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Inhibition of Reactive Oxygen Species (ROS) and Nitric Oxide (NO) by *Gelidium elegans* Using Alternative Drying and Extraction Conditions in 3T3-L1 and RAW 264.7 Cells

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Abstract

***Gelidium* (*G.*) *elegans* is a red alga inhabiting intertidal areas of North East Asia. We examined anti-oxidative and anti-inflammatory effects of *G. elegans*, depending on drying and extraction conditions, by determining reactive oxygen species (ROS) and nitric oxide (NO) in 3T3-L1 and RAW 264.7 cells. Extraction yields of samples using hot air drying (HD) and far-infrared ray drying (FID) were significantly higher than those using natural air drying (ND). The 70% ethanol extracts showed the highest total phenol and flavonoid contents compared to other extracts (0, 30, and 50% ethanol) under tested drying conditions. The scavenging activity on 2,2-diphenyl-1-picrylhydrazyl (DPPH) and nitrite correlated with total phenol or flavonoid content in the extracts. The greatest DPPH scavenging effect was observed in 70% ethanol extract from FID and HD conditions. The production of ROS and NO in 3T3-L1 and macrophage cells greatly decreased with the 70% ethanol extraction derived from FID. This study suggests that 70% ethanol extraction of *G. elegans* dried by FID is the most optimal condition to obtain efficiently antioxidant compounds of *G. elegans*.**

Key words: *Gelidium elegans*, radical scavenging activity, ROS/RNS production, far-infrared ray drying, ethanol extraction

INTRODUCTION

Red seaweed is a commonly eaten edible plant in East Asia (1), and a major source of commercial products such as agar and carageenans. In particular, red algae, such as *Palmaria palmata* and *Porphyra umbilicalis* (nori/gim), are traditional foods in Europe and Asia (2). *Gelidium* (*G.*) *elegans*, also a red alga belonging to the family *Gelidiceae*, phylum *Rhodophyta* (3), is distributed across Korea, Japan, and Russia. Although *G. elegans* is an edible seaweed, its biological functions have yet to be investigated.

Seaweed requires processing to conserve whole compounds prior to ingestion. The extraction of seaweed components depends on the drying conditions, an important processing method directly affecting the quality of the seaweed products, and specific components, which can be altered during this process. Therefore, selection of the drying method and the use of optimal extraction conditions should be considered to obtain target compounds from seaweed. Natural air drying (ND) is the most economical and prevalent traditional drying method requiring no energy. Hot air drying (HD), which has been widely adopted in the manufacture of conventional dried foods, can reduce drying time. Far infrared-ray drying (FID) is often used to obtain high-quality and/or heat-sensitive products. Although FID has several disadvantages, such as low productivity and high cost (4), it minimizes component loss and has an efficient drying rate, resulting in increased quality, among its many advantages (5). In this study, we examined ND, HD, and FID as optimized drying methods for *G. elegans* extraction.

Reactive oxygen species (ROS) are produced as a general byproduct of cellular metabolism, but are considered to be toxic. The imbalance in ROS status has been broadly defined as oxidative stress. Over-production of ROS can damage proteins, lipids, and DNA (6). ROS accumulation has been implicated in a variety of human diseases, including atherosclerosis, cancer, neurodegenerative diseases, and aging (7,8). Moreover, ROS tend to work with reactive nitrogen species (RNS) to damage cells. RNS such as NO also have deleterious effects on the body due to their reactivity in cells, playing an important role in inflammation (9). Therefore, controlling ROS and RNS levels is important to protect against degenerative

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diseases.

Recent studies suggest that bioactive compounds from seaweed have a variety of biological effects. Fucoidan, a sulfated polysaccharide, has various biological activities, including anti-cancer, anti-proliferation, anti-viral, anti-inflammatory, and anti-coagulant activities (10-12). In addition, active compounds from several algae have antioxidant or anti-inflammatory effects (13,14). These natural antioxidants have been considered to be safe, unlike synthetic antioxidants, which can be toxic (15). Thus, edible seaweed-derived antioxidants could be used to prevent ROS/RNS-related diseases.

