

Radical Scavenging and Antioxidant Activities of Fermented *Laminaria japonica* Extracts

Min-Jung Park and Ji-Sook Han[†]

Department of Food Science and Nutrition, Pusan National University, Busan 609-735, Korea

Abstract

Radical scavenging and antioxidant activities of *Laminaria japonica* and fermented its extracts were evaluated. Freeze-dried *L. japonica* was fermented by *Aspergillus oryzae* and extracted with distilled water. The extract solution was mixed with ethanol and centrifuged. The supernatant was ethanol soluble fraction, non-polysaccharide fraction (ESF), and residue was ethanol insoluble precipitation, polysaccharide fraction (EIP). ESF was subjected to sequential fractionation with dichloromethane, ethyl acetate, butanol and water. To determine the radical scavenging and antioxidant activities of these, DPPH radical, hydroxyl radical, superoxide anion radical scavenging activities and linoleic acid oxidation were tested. Among the extracts, ESF of fermented *L. japonica* showed the highest radical scavenging activity. The ESF showed DPPH radical scavenging activity of 64.33% at concentration of 50 µg/mL. It was higher than 57.70% of vit. C. Ethyl acetate and butanol fraction had high value of radical scavenging and antioxidant activities, especially butanol fraction of fermented *L. japonica* was 79.48% of hydroxyl radical scavenging activity at concentration of 50 µg/mL. The fermented *L. japonica* had radical scavenging and antioxidant activities higher than *L. japonica*. These results suggest that fermented *L. japonica* is healthy food having radical scavenging and antioxidant activities.

Key words: antioxidant activity, fermentation, *Laminaria japonica*

INTRODUCTION

Seaweeds have demonstrated free radical scavenging activities, and thus may help prevent aging and some chronic diseases. Almost all seaweed species have substantial ability to scavenge hydroxyl radicals (1). *Hijikia fusiformis*, a kind of fresh brown algae, showed strong 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity. The major active compound from *H. fusiformis* was identified as fucoxanthin by ¹³C-nuclear magnetic resonance spectroscopy (2). Sulfated polysaccharides from edible seaweeds potentially could be used as natural antioxidants by the food industry (3).

Laminaria japonica, a kind of brown algae, has long been used as a food to promote health. Recently, as a dietary supplement it has been known for several biological activities: scavenging activity against DPPH radicals (4), antimutagenic activity (5) and down-regulation of blood glucose in diabetic rats (6). *L. japonica* usually contain alginic acid, fucoidan and laminaran. These are the most abundant polysaccharides of *L. japonica*. These polysaccharides have been reported to exhibit a variety of biological activities (3,7).

Fermentation is a chemical reaction that splits complex organic compounds into relatively simple substances. *Laminaria japonica*'s active compounds may be packed in its rigid structural matrix. During fermentation, the active compounds in *L. japonica* will be exposed and these may have effectiveness such as antioxidant activity.

Antioxidant activity is intensively focused due to the currently growing demand from the functional food industry. Almost all photosynthesizing plants including seaweeds are exposed to a combination of light and high oxygen concentrations, which lead to the formation of free radicals and other strong oxidizing agents, but they seldom suffer any serious photodynamic damage during metabolism. This fact implies that their cells have some protective antioxidative mechanisms and compounds (8). Seaweeds are considered to be rich source of antioxidants (9). Recently, the potential antioxidant compounds were identified as some pigments and polyphenols. Those compounds are widely distributed in plants or seaweeds and are known to exhibit high antioxidant activity.

Many researchers have indicated that reactive oxygen species (ROS) and lipid oxidation in human body increase

