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Antioxidant Activity of a Red Seaweed *Polysiphonia morrowii* Extract

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Abstract Antioxidant activities of the extract of red seaweed, *Polysiphonia morrowii*, were evaluated using several *in vitro* assay systems. Activity-guided fractionation revealed that the 90% MeOH fraction of the *P. morrowii* extract exhibited the highest antioxidant activity, and that this fraction had a high total phenolic content (135.7±5.0 mg gallic acid/g extract). Therefore, the antioxidant activities of the 90% MeOH fraction against 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl radical, reducing power, ferrous chelating, and hydrogen peroxide were investigated. The results revealed that the antioxidant activities of the 90% MeOH fraction were similar and/or superior to that of commercial antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). In addition, the ability of the 90% MeOH fraction to inhibit oxidative damage to DNA was assessed by measuring the conversion of the supercoiled pBR322 plasmid DNA to the open circular form. The 90% MeOH fraction was found to significantly protect this hydroxyl radical-induced DNA damage in a dose-dependent manner. Taken together, these findings suggest that the 90% MeOH fraction of *P. morrowii* extract and/or its constituents has the potential for use as a new bioresource of antioxidants.

Keywords: seaweed, *Polysiphonia morrowii*, antioxidant activity, free radical, DNA damage

Introduction

Free radical-mediated lipid peroxidation, oxidative stress and antioxidants have recently been widely discussed. Reactive oxygen species (ROS) such as superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide, and hydroxyl radical (·OH) are physiological metabolites that are formed in organisms as an unavoidable consequence of aerobic respiration. Under normal conditions, ROS are effectively eliminated by antioxidant enzymes such as catalase, superoxide dismutase, and glutathione peroxidase as well as by non-enzymatic factors such as vitamin E, vitamin C, and β-carotene. However, ROS are very unstable and react rapidly with other groups or substances in the body including DNA, membrane lipids and proteins. As a result, it is believed that ROS are involved in many health disorders such as diabetes mellitus, cancer, neurodegenerative diseases, gastric ulcers, ischemic reperfusion, arthritis, and inflammatory diseases (1-4). Antioxidants are substances that delay or prevent the oxidation of cellular oxidizable substrates by scavenging free radicals and ROS, preventing the generation of free radicals and ROS, and/or activating a battery of detoxifying proteins. Until recently, several synthetic antioxidants including butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), and *t*-butyl hydroquinone (TBHQ) were commonly used to maintain foodstuffs; however, the use of these products has been restricted because they are now suspected to be carcinogenic (5). As a result, naturally occurring antioxidants from bioresources such as medicinal plants and seaweed have received a great deal of attention during the past decade.

Seaweeds provide essential bioactive compounds such as carotenoids, dietary fiber, protein, essential fatty acids, vitamins, and minerals, and are good for the growing children and pregnant women (6). In addition, many studies have reported that extracts of seaweed have biological activities such as anticoagulation, protection against oxidative cell damage, enzyme inhibition, and antioxidant and immunomodulatory effects (7-11). As a result, seaweeds are generally believed to be good candidates for the production of safe biologically active substances. During our screening of seaweeds for antioxidants, the 80% methanolic extract of *Polysiphonia morrowii*, red seaweed, was found to have significant 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. The occurrence of *P. morrowii* is widespread along the coast of the Korean peninsula. However, little information regarding the biological activity of *P. morrowii* has been available to date (12,13), and no studies have been conducted to evaluate its antioxidant activity. In the present study, we conducted an activity-guided fractionation using various organic solvents that resulted in the recovery of a highly active solvent fraction from the 80% methanolic extract of *P. morrowii*. Therefore, we evaluated the antioxidant activities of the resultant active solvent fraction by determining its reducing power, metal ion chelating activity, and the scavenging activities against DPPH free radicals, hydroxyl radicals, and hydrogen peroxide. In addition, the protective effect of the active fraction on hydroxyl radical-induced DNA damage was investigated.

Materials and Methods

Chemicals 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2-deoxy-D-ribose, gallic acid, ethylenediamine tetraacetic acid (EDTA), Folin-Ciocalteau's phenol reagent, hydrogen peroxide, 2,2-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS), thiobarbituric acid (TBA), trichloroacetic acid (TCA), peroxidase, potassium ferricyanide, and ferrozine were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents used in this study

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were of analytical grade and commercially available.