In this study, we examined various drying methods and extraction conditions for the antioxidant compounds of *G. elegans*. The antioxidant properties of *G. elegans* were assessed in DPPH radical scavenging activity, NBT assay and nitrite scavenging ability, as well as the production of ROS/RNS, total phenol and flavonoid contents. This study will provide useful data for the commercial use of *G. elegans* as marine antioxidants in company with optimal drying and extraction conditions.

MATERIALS AND METHODS

Seaweed materials

G. elegans was collected from Jeju Island, off the coast of Korea, from March 2006 to March 2008. Epiphytes, sand and salt were removed using tap water. Finally, the specimens were gently washed with distilled water and stored at -20°C for further experiments.

Drying of *G. elegans* and extract preparation

G. elegans was dried by ND, HD and FID. ND was performed outside in a sunny location for three days. During drying, the specimens were turned inside out every 12 hr. HD was performed in a dry oven (VS-1202D3N, VISION, Daejeon, Korea) for 2 hr at 100°C. During drying, the specimens were turned inside out every 30 min. FID was performed using a ceramic radiator (110 V) of the FIR system (JOURI-Q, KEC, Gyeongnam, Korea) for 4 hr at 80°C. During drying, the specimens were turned inside out every 1 hr. Water contents of specimens derived from ND, HD and FID were 8.7, 4.8, and 5.2%, respectively. All dried samples were pulverized using a blender and extracted with ten volumes of ethanol (30, 50, and 70%) and distilled water (100 g/L) by stirring at room temperature for 24 hr in a dark place. Each extract was filtered using Whatman No. 2 paper and concentrated using a vacuum evaporator (BUCHI Rotavapor R-200, New Castle, DE, USA). The condensed extracts were powdered using a freeze-dryer (DC1316, ilShin Lab Co, Yangju, Korea) and sealed in a bottle. The crude powders were weighed and used as samples in the following experiments. Extraction yields were obtained by measuring the weights of specimens before ethanol extraction and crude powder after extraction.

Materials

Dulbecco's modified Eagle's medium (DMEM), bovine calf serum (BS), fetal bovine serum (FBS), penicillin-streptomycin (P/S), phosphate-buffered saline (PBS), and trypsin-EDTA were purchased from Gibco (Gaithersburg, MD, USA). Dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX), insulin, nitroblue tetrazolium (NBT), and N-acetyl cysteine (NAC) were purchased from Sigma (St. Louis, MO, USA). Ethanol (95%) was purchased from Samchun chemicals (Gyeonggi, Korea). Unless noted, all other chemicals were purchased from Sigma and reagents were of the highest grade available commercially.

Cell culture

3T3-L1 preadipocytes obtained from the American Type Culture Collection (ATCC, CL-173) were maintained in high-glucose DMEM containing 10% BS and 1% P/S at 37°C in a humidified atmosphere containing 5% CO_2 (16). Post-confluent preadipocytes were briefly treated with a hormonal cocktail (0.25 μmol/L DEX, 0.5 mmol/L IBMX, and 10 μg/mL insulin) for two days in 10% FBS containing DMEM. The cells were then placed in 10% FBS/DMEM containing insulin for four days, changing media every other day. The cells were placed in 10% FBS/DMEM without insulin for two days or longer to induce adipocyte formation at day 8. The adipocytes were treated with the crude samples described above to determine ROS production. Raw 264.7 cells (ATCC, TIB-71) were maintained in DMEM containing 10% FBS and 1% penicillin/streptomycin and were plated in a 96-well microplate at a concentration of 1×105 cells/well and cultured for 24 hr at 37°C in the presence of 5% CO_2.

Determination of the total phenol and flavonoid contents in the *G. elegans* extracts

The total phenol content was determined according to the modified Folin-Ciocalteu method (17). The sample (100 μg) was placed in a test tube with distilled water (7 mL) and Folin-Ciocalteu reagent (200 mg/L, 0.5 mL) saturated with sodium carbonate solution (1 mL), and allowed to stand for 30 min. The reaction was monitored by measuring the absorbance at 715 nm. For determination of the total flavonoid content (18), 5 mL of 50% methanol, 1 mL of sample, and 10 mL of diethylene glycol were mixed with 1 mL of 1 M NaOH and incubated for 1 hr at 37°C. The reaction color was measured at

420 nm. Naringin (Sigma) was used as a standard marker for total flavonoids.

