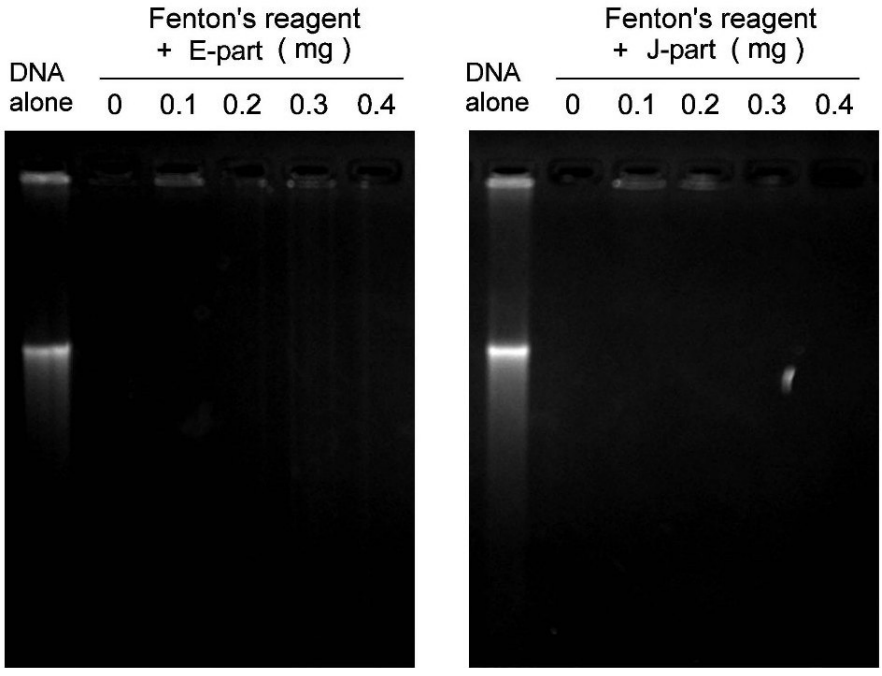

Figure.6. Effects of extracts prepared from edible and joint parts of lotus root on radical-induced DNA degradation. Calf thymus DNA was incubated with different amounts of either edible part (E-part) or joint part (J-part) extract in a Fenton's reaction mixture at 37°C for 30 min. The DNA degradation was analyzed using an agarose-gel electrophoresis as described in the text.

Since the radical scavenging activity of the joint part extract was shown to be markedly higher as compared to that of the edible part extract (Fig. 2), these extracts were expected to show the different potencies of their protective effect against the iron-induced ROS-dependent cytotoxicity, but on the contrary the protective effects of these extracts were shown to have the same potencies (Fig. 4). These findings seem to provide further evidence for suggesting that the radical scavenging activity may not be directly related to the protective effects of the extracts against the iron-induced cytotoxicity. On the other hand, both the edible part and the joint part extracts were shown to have the same antioxidant potencies (Fig. 3). Therefore, it seems possible that the extracts may inhibit the oxidation of ferrous ions by hydrogen peroxide (Fenton's reaction) within the cells probably through their antioxidant activities, thus resulting in the prevention of radical generation in the cells.

Based on the findings presented here, it seems possible to assume that the protective effects of the lotus root extracts against the iron-induced cytotoxicity may be attributed, even at least partly, to their antioxidant activities, and polyphenolic compounds contained in the extracts are considered to contribute to their protective effect against the iron-induced oxidative damage to the glioma cells. However, it seems likely that the lotus root extracts may contain a variety of the antioxidants, such as carotenoids, lipoic acid, uric acid and others, and they may also contribute to the protective effects of these extracts against the iron-induced cell death observed here. Thus, the aqueous extracts of lotus root are clearly shown to prevent the iron-induced oxidative damage to C6 glioma cells, and suggested to contain potential cytoprotective substance(s), which may be able to protect glial cells against the oxidative damage brought by the abnormal accumulation of iron in the brain, thereby playing a beneficial role for preserving the brain function particularly in elderly people. However, it seems still necessary to determine active substance(s) responsible for the cytoprotective effect of the lotus root extract, and therefore these extracts are now subjecting to a HPLC analysis to identify water-extractable active component(s).

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Conflict of Interest statement

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

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