Preparation of seaweed extract and fractionations The alga, *P. morrowii*, was collected from the Yeosu coast in the Korea between March and April of 2006. A voucher specimen has been deposited in our laboratory at Chonnam National University. After cleaning the surface of the thalli of *P. morrowii* to remove visible epiphytes and soil, the sample was freeze-dried and then ground prior to extraction. The sample was then extracted using a method that has been described in detail in a previous report (12), with slight modification. Briefly, the freeze-dried alga (15.0 g) was extracted with 1 L of 80% methanol (in water, 80% MeOH) at 80°C 10 times. Each extraction lasted for 1 hr to give a total extraction time of 10 hr. Upon removal of the solvent *in vacuo*, the extract yielded 3.77 g. This total extract was then suspended in H_2O and partitioned successively with CH_2Cl_2. The CH_2Cl_2 fraction was then evaporated to dryness *in vacuo* to give 0.55 g. Next, the CH_2Cl_2 fraction was resolved in 90% MeOH and partitioned with *n*-hexane. The resultant *n*-hexane fraction was then evaporated *in vacuo* to give 0.26 g. The residual 90% MeOH fraction and aqueous fractions were 0.29 and 3.22 g, respectively.

Total phenolic content (TPC) The TPC was determined using the method described by Singleton *et al.* (14). Briefly, 40 μL of a sample solution (1 mg/mL) was mixed with 200 μL of Folin-Ciocalteau reagent and 1,160 μL of distilled water for 3 min, after which 600 μL of 20% sodium carbonate (Na_2CO_3) was added. The mixture was then shaken for 2 hr at room temperature, after which a 200 μL aliquot of the mixture was added to each well of a 96-well microplate. The absorbance was then measured at 720 nm using a microplate reader (ELx 808™; BioTek, Winooski, VT, USA). All tests were performed in triplicate and gallic acid was used as a standard. The concentration of total phenolic compounds in the sample was expressed as mg gallic acid equivalents (GAE)/g of extract.

DPPH scavenging activity The DPPH scavenging activities of the solvent extracts were measured according to of the method described by Blois, with slight modification (15). Briefly, DPPH solution (1.5×10^{-4} M, 100 μL) was mixed with and without each extract (100 μL), after which the mixture was incubated at room temperature for 30 min. The absorbance of the mixture at 517 nm was then determined using a microplate reader (ELx 808™; BioTek).

Hydroxyl radical scavenging activity The deoxyribose non site-specific hydroxyl radical scavenging activities of the solvent extracts were determined using the method described by Chung *et al.* (16). Briefly, hydroxyl radicals were generated by a Fenton reaction in the presence of $FeSO_4$. A reaction mixture comprised of 0.1 mL each of 10 mM $FeSO_4$, 10 mM EDTA, and 10 mM 2-deoxyribose was then mixed with 0.1 mL of the extract solution. Next, 0.1 M phosphate buffer (pH 7.4) was added to the reaction mixture until the total volume reached 0.9 mL. Finally, 0.1 mL of 10 mM H_2O_2 was added to the reaction mixture, which was then incubated at 37°C for 4 hr. After incubation, 0.5 mL each of 2.8%(w/v) TCA and 1.0%(w/v) TBA was added, after which the mixture was placed in a boiling water bath for 10 min. The absorbance at 532 nm was then measured.

Hydrogen peroxide scavenging activity The hydrogen peroxide scavenging activity was determined according to the method described by Müller (17). Briefly, 100 μL of 0.1 M phosphate buffer (pH 5.0) and the sample solution were mixed in a 96-well microplate. Next, 20 μL of hydrogen peroxide was added to the mixture, after which it was incubated at 37°C for 5 min. Following incubation, 30 μL of 1.25 mM ABTS and 30 μL of peroxidase (1 unit/mL) were added to the mixture, which was subsequently incubated at 37°C for 10 min. The absorbance at 405 nm was then measured using a microplate reader.

Reducing power The reducing powers of the solvent extracts were determined using the method described by Oyaizu (18). Briefly, a sample solution was mixed with 0.5 mL of 0.2 M phosphate buffer (pH 6.6) and 0.5 mL potassium of ferricyanide (1%, w/v). The mixture was then incubated at 50°C for 20 min. Next, 0.5 mL TCA (10%, w/v) was added to the mixture, which was then centrifuged at 1,036×g for 10 min. A 0.5 mL aliquot of the upper layer of the solution was then mixed with 0.5 mL distilled water and 0.1 mL $FeCl_3$ (0.1%, w/v), after which the absorbance was measured at 700 nm. A higher absorbance was taken to indicate a greater reducing power.