DPPH radical scavenging activity of the *G. elegans* extracts

The DPPH radical scavenging activities of the *G. elegans* extracts were determined using the Blois method (19). DPPH (0.15 mol/L) and the sample were mixed (10:1, v/v) and incubated for 30 min at room temperature in the dark. The reaction color was determined at 517 nm using a spectrophotometer. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), a water-soluble derivative of vitamin E, was used as a positive control.

Nitrite scavenging ability of the *G. elegans* extracts

The nitrite scavenging ability in each sample was determined using the Gray and Dugan method (20). The reaction, composed of 1 mL of sample and 1 mL of 1 mM $NaNO_2$, was adjusted to pH 1.2 with 0.1 N HCl to produce a final volume of 10 mL and incubated at 37°C in a water bath for 2 hr. The reaction mixture (1 mL) was combined with 5 mL of 2% acetic acid and 0.4 mL of Griess reagent (1% sulfanilic acid and 1% naphthylamine in 30% acetic acid, mixed at a 1:1 ratio), and vortexed. The sample was then incubated at room temperature for 15 min. The remaining nitrite was then measured at 520 nm. The control group was incubated with 0.4 mL of distilled water instead of Griess reagent.

Nitric oxide (NO) assay

Raw 264.7 cells were pre-treated with the *G. elegans* samples (100 μg/mL) for 1 hr followed by lipopolysaccharide (LPS; 10 μg/mL) treatment for an additional 24 hr at 37°C. The cultured cell supernatants (100 μL) were mixed with an equal volume of Griess reagent (Sigma) and incubated at room temperature for 10 min. The amount of NO was measured at 540 nm.

Effects of the *G. elegans* extracts on ROS production in preadipocytes and adipocytes

3T3-L1 preadipocytes were grown to confluence and induced to differentiate into adipocytes, as described previously (21). ROS production was detected using the NBT assay (22). At day 8 after induction, the cells were incubated for 90 min in PBS containing 0.2% NBT. The dark-blue formazan was dissolved in 50% acetic acid, and its absorbance was determined at 570 nm. To monitor ROS production in mature adipocytes, the 3T3-L1 cells differentiated for eight days were further cultured with fresh medium for two days. ROS production was then measured using an OxiSelect Intracellular ROS Assay Kit (Cell Biolabs, Inc., San Diego, CA, USA).

Statistical analysis

The results were statistically analyzed by ANOVA and Duncan's multiple range test using SAS software (23). A p-value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Drying methods and extraction conditions for *G. elegans*

We examined the optimal drying methods and extraction conditions for obtaining bioactive compounds with ROS/RNS scavenging activity. We selected ethanol as the *G. elegans* extraction solvent in this study because it can be metabolized in the human body with limited dose (unlike other solvents), and has been used as a material for foods such as alcoholic drinks (24). As shown in Table 1, different ethanol concentrations (30, 50, and 70%) were used for the extraction of *G. elegans* dried by ND, HD and FID. Ethanol (100%) was also evaluated for *G. elegans* extraction, but resulted in low extraction yields (<5%) in all drying conditions (data not shown).

Each drying method showed the highest extraction yields with pure water extraction. HD (31.8%) and FID (30.9%) showed higher extraction yields than ND (25.0 %). The extraction yields decreased as the concentration of ethanol increased, suggesting high ethanol concentrations extract low-molecular weight phenol compounds and remove high-weight molecules, such as proteins and carbohydrates. In general, the extraction yields were higher in the HD and FID groups than in the ND group in all ethanol concentrations. This suggests that the FID and HD specimens were more efficiently extracted than the ND samples under the tested extraction conditions,