[†]Corresponding author. E-mail: hanjs@pusan.ac.kr
Phone: +82-51-510-2836. Fax: +82-51-583-3648

oxidative stress. ROS and lipid oxidation such as superoxide anion radical ($\cdot O_2^-$), hydroxyl radical ($\cdot OH$) and linoleic acid oxidation are physiological metabolites formed during aerobic life as a result of the metabolism of oxygen. It is well known that ROS are involved in the signaling of various cellular events. Attack by ROS, common to many kinds of cell/tissue injury, has been implicated as the cause of diabetes, atherosclerosis and other vascular diseases, and treatments with various antioxidants are known to inhibit these events. A human body has several mechanism of defence against free radical and other ROS. The radical scavenging activity can help mechanism of defence in human body on free radical. Antioxidants are essentially needed in body system. Natural antioxidants has advantage against chemical ones, since natural antioxidants are considered to be safe by the consumer and practically no safety tests are required by the legislation if the food component is Generally Recognized As Safe (10). The aim of this work was to evaluate the radical scavenging and antioxidant activities of fermented *L. japonica* for functional food. *L. japonica* was fermented by *Aspergillus oryzae* and potential antioxidant activities of the resultant fermented extracts were evaluated using three different ROS scavenging assays such as DPPH radical, superoxide anion and hydroxyl radical, and linoleic acid oxidation assay. So that we suggest the using possibilities of fermented *L. japonica* as functional food having antioxidant activity.

MATERIALS AND METHODS

Material

Laminaria japonica was purchased at local market in Pusan, Korea and used in this study.

Chemicals

DPPH, nitroblue tetrazolium (NBT), ethylenediaminetetra acetic acid disodium salt (Na_2 EDTA), 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), linoleic acid, ascorbic acid and other reagents of analytic grade were purchased from Sigma, USA.

Sample preparation and extraction

Freeze-dried *L. japonica* was fermented by *Aspergillus oryzae* at $35 \pm 1^\circ C$ for 72 h. *L. japonica* and fermented *L. japonica* were extracted with distilled water. The extract solutions were mixed with ethanol and centrifuged. The supernatant is ethanol soluble fraction, non-polysaccharide fraction (ESF) and the residue is ethanol insoluble precipitation, polysaccharide fraction (EIP) (11). ESF was subjected to sequential fractionation with dichloromethane, ethyl acetate, butanol and water.

Each extract was dried and placed in a plastic bottle, and then stored at $-80^\circ C$ until used (12,13).

DPPH radical scavenging activity

The scavenging activity of fermented *L. japonica* extracts on DPPH radical was studied, employing the modified method described earlier by Yamaguchi et al. (14,15). Briefly, dissolved sample in ethanol, 1.5 mL of DPPH solution (0.1 mM, in 95% ethanol) was incubated with varying concentrations of the sample. The reaction mixture was shaken well and incubated for the resulting solution. The absorbance of the solution was read at 517 nm against a blank. IC_{50} (inhibitory concentration 50%) is concentration of a sample that inhibits a standard 50% response. The individual IC_{50} was calculated as the concentration.

Superoxide anion radical scavenging activity

The scavenging potential for superoxide anion radical was analyzed via a hypoxanthine/xanthine oxidase generating system coupled with nitroblue tetrazolium (NBT) reduction following the method of Kirby and Schmidt (16). The reaction mixture contained 125 μL of buffer (50 mM K_2HPO_4/KOH , pH 7.4), 20 μL of 15 mM Na_2EDTA in buffer, 30 μL of 3 mM hypoxanthine in buffer, 50 μL of xanthine oxidase in buffer (1 unit per 10 mL buffer) and 25 μL of plant extract in buffer (a diluted sonicated solution of 10 μg per 250 μL buffer). The absorbance of the solution was measured at 540 nm. Superoxide scavenging activity was expressed as % inhibition compared to the blank containing buffer in place of extract.

Hydroxyl radical scavenging activity

The reaction mixture contained 0.45 mL of 0.2 M sodium phosphate buffer (pH 7.0), 0.15 mL of 10 mM 2-deoxyribose, 0.15 mL of 10 mM $FeSO_4-EDTA$, 0.15 mL of 10 mM H_2O_2 , 0.525 mL of H_2O , and 0.075 mL of sample solution. The reaction was started by the addition of H_2O_2 . After incubation at $37^\circ C$ for 4 h, the reaction was stopped by adding 0.75 mL of 2.8% trichloroacetic acid and 0.75 mL of 1.0% of 2-tribarbituric acid in 50 mM NaOH. The solution was boiled for 10 min, and then cooled in water. The absorbance of the solution was measured at 520 nm. Hydroxyl radical scavenging activity was evaluated as the inhibition rate of 2-deoxyribose oxidation by $\cdot OH$ (17).