Ferrous ion chelating activity The ferrous ion chelating activity was determined using the method described by Singh and Rajini (19). Briefly, the sample solution was mixed with 0.1 mM $FeCl_2$ and then allowed to rest at room temperature for 30 sec. The reaction was then initiated by the addition of 0.25 mM ferrozine. The absorbance was then measured at 562 nm after 10 min.

Protective effect against hydroxyl radical-induced DNA damage To evaluate the protective effects of the 90% MeOH fraction against DNA damage caused by hydroxyl radicals, a reaction was induced by placing the following reagents (total volume, 12 μL) in an eppendorf tube: 0.5 μg pBR322 DNA, 2 mM $FeSO_4$ and various concentrations of the 90% MeOH fraction. The mixture was then incubated at 37°C for 30 min, after which 4 μL of 30% H_2O_2 were added (20). Next, the mixture was subjected to 0.8% agarose gel electrophoresis, after which the DNA bands (supercoiled, linear, and open circular) were stained with ethidium bromide.

Statistical analysis Data were evaluated for statistical significance using the SPSS package for Windows (Version 14.0). Values were expressed as the mean± standard error (SE). The mean values were compared using an one-way analysis of variance (ANOVA) followed by Tukey's or Duncan's test. A *p*-value of less than 0.05 was considered to be significant.

Results and Discussion

Total phenolic content (TPC) and DPPH radical scavenging activity of the extract and fractions of *P. morrowii* The TPCs of the total extract and fractions of

Table 1. Total phenolic content of total extract and fractions obtained from *P. morrowii*

Extracts	mg GAE/g extract[1)]
Total	23.2±2.6
CH_2Cl_2	75.0±1.5[2)]
H_2O	15.5±5.0
n-Hexane	7.8±4.5
90% MeOH	135.7±5.0[2,3)]

[1)]Results are mean±SE of 3 independent experiments.
[2)]The value is significantly different from the value of total extract (p<0.05).
[3)]The value is significantly different from the value of CH_2Cl_2 fraction (p<0.05).

Table 2. DPPH radical scavenging activity of total extract and fractions obtained from *P. morrowii*

Extracts	Scavenging activity[1)] (%)
Total	47.5±1.3
CH_2Cl_2	72.7±1.7[2)]
H_2O	17.5±1.2
n-Hexane	30.1±1.3
90% MeOH	92.0±1.6[2,3)]

[1)]Results are mean±SE of 3 independent experiments.
[2)]The value is significantly different from the value of total extract (p<0.05).
[3)]The value is significantly different from the value of CH_2Cl_2 fraction (p<0.05).

P. morrowii are shown in Table 1. The 90% MeOH fraction was found to have the highest TPC, while the *n*-hexane fraction had the lowest. As shown in Table 2, the DPPH radical scavenging activities of the total extract and the fractions differed. Specifically, the 90% MeOH fraction exhibited stronger scavenging activity than the other fractions. These findings are consistent with the results of several studies that have reported that free radical scavenging activity was augmented by increasing the TPC (21,22).

DPPH radical scavenging activity of the 90% MeOH fraction of *P. morrowii* DPPH is a free radical donor that is stable and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (23). The DPPH radical model is a widely-used, relatively quick method for evaluating free radical scavenging activity. The effect of antioxidants on DPPH radical scavenging is believed to occur due to their hydrogen donating ability (24), and this scavenging is visually noticeable as a change in color from purple to yellow. Therefore, DPPH is often used as a substrate to evaluate antioxidant activity.

The 90% MeOH fraction showed a higher DPPH radical scavenging activity than any other fractions. Therefore, the dose-dependent DPPH radical scavenging activity of the 90% MeOH fraction was further evaluated (Fig. 1). This fraction exhibited a high potential for DPPH radical scavenging that ranged from 27.8 to 92.0% at concentrations ranging from 5 to 100 μg/mL. BHA and BHT, which are powerful synthetic antioxidants, were used as positive controls. At a concentration of 50 μg/mL, the scavenging activity of BHA was 88.3%, which was lower than that of the 90%