Table 1. Drying and extraction conditions of *G. elegans*

Extract number	Drying method	Ethanol conc. (%)	Extract yields (%)
1	ND	0	25.8 ± 0.15^{d}
2		30	21.9 ± 0.20^{f}
3		50	19.6 ± 0.13^{h}
4		70	19.5 ± 0.14^{i}
5	HD	0	31.8 ± 0.14^{a}
6		30	25.7 ± 0.27^{d}
7		50	23.6 ± 0.08^{e}
8		70	21.3 ± 0.12^{g}
9	FID	0	30.9 ± 0.04^{b}
10		30	26.2 ± 0.29^{c}
11		50	23.4 ± 0.07^{e}
12		70	22.3 ± 0.13^{f}

The values represent the mean±SD (n=3). Means in the same column not sharing a common letter (a-i) are significantly different ($p<0.05$) based on Duncan's multiple range test. All experiments were performed in triplicate. ND: natural air drying; HD: hot air drying at 100°C; FID: far-infrared drying at 80°C; 1, 5, 9: water extract; 2, 6, 10: 30% ethanol extract; 3, 7, 11: 50% ethanol extract; 4, 8, 12: 70% ethanol extract.

although the specimen tissue structure could be modified during drying, thus affecting the extraction efficiency. Far-infrared rays infiltrate and activate deep cell tissues through minute vibrations, releasing heat energy to remove internal water (25). Unlike heat transfer and convection currents, far-infrared rays directly heat the subject without the use of a secondary medium (25). Thus, FID offers superior drying efficiency.

HD is also an efficient method for the rapid removal of water because of the high temperature used. However, sample tissues can be modified by hot air transfer as reported by Arun et al., who showed detrimental effects to tissue specimens by hot air (26). Moreover, a recent study demonstrated that far-infrared rays and temperature effect tissue structure pore size (27). Exposure to far-infrared rays or hot air during drying increases the tissue pore diameter, and the porosity is enhanced as temperature increases. Therefore, FID and HD can remove water efficiently and provide a favorable tissue environment for extraction.

In comparison, ND specimens harness a small amount more of water content than specimens from HD and FID. Higher water content could cause overestimation on the weight of ND specimens and affect extraction yields, even if the difference of water content among specimens is small. In addition, ND produces closely packed specimen tissues during drying (28), and these tissue structures may be unfavorably modified for extraction by sunlight and wind. Therefore, FID and HD produce better extraction yields as drying methods than ND.

Freeze drying was not addressed in this study due to increased time and cost compared to the other three drying methods, although it generates similar drying conditions with higher quality (4). Moreover, previous studies showed that FID was better suited for drying time, extraction yield and polyphenol content compared to FD (29,30). In addition, we performed FID at 80°C due to a better extraction yield compared to lower temperatures (29,30), but 100°C FID condition was not feasible by our temperature system. Next, we examined the total phenol and flavonoid contents of extracts with differing extraction conditions under ND, HD and FID.

Total phenol and flavonoid contents

Polyphenol is a secondary metabolite in photosynthesizing plants that plays an important role in protection from oxidative stress (31). Flavonoids, a group of polyphenols with strong antioxidant activity, include such compounds as catechin, flavones, and isoflavones (32). Since phenolic compounds generally have antioxidant activity, we initially examined the polyphenol and flavonoid contents in our *G. elegans* extracts. Under all

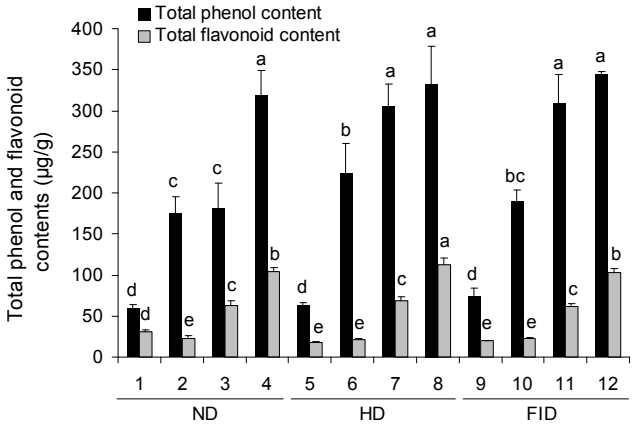

Fig. 1. Total *G. elegans* phenol and flavonoid contents. The total phenolic content was measured using the Folin-Ciocalteu method. The total flavonoid content was measured using methanol, diethylene glycol, and 1 N NaOH. The total phenol and flavonoid absorbances were measured at 715 and 420 nm, respectively. Means in the same column not sharing a common letter (a-e) are significantly different ($p<0.05$) based on Duncan's multiple range test. All experiments were performed in triplicate.