Prevention of linoleic acid oxidation (FTC method)

The oxidation test was conducted by using the linoleic acid model system. A 0.2 mL of sample solution and 0.5 mL of 0.2 M sodium phosphate buffer (pH 7.0) were mixed with 0.5 mL of 2.5% linoleic acid in ethanol. The

peroxidation was initiated by the addition of 50 μL of 0.1 M 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) and carried out at 37°C for 200 min in the dark. The degree of oxidization was measured according to the thiocyanate method (18) for measuring peroxides by reading the absorbance at 500 nm after coloring with FeCl_2 and ammonium thiocyanate. A control test was performed with linoleic acid but without sample solution. Ascorbic acid was used as positive control.

Statistical analysis

The data are presented as mean \pm SD. The values were evaluated by one-way analysis of variance (ANOVA) followed by post-hoc Duncan's multiple range tests.

RESULTS AND DISCUSSION

Scavenging activity of DPPH radical

DPPH is a free radical donor, which has been widely used to evaluate the free radical scavenging effect of natural antioxidants (8,19). The scavenging activities of *L. japonica* extracts on DPPH radical was shown in Fig. 1. The fermented *L. japonica* extracts showed higher scavenging activity than *L. japonica* extracts overall. Especially, ESF that was non-polysaccharide fraction of fermented *L. japonica* showed the free radical scavenging activity of 64.33% at concentration of 50 $\mu\text{g}/\text{mL}$, it was higher than 57.70% of vitamin C, positive control and it was the highest value compared with other samples. The free radical scavenging activities of WE and EIP of fermented *L. japonica* was 48.33% and 35.27% at the same concentration respectively, and they were higher than those of *L. japonica*. Among the

fractionated extracts of ESF, ethyl acetate and butanol fraction of fermented *L. japonica* at concentration of 50 $\mu\text{g}/\text{mL}$ on DPPH radical had high values of the scavenging activities of 68.06% and 73.21%, respectively. It could be concluded that fermented of *L. japonica* increased free radical scavenging activity. Generally, many studies reported that polysaccharide of seaweed was effective in free radical scavenging, but it is found in this study that the free radical scavenging activity are also contained in non-polysaccharide fraction.

The IC_{50} values of the DPPH radical scavenging activity of ESF fractions extracted from *L. japonica* and fermented *L. japonica* are shown in Table 1. Butanol fraction of fermented *L. japonica* had the highest DPPH radical scavenging activity among the fractions tested with IC_{50} of 28.58 ± 1.02 $\mu\text{g}/\text{mL}$. Many researchers have reported positive correlation between free radical scavenging activity and antioxidant compound. In this study, the extracts of fermented *L. japonica* increased free radical scavenging activity comparison with the extracts of *L. japonica*. This result suggest that the amount of antioxidant compounds with scavenging free radical was increased during fermentation of *L. japonica*.

Scavenging activity of superoxide anion radical

Superoxide anion radical scavenging activity of *L. japonica* extracts were estimated using hypoxanthine-xanthine oxidase system (NBT method). Each sample showed the superoxide anion radical scavenging activity and the activities were decreased in the order $\text{ESF} > \text{WE} > \text{EIP}$ (Fig. 2). Among them, ESF of fermented *L. japonica* scavenged the superoxide anion radical of 60.63%