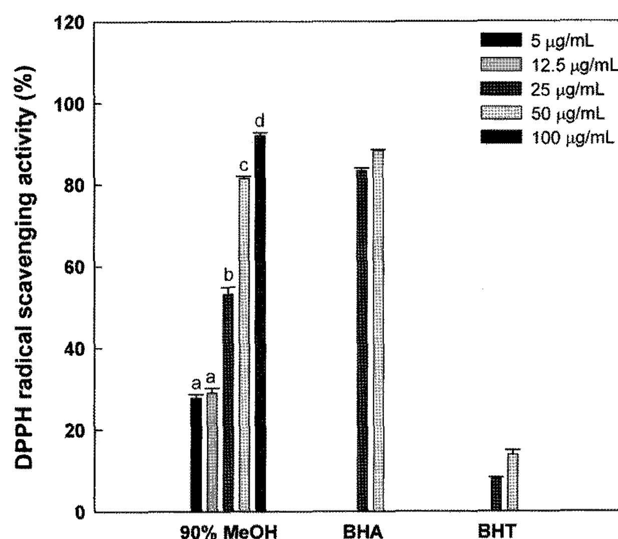

Fig. 1. DPPH radical scavenging activity of the 90% MeOH fraction obtained from *P. morrowii*. BHA and BHT were used as positive controls. Results are the mean±SE of 3 independent experiments. [a-d]Values with different subscripts indicate a significant difference (p<0.05).

MeOH fraction at 100 μg/mL. At a concentration of 50 μg/mL, BHT also exhibited scavenging activity that was weaker than that of the 90% MeOH fraction at 5 μg/mL. Many studies have been conducted to evaluate the antioxidant activities of seaweed extracts obtained by solvent and enzymatic extraction methods. For example, Heo *et al.* (25) extracted brown seaweeds using enzymatic hydrolysis and found that the DPPH radical scavenging activity was dependent on the types of enzymes used. In addition, Chandini *et al.* (26) reported that the antioxidant activity of extracts of brown seaweeds obtained using the solvent extract method was dose- and solvent-dependent. Furthermore, Ganesan *et al.* (11) found that solvent extracts of red seaweeds had weak scavenging activity. In this study, the DPPH scavenging activity of the 90% MeOH fraction of *P. morrowii* extract was superior to any other activities that have been reported to date (11,25,26).

Hydrogen peroxide scavenging activity of the 90% MeOH fraction of *P. morrowii* Measuring the hydrogen peroxide scavenging activity is known to be one of the most useful methods of determining the ability of an antioxidant to decrease the level of pro-oxidants such as hydrogen peroxide (27). Scavenging of hydrogen peroxide by antioxidants can be attributed to their electron donating ability. The hydrogen peroxide scavenging activity of the 90% MeOH fraction is shown in Fig. 2. The activity increased as the concentrations of the 90% MeOH fraction increased. The scavenging rate observed when the concentration was 100 μg/mL was with an 85.7%, which was only slightly lower than the scavenging activity of BHA and BHT. Hydrogen peroxide, which is a reactive non radical, is very important because it can penetrate biological membranes. Although hydrogen peroxide itself is not very reactive, it is easily converted into more reactive species such as singlet oxygen and hydroxyl radicals, which can then initiate lipid peroxidation or induce toxic

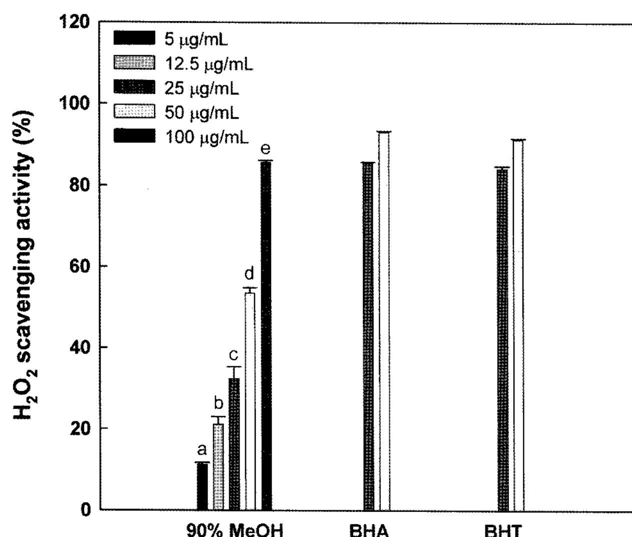

Fig. 2. Hydrogen peroxide (H_2O_2) scavenging activity of the 90% MeOH fraction obtained from *P. morrowii*. BHA and BHT were used as positive controls. Results are the mean±SE of 3 independent experiments. [a-e]Values with different subscripts indicate a significant difference ($p<0.05$).