drying conditions, the total phenol content increased with higher ethanol concentrations (Fig. 1). However, 50% ethanol extraction yielded similar phenol contents as 70% ethanol extraction in FID and HD. Ethanol extraction (50 or 70%) with FID or HD increased the total phenol content by 40% compared to 50% ethanol extract of ND. The total phenol content in the 30% ethanol or water extract did not differ under different drying conditions. The flavonoid content under each drying condition showed a similar pattern to the phenol content, an inverse relationship with the extraction yield as shown in Table 1. Since proteins and carbohydrates are more soluble in water, and given that phenolic compounds tend to be dissolved in solvents, more phenols or flavonoids were present at higher ethanol concentrations, which corresponds to lower extraction yields.

DPPH scavenging activity and ROS production

We examined the radical scavenging activities of each extract under different drying conditions using DPPH. DPPH has a deep violet color as a radical indicator, but becomes pale yellow when it is neutralized by antioxidants. As expected, the color change of DPPH was most significant in the 70% ethanol extract under HD and FID (Fig. 2A), suggesting that the 70% ethanol extracts contained the most radical scavenging compounds. In the ND group, the 50 and 70% ethanol extracts showed similar scavenging activities. On the other hand, the water extracts contained negligible radical scavenging compounds under each drying condition. This suggests that 70% ethanol extraction combined with FID (or HD)