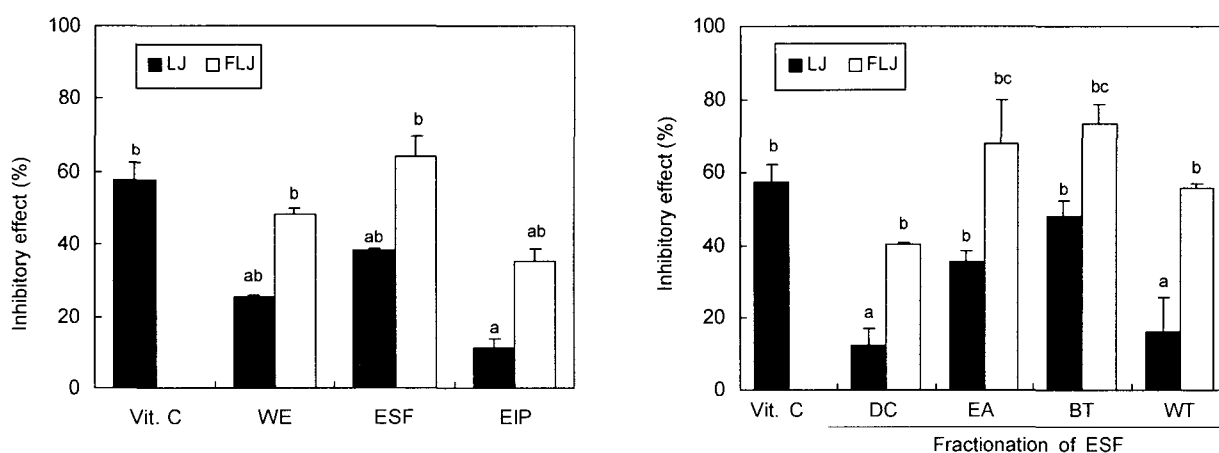


Fig. 1. Scavenging activity of *Laminaria japonica* extracts on DPPH.

LJ, *Laminaria japonica*; FLJ, fermented *Laminaria japonica*. WE, water extract; ESF, ethanol soluble fraction; EIP, ethanol insoluble precipitation; DC, dichloromethane fraction; EA, ethyl acetate fraction; BT, butanol fraction; WT, water fraction; Vit. C, vitamin C. The final concentration of samples and vitamin C in reaction mixture were 50 $\mu\text{g}/\text{mL}$.

Each value is expressed as mean \pm SD in triplicate experiments.

^{a-c}Values with different alphabets are significantly different at $p < 0.05$ as analyzed by Duncan's multiple range test.

Table 1. IC₅₀ value¹⁾ of radical scavenging and antioxidant activities of ESF fractions of *Laminaria japonica* (µg/mL)

	ESF fractions ⁴⁾	<i>L. japonica</i>	Fermented <i>L. japonica</i>
DPPH radical scavenging	Dichloromethane	179.01 ± 5.04 ^{2)b3)}	65.18 ± 2.54 ^{ab}
	Ethyl acetate	75.16 ± 1.67 ^{ab}	35.24 ± 0.67 ^a
	Butanol	57.10 ± 0.28 ^{ab}	28.58 ± 1.02 ^a
	Water	214.57 ± 0.94 ^b	47.24 ± 1.57 ^a
Superoxide anion radical scavenging	Dichloromethane	119.21 ± 5.24 ^b	64.75 ± 6.54 ^{ab}
	Ethyl acetate	59.50 ± 4.30 ^{ab}	25.14 ± 0.25 ^a
	Butanol	48.92 ± 1.47 ^{ab}	40.35 ± 2.50 ^{ab}
	Water	98.24 ± 3.21 ^b	42.51 ± 1.47 ^{ab}
Hydroxyl radical scavenging	Dichloromethane	236.67 ± 0.57 ^c	87.02 ± 2.44 ^b
	Ethyl acetate	87.25 ± 0.46 ^b	51.50 ± 3.26 ^{ab}
	Butanol	63.02 ± 2.67 ^{ab}	23.49 ± 3.22 ^a
	Water	164.25 ± 8.24 ^c	41.57 ± 4.27 ^{ab}
Antioxidant activity on linoleic acid oxidation	Dichloromethane	229.15 ± 6.59 ^c	88.14 ± 1.54 ^b
	Ethyl acetate	91.47 ± 4.25 ^b	47.47 ± 5.12 ^a
	Butanol	49.24 ± 3.31 ^a	39.89 ± 0.63 ^a
	Water	97.20 ± 4.84 ^b	67.64 ± 3.41 ^{ab}

¹⁾IC₅₀ value is the concentration of sample required for 50% inhibition.

²⁾Each value is expressed as mean ± SD in triplicate experiments.

³⁾Values with different alphabets are significantly different at p<0.05 as analyzed by Duncan's multiple range test.