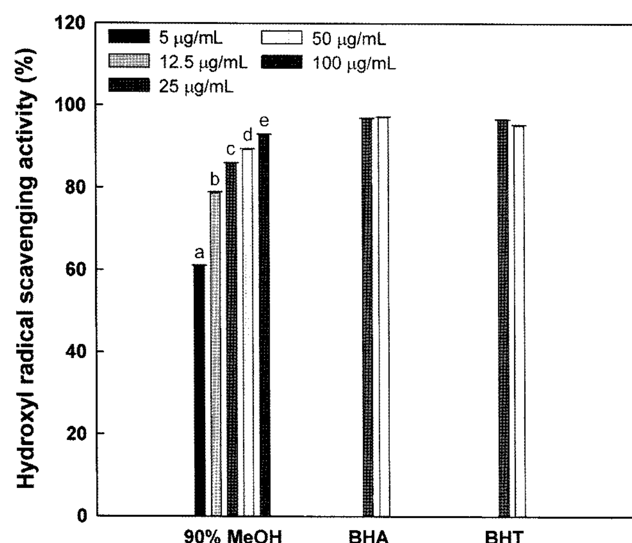

Fig. 3. Hydroxyl radical scavenging activity of the 90% MeOH fraction obtained from *P. morrowii*. BHA and BHT were used as positive controls. Results are the mean±SE of 3 independent experiments. [a-e]Values with different subscripts indicate a significant difference ($p<0.05$).

effects in cells. Many species of seaweed are known to possess scavenging activity for hydrogen peroxide (25, 28, 29); however, the scavenging activity of these seaweeds is lower than that of the 90% MeOH fraction of *P. morrowii* observed in this study.

Hydroxyl radical scavenging activity of the 90% MeOH fraction of *P. morrowii* The hydroxyl radical scavenging activity of the 90% MeOH fraction was then evaluated (Fig. 3). It ranged from 60.9 to 92.7% when the 90% MeOH fraction was evaluated at concentrations of 5 to 100 μg/mL. In addition, the hydroxyl radical scavenging activities of BHA and BHT were similar to those of the

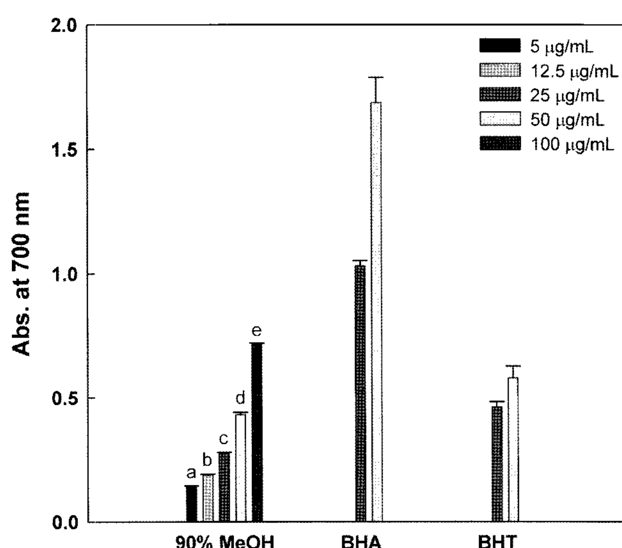

Fig. 4. Reducing power of the 90% MeOH fraction obtained from *P. morrowii*. BHA and BHT were used as positive controls. Results are the mean±SE of 3 independent experiments. [a-e]Values with different subscripts indicate a significant difference ($p<0.05$).

90% MeOH fraction. Most seaweed extracts have weak hydroxyl radical scavenging activities that are typically around 40% at mg levels (25,29,30). However, the 90% MeOH fraction evaluated in this study exhibited high scavenging activity at concentrations at the μg level. Indeed, at a concentration of 5 μg/mL this fraction was found to have a hydrogen peroxide scavenging activity of 11.3% (Fig. 2) and a 60.9% scavenging activity against hydroxyl radical (Fig. 3). These results suggest that the 90% MeOH fraction is a potent hydroxyl radical scavenger. In addition, a ferrous ion chelating assay revealed that the 90% MeOH fraction had a weak chelating activity (11.3%) at a concentration of 250 μg/mL. This result supports the finding that the 90% MeOH fraction is able to directly scavenge hydroxyl radicals. Among the ROS, the hydroxyl radical shows the strongest chemical reactivity and reacts most easily with amino acids, DNA and membrane components. Therefore, removal of hydroxyl radicals is one of the most effective defenses in a living body against various diseases.