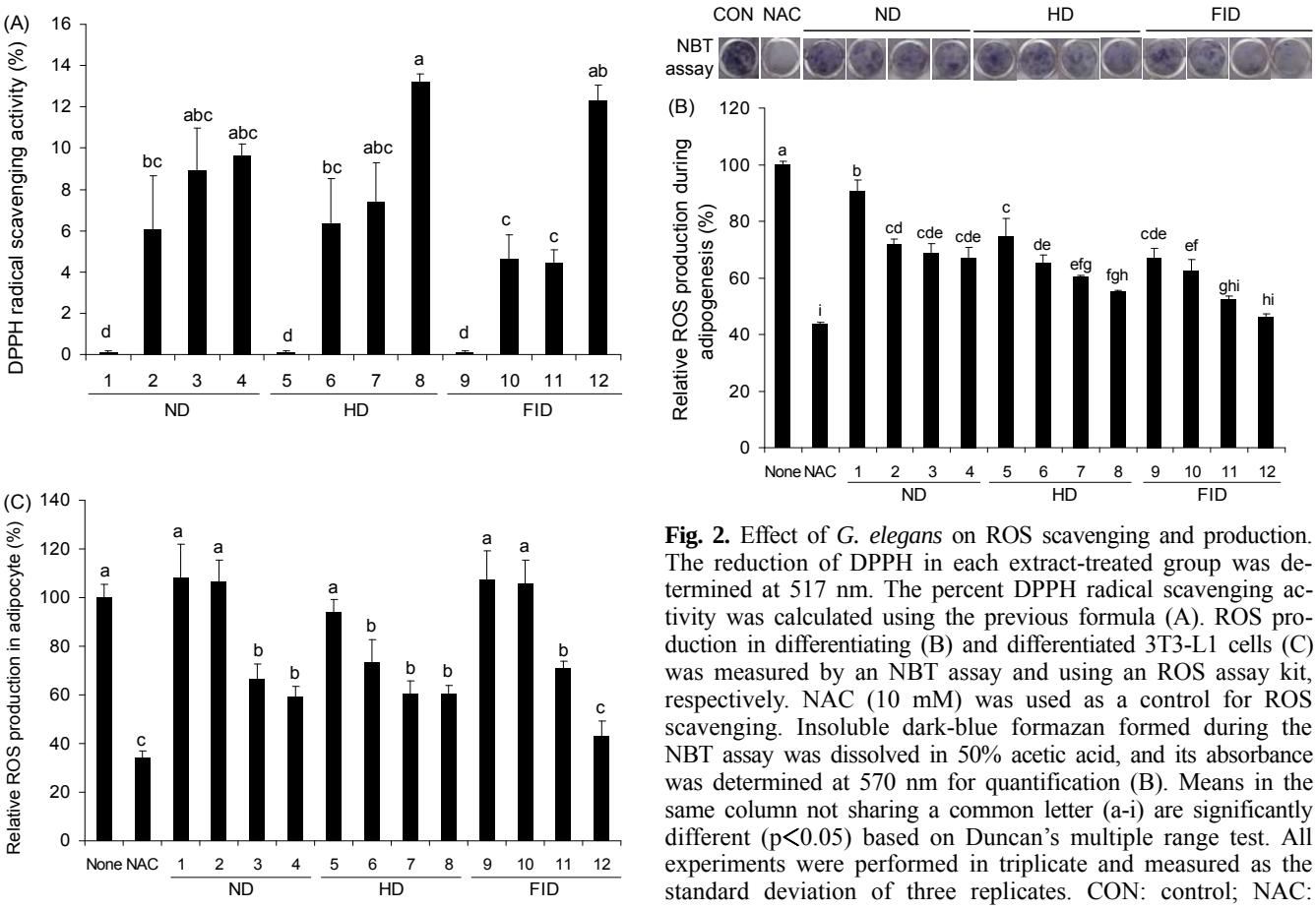

Fig. 2. Effect of *G. elegans* on ROS scavenging and production. The reduction of DPPH in each extract-treated group was determined at 517 nm. The percent DPPH radical scavenging activity was calculated using the previous formula (A). ROS production in differentiating (B) and differentiated 3T3-L1 cells (C) was measured by an NBT assay and using an ROS assay kit, respectively. NAC (10 mM) was used as a control for ROS scavenging. Insoluble dark-blue formazan formed during the NBT assay was dissolved in 50% acetic acid, and its absorbance was determined at 570 nm for quantification (B). Means in the same column not sharing a common letter (a-i) are significantly different ($p<0.05$) based on Duncan's multiple range test. All experiments were performed in triplicate and measured as the standard deviation of three replicates. CON: control; NAC: n-acetyl-cysteine.

is best for obtaining DPPH radical scavenging compounds from *G. elegans*. The ROS/RNS status in adipocytes (mature fat cells) and macrophages plays an important role in lipid accumulation and inflammation, respectively (33,34). Thus, we next analyzed the effect of each extract on ROS production and the ROS/RNS content using adipocytes (3T3-L1) (Fig. 2) and immune (macrophage) cells (Fig. 3).

ROS production in cultured fat cells decreased with increasing ethanol concentrations under all drying conditions (Fig. 2B,C). In general, all of the extract groups showed inhibitory effects on ROS production, although the HD and FID extracts had a more significant inhibitory effect than the ND extracts. In particular, the 70% ethanol extract produced by FID showed the most significant inhibition (55%) of ROS production in fat cells compared to the control group (Fig. 2B); further, this extract showed a similar inhibitory effect as *N*-acetylcysteine, a known antioxidant. On the other hand, the ND group showed no differences in ROS production between the different ethanol extracts, except for the water extract, which exerted the smallest inhibitory effect on ROS production in each group. This suggests that 70% ethanol extraction with FID is the best condition for obtaining bioactive compounds that inhibit ROS production in fat cells. We also examined the ROS contents in 3T3-L1 mature fat cells treated with each extract. The ROS contents decreased in the 50 and 70% ethanol extracts, as shown in Fig. 2B, under all drying conditions. The ROS content in the 30% ethanol extract produced with HD was reduced by >30% compared to ND and FID (Fig. 2C). In particular, the 70% ethanol extract produced with FID showed the greatest reduction (>60%) in ROS content compared to control (None). Therefore, 70% ethanol extraction with FID can be used to obtain ROS reducing compounds from *G. elegans*.