⁴⁾Ethanol soluble fraction.

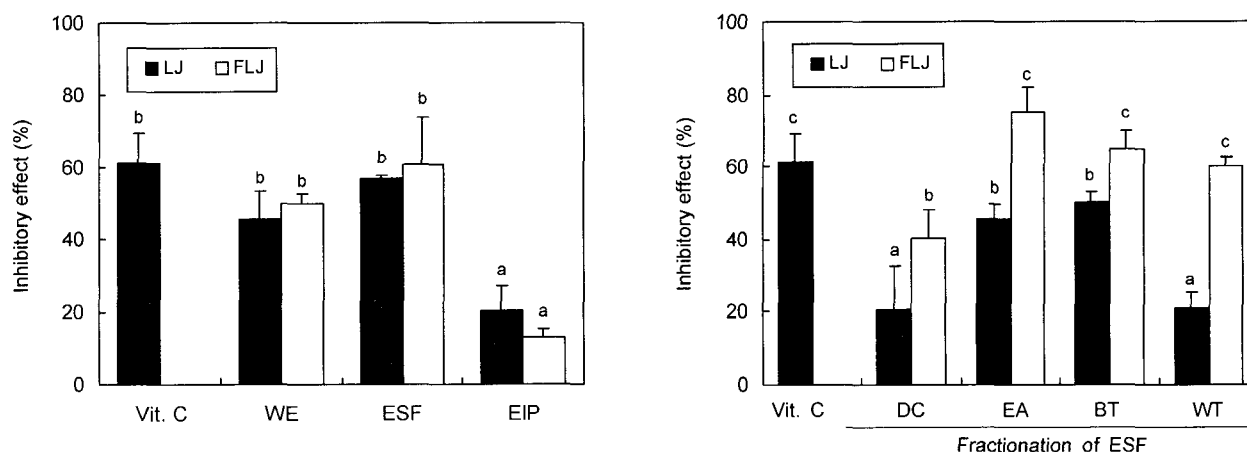


Fig. 2. Scavenging activity of *Laminaria japonica* extracts on the superoxide anion radical.

See the abbreviations in Fig. 1. The final concentration of samples and vitamin C in reaction mixture were 50 µg/mL.

Each value is expressed as mean ± SD in triplicate experiments.

^{a-c}Values with different alphabets are significantly different at p<0.05 as analyzed by Duncan's multiple range test.

at concentration of 50 µg/mL, it was similar to 61.40% of vitamin C.

Superoxide anion ($\cdot\text{O}_2^-$) is formed in viable cells during several biochemical reaction and its effect can be magnified because it produces other types of free radicals and oxidizing agent that can induce cell damage (20). In this study, some extracts showed higher superoxide anion scavenging activities than the commercial antioxidant, vitamin C. In particular ethyl acetate fraction (IC₅₀=25.14 ± 0.25 µg/mL, Table 1) of fermented *L. japonica* among the fractionated extracts of ESF, indicated the highest superoxide anion radical scavenging activity

of 75.28% at concentration of 50 µg/mL.

In a study with *Scytosiphon lomentaria*, a brown algae, water extract solution has antioxidant activity, but ethanol extract solution did not show superoxide anion scavenging activity (21). However, in this study showed superoxide anion radical scavenging activity of ESF that was extracted with distilled water and then mixed with ethanol and centrifuged, and was higher than those of WE and EIP. Therefore, ESF of fermented *L. japonica* need to study further to clarify the discrepancies.

Scavenging activity of hydroxyl radical

Hydroxyl radical scavenging activity of the extracts

of *L. japonica*. was measured as the percentage of inhibition of hydroxyl radicals generated in the Fenton reaction mixture. 2-Deoxyribose is oxidized by $\cdot\text{OH}$ that formed by the Fenton reaction and degraded to malondialdehyde. The scavenging activity of *L. japonica* extracts on hydroxyl radical is shown in Fig. 3.

