

Antioxidative Activities and Liver-protective Effects of *Myelophycus simplex* Extract

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Abstract The free radical scavenging activity of extracts prepared from *Myelophycus simplex* was evaluated by using an ESR spectrometer. For this study, *Myelophycus simplex* was 70% ethanol extraction was performed. *Myelophycus simplex* extracts scavenged DPPH, hydroxyl, superoxide, and alkyl radicals. Especially, the extracts exhibited the highest scavenging activity on hydroxyl radical, which is deemed of the strongest radical. In addition, we also evaluated the protective effects of *Myelophycus simplex* extract on 3.5% alcohol-induced damage (oxidative stress) in normal liver cells. The *Myelophycus simplex* extract inhibited productions of reactive oxygen species (ROS), and cell death against alcohol-induced liver cell damage. It was presumed that the *Myelophycus simplex* extract was involved in regulation of apoptosis-related pathway in the cell environments. These results indicate that *Myelophycus simplex* extract possess antioxidative activity on liver cells.

Keywords: *Myelophycus simplex*, antioxidant, free radical, liver cells, flow cytometer

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1. Introduction

Free radicals are highly related to aging promotion, and recently, attempts to obtain antioxidants harmless to the human body from natural plants while enhancing the antioxidant enzyme system in vivo have been performed from various angles [1,2]. Known antioxidants include synthetic antioxidants such as BHA or BHT, and natural antioxidants such as polyphenols, flavonoids, carotenoids, ascorbic acid, tocopherols, and phospholipids [3]. The demand for agents tends to increase day by day [4]. Natural antioxidants are mostly antioxidants of plant origin and are present in all parts of trees, bark, stems, leaves, fruits, roots, flowers, fruits, seeds, etc. These are mainly phenolic compounds that delay the formation of free radicals or an antioxidant by inhibiting activity [5,6,7].

Myelophycus simplex is a brown algae belonging to the broad seaweed family. It is a brown, needle-shaped, single-stranded, forming a bunch. It is about 5~15 cm in height and 1~2 mm in thickness. The main distribution of *Myelophycus simplex* is known to be distributed all over the western coast of the North Pacific and the southern coast of Korea and Japan [8]. Brown algae are known to contain the components of fucoïdan and laminarin, which have anti-cancer and anti-inflammatory physiological activities [9,10]. *Myelophycus simplex* also contains fucoïdan, so it seems to have several physiological activities. However, research on the functionality of *Myelophycus simplex* has been rarely conducted except for studies on neuronal protective effects [11].

However, studies using *Myelophycus simplex* extracts have little information until now. Therefore, in this study, the antioxidant activity through the free radical scavenging ability of the *Myelophycus simplex* extract and the toxicity evaluation using normal liver cells are to be used as basic data for the development of functional materials.

2. Materials and Methods

2.1. Materials

Myelophycus simplex was purchased from a local market (Yeosu, Korea). 2,2-azobis (2-amidinopropane) (4-pyridyl-1-oxide)-N-tert-butyl nitron (4-POBN), 5,5-Dimethyl-1-pyrroline N-oxide (DMPO), hydrochloride (AAPH), 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (St Louis, USA). In addition, fetal bovine serum (FBS), penicillin, streptomycin and Dulbecco's modified Eagle's medium (DMEM) were purchased from Hyclone (Logan, UT, USA). All other reagents were of the highest grade available commercially.

2.2. DPPH Radical Scavenging Activity

DPPH radical scavenging activity was measured using the method described by Nanjo *et al.* [9]. An ethanol solution of 60 μ L of each sample (or ethanol itself as control) was added to 60 μ L of DPPH (60 mM) in ethanol solution. After mixing vigorously for 10 s, the solution

was then transferred into a 100 mL quartz capillary tube, and the scavenging activity of JRSF extract on DPPH radical was measured using a JES-FA ESR spectrometer (JEOL Ltd., Tokyo, Japan). ESR spectrum was measured on an ESR spectrometer exactly 2 min later. Experimental conditions were as follows: central field, 3475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 5 mW; gain, 6.3×10^5 and temperature, 298 K.