Nitrite scavenging activity and NO production

We examined the RNS scavenging effect of each extract by analyzing nitrite (NO_2^-). The nitrite scavenging activity in the 30 to 70% ethanol extracts significantly increased compared to water extraction, which showed relatively low scavenging activity. However, in all drying conditions, there was no significant difference in nitrite scavenging activity from 30 to 70% ethanol extracts (Fig. 3A). We examined the effect of each extract on NO production in cultured macrophages stimulated with LPS. HD and FID showed the tendency to decrease NO pro-

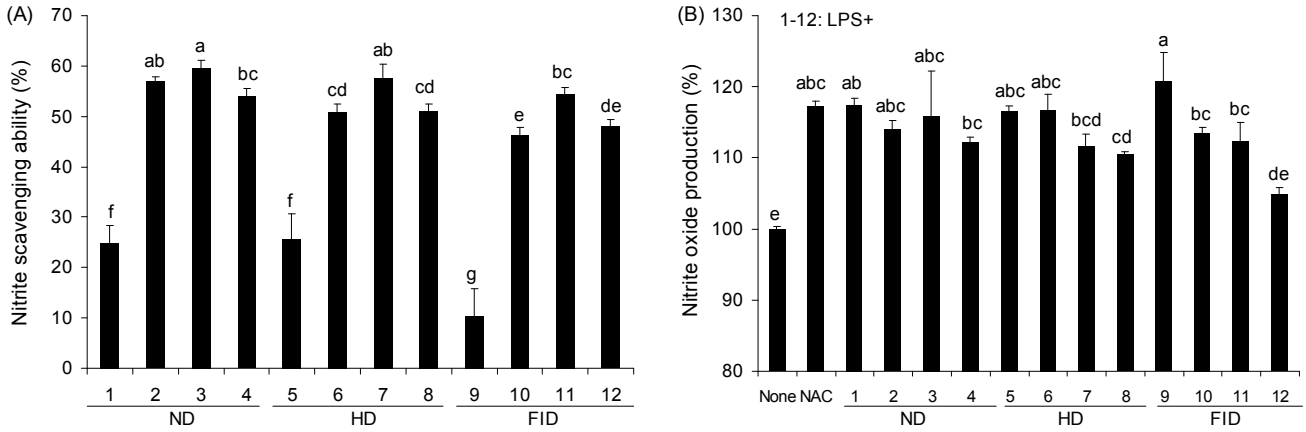

Fig. 3. Effect of *G. elegans* on RNS scavenging and production. The nitrite concentrations in the sample-treated reactions were determined at 520 nm. Nitrite scavenging activity was calculated using the previous formula (A). Cultured Raw 264.7 macrophages were treated with *G. elegans* extracts; LPS treatment induced NO production. The culture medium (supernatant) was used for the NO assay (B). Means in the same column not sharing a common letter (a-g) are significantly different ($p<0.05$) based on Duncan's multiple range test. All experiments were performed in triplicate and measured as the standard deviation of three replicates. LPS: lipopolysaccharide.

duction steadily as the ethanol concentration increased. In addition, the 70% ethanol extracts significantly decreased NO production (Fig. 3B) compared to the water extract under all drying conditions. In particular, the 70% ethanol extract of FID showed the most reduced NO production (Fig. 3B). Thus, 70% ethanol extraction with FID is most effective in NO reduction.

CONCLUSION

In this study, we examined the drying methods and extraction conditions required to obtain beneficial compounds from *G. elegans* by analyzing anti-oxidative and anti-inflammatory effects in adipocytes and macrophage cells. We found that FID and HD are drying conditions presenting higher extraction yields than ND, and ethanol extraction (70%) is better to obtain the total phenol and flavonoid contents, which have ROS/RNS scavenging antioxidant activities, under HD and FID conditions. In DPPH radical and nitrite scavenging activities, we did not observe significant differences between FID and HD. However, ethanol extraction (70%) combined with FID significantly reduced ROS and NO production in cultured cells compared to the other drying conditions. In this study, the extraction of *G. elegans* dried by FID with 70% ethanol was the best way to obtain polyphenols with antioxidant activity.

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