Reducing power of the 90% MeOH fraction of *P. morrowii* The reducing power, which provides an estimate of the ability of a compound to reduce ferric iron (III) to ferrous iron (II), is determined using a redox-linked colorimetric reaction (18). In addition, the reducing capacity of a compound may serve as a significant indicator of its potential for use as an antioxidant (31). As shown in Fig. 4, the 90% MeOH fraction was found to have a good ability to reduce ferric iron (III) to ferrous iron (II). In addition, the reducing power of the 90% MeOH fraction increased in a dose-dependent manner. Furthermore, its activity was superior to that of BHT, but inferior to that of BHA. Several studies reported that the reducing power of solvent extracts from red and brown seaweeds were low at the mg level, as indicated by an optical density (OD) of <0.2 (11,26,32). However, in the present study, the 90%

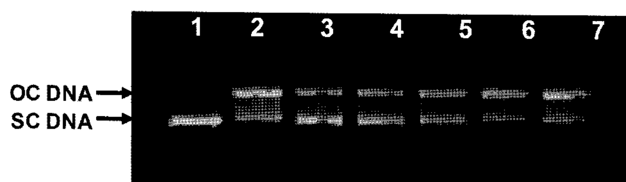

Fig. 5. Agarose gel electrophoretic patterns of plasmid DNA breaks induced by hydroxyl radicals generated by a Fenton reaction in the presence of the 90% MeOH fraction. The 0.5 μg of pBR 322 DNA were incubated at 37°C for 30 min in 2 mM $FeSO_4$ and 30% H_2O_2 with the following additive combinations: lane 1, no addition (plasmid DNA); lane 2, $FeSO_4$ and H_2O_2 (DNA damage control); lane 3-7, $FeSO_4$ and H_2O_2 in the presence of the 90% MeOH fraction at concentrations of 125, 62.5, 25, 12.5, and 5 μg/mL, respectively.

MeOH fraction of *P. morrowii* was found to have strong reducing power, with an OD of 0.72 being observed when the concentration of 100 μg/mL was evaluated. These findings indicate that the extract evaluated here is a potent antioxidant.

***In vitro* protective effect of the 90% MeOH fraction of *P. morrowii* against hydroxyl radical-induced DNA damage** The antioxidant effect of the 90% MeOH fraction was also investigated by evaluating its protective effect against hydroxyl radical-induced DNA damage *in vitro*. A single-strand break in supercoiled (SC) plasmid DNA leads to the formation of open circular (OC) DNA when the DNA is exposed to hydroxyl radicals derived from the Fenton reaction (20). As shown in Fig. 5, incubation of plasmid DNA with Fe^{2+}/H_2O_2 resulted in increased formation of OC DNA, indicating that DNA single-strand breaks were induced by treatment with Fe^{2+}/H_2O_2. However, addition of the 90% MeOH fraction to Fe^{2+}/H_2O_2 resulted in a decreased conversion of SC DNA to OC DNA, and this decrease occurred in a dose-dependent manner. These findings indicate that the 90% MeOH fraction is a potent scavenger of hydroxyl radicals.

DNA is another sensitive biotarget for ROS-mediated oxidative damage (33). DNA damage caused by ROS can initiate carcinogenesis or features common to the pathogenesis of neurodegenerative diseases such as Parkinson's and Alzheimer's disease. Among the ROS, the hydroxyl radical is recognized as a DNA damaging agent of physiological significance (34). As shown in Fig. 5, the 90% MeOH fraction effectively protected DNA from hydroxyl radical-induced damage in a dose-dependent manner.

In summary, solvent fractionation of the extract of *P. morrowii* yielded a 90% MeOH fraction that exhibited high antioxidant activity *in vitro*. Specifically, this fraction showed excellent antioxidant abilities toward DPPH, hydroxyl radical, and hydrogen peroxide. In addition, this fraction exerted a protective effect against hydroxyl radical-induced DNA damage and was found to have a high reducing power. Although additional *in vitro* and *in vivo* studies related to the toxicity and other biological activities of this compound are needed, the results of the present study suggest that *P. morrowii* extract has the possibility for use as a new bioresource of antioxidants. Currently, we are conducting additional studies to identify the antioxidant compounds responsible for the activities reported here.

Acknowledgments

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