2.3. Hydroxyl Radical Scavenging Activity

Hydroxyl radicals were generated by iron-catalyzed Haber-Weiss reaction (Fenton driven Haber-Weiss reaction) and the generated hydroxyl radicals rapidly reacted with nitron spin trap DMPO [10]. The resultant DMPO-OH adducts was detectable with an ESR spectrometer. JRSF extract (0.2 mL) with various concentrations was mixed with DMPO (0.3 M, 0.2 mL), Fe_2SO_4 (10 mM, 0.2 mL) and H_2O_2 (10 mM, 0.2 mL) in a phosphate buffer solution (pH 7.2), and then transferred into a 100 mL quartz capillary tube. After 2.5 min, the ESR spectrum was recorded using an ESR spectrometer. Experimental conditions were as follows: central field, 3475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 1 mW; gain, 6.3×10^5 and temperature, 298 K.

2.4. Alkyl Radical Scavenging Activity

Alkyl radicals were generated by AAPH. The PBS (pH 7.4) reaction mixtures containing 40 mM AAPH, 40 mM 4-POBN and indicated concentrations of tested samples, were incubated at 37°C in a water bath for 30 min, and then transferred to a 60 μL Teflon capillary tube. The spin adduct was recorded on an ESR spectrometer. Measurement conditions were as follows: central field, 3475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 1 mW; gain, 6.3×10^5 and temperature, 298 K.

2.5. Cell Viability

Liver cells were seeded in 96-well plate at a concentration of 4.0×10^5 cells/mL. After 20 h, the cells were treated with different concentrations of various ethanol extracts from *Myelophycus simplex*, and incubated in a humidified incubator at 37°C for 1 h. Then, 3.5% alcohol was added as final concentration, and incubated for 24 h. Thereafter, a 100 μL of MTT stock solution (0.5 mg/mL) was added and incubated for 4 h. Then, the supernatants were aspirated and the formazan crystals in each well were dissolved in 150 μL of DMSO. Absorbance was measured by spectrofluorometer (SpectraMax M2/M2e, CA, USA) at a wavelength of 540 nm. The optical density of the formazan formed in the control cells was taken as 100% [5].

2.6. Cell Cycle and Death Analysis

For cell cycle analysis, the harvested cells were fixed with 80% ethanol (containing 0.5% Tween-20) for 24 h, incubated with 50 $\mu\text{g/mL}$ PI and 1 $\mu\text{g/mL}$ RNase A at

37 °C for 30 min, and analyzed by flow cytometry, using a fluorescence activated cell sorter (FACS) (BD, Franklin Lakes, NJ, USA) Calibur using 'Cell Quest pro' software. At least 10,000 events were evaluated [12].

2.7. Statistical Analysis

Study data are expressed as mean \pm standard error of mean (SEM). Statistical analyses of differences between treatment groups were conducted using Student's *t*-test for paired data, and $p < 0.05$ was considered to have statistical significance. All analyses were carried out in triplicate using Graph Pad Prism software version 4.00 (Graph Pad Software Inc., San Diego, CA).