Each sample showed the hydroxyl radical scavenging activity and its activity was decreased in the order of ESF, WE, and EIP. Specifically, ESF of fermented *L. japonica* showed higher scavenging activity of 63.04% than ESF of *L. japonica* at concentration of 50 $\mu\text{g}/\text{mL}$. Among the fractionated extracts of ESF, butanol fraction of fermented *L. japonica* had the highest hydroxyl radical scavenging activity of 79.48% at concentration of 50 $\mu\text{g}/\text{mL}$. IC_{50} value of this fraction was 23.49 ± 3.22 $\mu\text{g}/\text{mL}$ (Table 1). The cell-damaging action of hydroxyl radical is well known, as it is the strongest among free radicals (22). Some seaweed extracts have exhibited positive effects on hydroxyl radical, reaching around 60% (23,24).

There are several reports about antioxidant activity of *L. japonica*, but most of them were fresh and raw *L. japonica* (4,6). The results in this study indicate that fermented *L. japonica* has a noticeable effect of scavenging hydroxyl radical, especially the butanol fraction has high potential to ameliorate oxidative stress.

Antioxidant activity on linoleic acid oxidation

The antioxidant activity of *L. japonica* extracts on the peroxidation of linoleic acid was investigated. The FTC method was used to measure the amount of peroxide in initial stages of lipid oxidation system. As a result, ESF and WE showed higher antioxidant activities than EIP overall (Fig. 4). The inhibitory effect of WE from fermented *L. japonica* on linoleic acid oxidation had high

value of 58.50% at 50 $\mu\text{g}/\text{mL}$ concentration in comparison with ESF and EIP showing 49.97% and 11.48% at the same concentration, respectively. Among the fractionated extracts of ESF, the inhibitory effect of ethyl acetate fraction of fermented *L. japonica* was 75.28% at 50 $\mu\text{g}/\text{mL}$ concentration. It was dose-dependant (data not shown) and high value compared with other fractionated extracts. Therefore, fermented *L. japonica* extracts had higher antioxidant activity than the one without fermentation. The antioxidant activity of ESF, the non-polysaccharide fraction, was higher than that of EIP, the polysaccharide fraction, and ethyl acetate and butanol fraction were higher than other fractionated extracts of ESF.

In the present study, we used *L. japonica* as a potential natural water soluble antioxidant source and confirmed its scavenging activities on free radical, superoxide anion radical and hydroxyl radical, and antioxidant activity on linoleic acid oxidation. Among the WE fractions, ESF, the non-polysaccharide fraction had higher radical scavenging and antioxidant activity than EIP, the polysaccharide fraction. Especially, the butanol fraction showed the highest radical scavenging and antioxidant activity among the fractionated extracts of ESF.

The extracts of fermented *L. japonica* showed relatively higher antioxidant activities than the commercial antioxidant such as vitamin C. In particular, hydroxyl radical scavenging activity of the extracts of fermented *L. japonica* was predominant and its butanol fraction showed the scavenging activity of 79.48% at concentration of 50 $\mu\text{g}/\text{mL}$. The extracts of fermented *L. japonica* also showed high scavenging activity on free radical, superoxide anion radical and linoleic acid

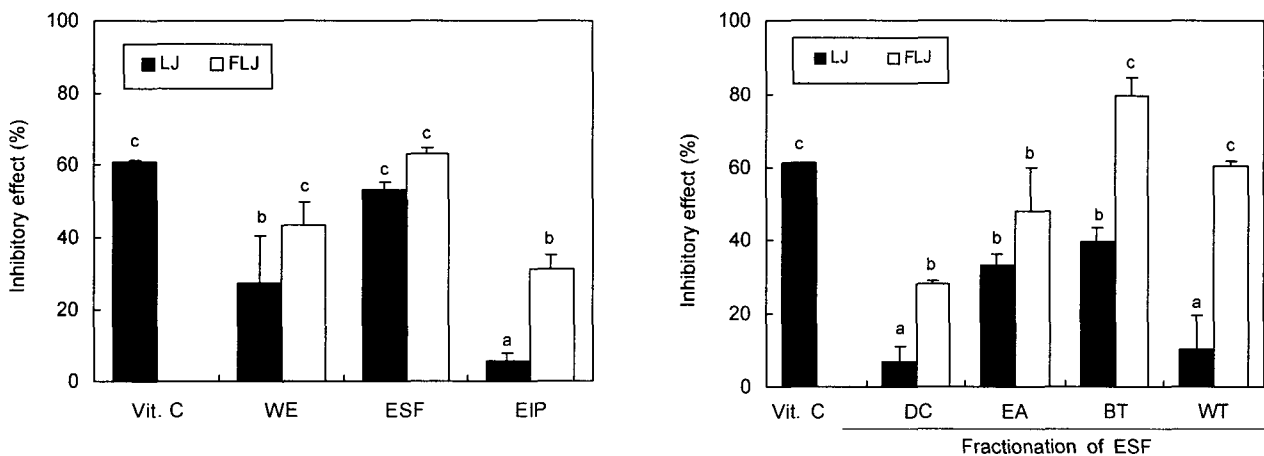


Fig. 3. Hydroxyl radical scavenging activity of *Laminaria japonica* extracts.

See the abbreviations in Fig. 1. The final concentration of samples and vitamin C in reaction mixture were 50 $\mu\text{g}/\text{mL}$. Each value is expressed as mean \pm SD in triplicate experiments.

^{a-c} Values with different alphabets are significantly different at $p < 0.05$ as analyzed by Duncan's multiple range test.

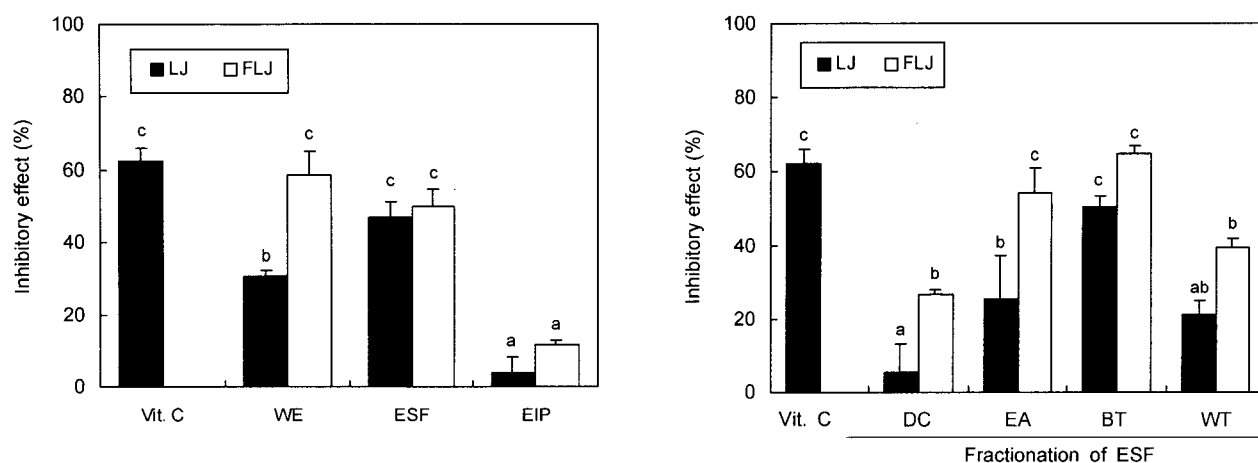


Fig. 4. Antioxidant activity of *Laminaria japonica* extracts on linoleic acid oxidation.

See the abbreviation in Fig. 1. The final concentration of samples and vitamin C in reaction mixture were 50 $\mu\text{g/mL}$. Each value is expressed as mean \pm SD in triplicate experiments.

^{a-c}Values with different alphabets are significantly different at $p < 0.05$ as analyzed by Duncan's multiple range test.

oxidation. Free radicals can have damaging effects directly on the cell, particularly on DNA, proteins and lipids, causing lipid peroxidation, ultimately leading to apoptotic cell death (25). As a result of this study, we confirm that fermented *L. japonica* has higher radical scavenging and antioxidant activity than intact *L. japonica*. Therefore it is suggested that the extracts of fermented *L. japonica* can be used as biological antioxidant in food and nutraceutical industry. But further studies are needed to identify the antioxidant compounds still in question after this study. This laboratory is in the process of preparation for the search of the active compounds.

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