3. Results and Discussion

DPPH is a stable free radical, and has been widely accepted as a tool for estimating the free radical scavenging activities of antioxidants. In this study, the DPPH radical scavenging activity of extract from *Myelophycus simplex* was shown in Table 1. The *Myelophycus simplex* extracts were capable of scavenging DPPH radicals in a dose-dependent manner. The ethanolic extracts showed similar DPPH radical scavenging activities compared to vitamin C. Also, the IC_{50} values was 17 $\mu\text{g/mL}$. Hydroxyl radicals generated in the ferrous ion/hydrogen peroxide system were trapped by DMPO, forming a spin adduct detected by an ESR spectrometer. In our study, the extract from *Myelophycus simplex* was observed that the IC_{50} value was 162 $\mu\text{g/mL}$. The alkyl radical spin adduct of 4-POBN/free radical was generated from AAPH, and a decrease in ESR signals was observed with the dose increment of extract. The extracts showed similar alkyl radical scavenging activities compared to that of vitamin C (Table 1), and the IC_{50} value was 22 $\mu\text{g/mL}$. ROS has been implicated in dozens of diseases, including malaria, AIDS, heart disease, stroke, atherosclerosis, diabetes, cancer and stomach ulcers. Therefore, the development and use of more effective antioxidants is desired. Antioxidants can protect the human body from free radical and ROS effects and delay the progression of many chronic diseases as well as lipid oxidative odors in food. The types of antioxidants used include antioxidant vitamins (e.g. ascorbic acid, α -tocopherol, β -carotene), inorganic antioxidants (e.g. selenium) and synthetic antioxidants (e.g. butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate). However, the use of these antioxidants in food is strictly regulated due to industrial efficiency or the potential health risks posed by these compounds. Therefore, there is a need to identify and characterize more effective natural antioxidants. In our study, indicate that the extract from *Myelophycus simplex* possess scavenging activity against various free radicals such as DPPH, hydroxyl and alkyl. The liver-protective effect of extract from *Myelophycus simplex* was determined by apoptosis analysis using a flow cytometer. The cells were treated with the extracts before alcohol (3.5%), the percentage of apoptotic cells was observed 27.6% at alcohol (3.5%), while the percentages of *Myelophycus simplex* extracts treated cells were 22.9 and 15.3% at

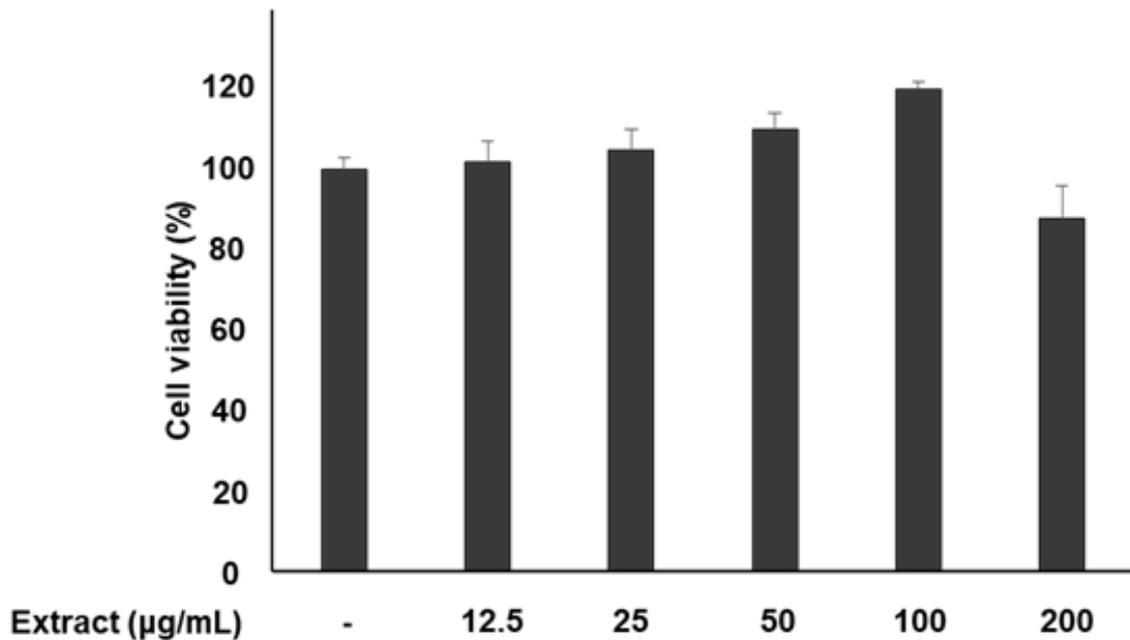
50 and 100 $\mu\text{g/mL}$, respectively (Figure 1B). The result of this study suggests that *Myelophycus simplex* extract could be utilised to develop physiologically functional

foods. In addition, it is expected that this will contribute to increase interest and potential applications of bioactive materials.

Table 1. DPPH, alkyl and hydroxyl radical scavenging activity of extract by electron spin resonance measurement

Sample	DPPH radical scavenging activity (IC ₅₀ mg/mL)	Alkyl radical scavenging activity (IC ₅₀ mg/mL)	Hydroxyl radical scavenging activity (IC ₅₀ mg/mL)
Ethanol extract	0.017 \pm 0.002	0.022 \pm 0.004	0.162 \pm 0.005
Vitamin C	0.004 \pm 0.001	0.014 \pm 0.002	0.029 \pm 0.002

A



B

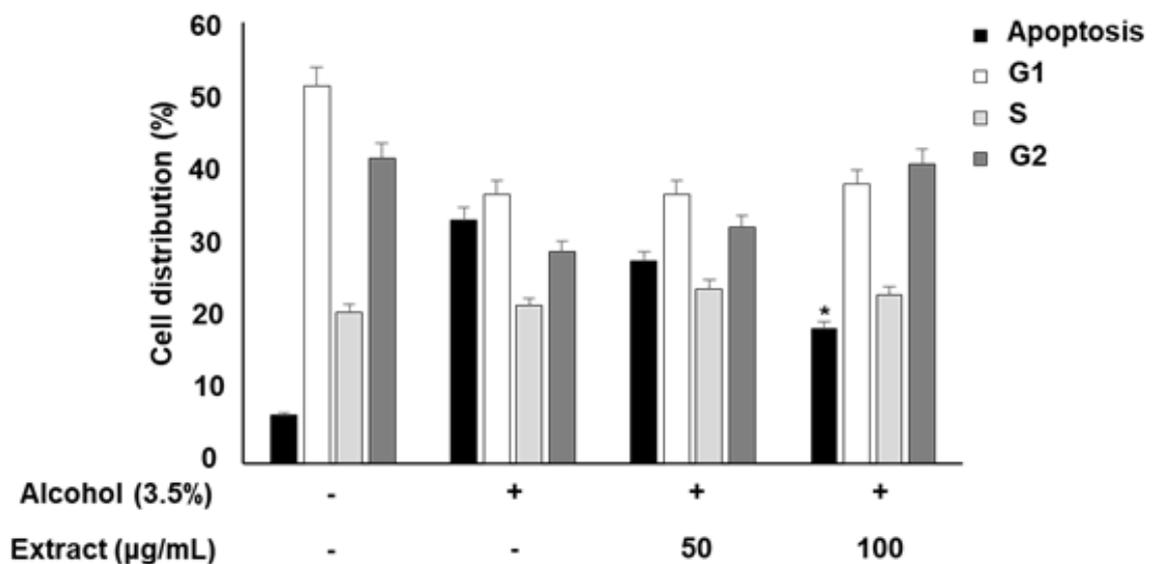


Figure 1. Dose-dependent effect of *Myelophycus simplex* extracts in Chang cells. The cytotoxicity was assessed using MTT assay (A). Cell apoptosis and cycle of Chang cells with extract of *Myelophycus simplex* prior to 3.5% alcohol treatment (B). Means \pm SD of determinations were made in triplicate experiments. *($p < 0.05$) are significantly different as analyzed by paired t-test that compared the oxidative damage group with the *Myelophycus simplex* extract treated group

4. Conclusions

This study was extracted with ethanol to investigate the antioxidant activity of *Myelophycus simplex* extract. As a result of measuring the radical scavenging ability using ESR, DPPH and alkyl radical scavenging ability excluding hydroxyl radicals showed high activity in ethanol extract. In particular, the alkyl radical scavenging activity showed similar activity to vitamin C. The results of measuring the radical scavenging activity using ABTS, the total antioxidant capacity using FRAP, and the antioxidant activity through reducing power also confirmed the excellent antioxidant efficacy of *Myelophycus simplex* extract. On the other hand, as a result of examining the survival rate of cells through MTT assay using normal liver cells (human liver, Chang cells) to examine the cytotoxicity, all of the ethanol extracts did not show any toxicity up to a concentration of 0.5 mg/mL. Therefore, based on this study, it is considered that further research into the potential as a natural antioxidant using *Myelophycus simplex* is needed.

Acknowledgments

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