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Antioxidant and Longevity-Related Properties of the Ethyl Acetate Fraction of *Cnidium officinale* Makino in *Caenorhabditis elegans*

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ABSTRACT: Reactive oxygen species (ROS) are produced from energy metabolism and may cause diseases or cell death. Antioxidation refers to the suppression of ROS production and is considered beneficial in preventing diseases. This study aimed to examine the antioxidative effects of *Cnidium officinale* Makino (COM) extracts and fractions using *Caenorhabditis elegans* as an experimental model. The COM ethanol extract was fractionated according to polarity. The results showed that the ethyl acetate fraction of COM showed powerful radical scavenging activities and increased the activities of superoxide dismutase (SOD) and catalase in *C. elegans* in a concentration-dependent manner. Moreover, the ethyl acetate fraction reduced the ROS production rate in *C. elegans* and increased the cell survival rate, suggesting oxidative and thermal stress resistance. In addition, the SOD-3::green fluorescent protein (GFP) expression level in the transformed cells of *C. elegans* (CF1553) increased, suggesting oxidative stress resistance. Similarly, the HSP-16.2::GFP expression level increased, suggesting thermal stress resistance. In conclusion, the ethyl acetate fraction of COM demonstrated the strongest antioxidative effects, indicating that it may help extend longevity.

Keywords: antioxidant, *Caenorhabditis elegans*, *Cnidium officinale* Makino, reactive oxygen species

INTRODUCTION

Reactive oxygen species (ROS) are produced during energy metabolism, which involves approximately 2%–3% of the inspired oxygen entering the body (de Zwart et al., 1999). ROS induce cellular damage via DNA, protein, and lipid peroxidation, and oxidative stress is considered as a risk factor for diseases including cancer, cardiovascular diseases, and neurological disorders (Bae, 2004). There are types of ROS that facilitate aging, including superoxide radicals, singlet oxygen, and hydrogen peroxide (Feng et al., 2015; Su and Wink, 2015). The use of antioxidants may help suppress ROS production and support disease prevention. Butylated hydroxyanisole and butylated hydroxytoluene are considered antioxidants with outstanding effects; however, their use has steadily decreased because of safety concerns (Branen, 1975). Thus, there is a need to develop novel antioxidants derived from natural materials or medicinal herbs, which may help prevent side effects. *Cnidium officinale* Makino (COM) is a perennial herbaceous plant of the Umbelliferae family. It is a medicinal herb originating from China and a plant resource cultivated in South Korea and Japan (Li et al., 2012). Approximately 2% of COM is essential oil comprising ligustilide, neocnidilide, butylphthalide, decanoic acid, and phthalides, which are responsible for the antifungal effects of ligustilide, a phthalide derivative (Lee, 2004). Several studies on COM have examined its antioxidant activities (Lee et al., 2002; Jeong et al., 2009b) and effects on brain diseases (Kim et al., 2003). Moreover, other studies have used COM as a food additive in rice-related food, such as sweet rice punch (Kim and Park, 2012) and pounded rice cake (Park, 2022).

Caenorhabditis elegans is a small nematode that can grow quickly; it has a transparent body that allows the observation of expressed proteins through fluorescence (Chalfie et al., 1994; Corsi et al., 2015). Moreover, having approximately 60% genetic homology with humans, *C. elegans* can be used as a research model (Oh et al., 2013) in studies of factors affecting longevity and metabolic syndromes (Bargmann, 1998; Kampkötter et al., 2008).

In this study, the value of COM as an antioxidant was investigated. COM ethanol extract fractions were prepared to analyze the polyphenol and flavonoid contents in the extract and fractions. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activities were measured to identify the ethyl acetate fraction that ex-

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hibited the highest radical scavenging activities with respective polyphenol and flavonoid contents. *C. elegans* was used as an experimental model to investigate antioxidation. First, the activities of superoxide dismutase (SOD) and catalase as antioxidant enzymes in *C. elegans* were examined, and their respective inhibitory effects on ROS were determined. Second, resistance against juglone-induced oxidative stress was monitored, and the effects on the expression levels of green fluorescent protein (GFP) in a GFP-fused transgenic strain (CF1553) that harbors the transformed gene SOD-3::GFP and on the longevity of *C. elegans* were determined. Moreover, thermal stress resistance was evaluated at high temperatures. The results of this study may contribute to the development of natural antioxidants.

MATERIALS AND METHODS

Sample extraction and fractionation

The COM used in this study was purchased from Doosonaeyakcho. About 300 g of ground dried COM powder was mixed with 2 L of ethanol, and the extraction was repeated thrice in a 50°C water bath. The resulting extract solution was filtered. Then, it was vacuum-concentrated in a 50°C water bath, and about 49.31 g of ethanol extract concentrate was obtained. The concentrate was suspended in 500 mL of water for fractionation using equal amounts of *n*-hexane (0.61 g), methylene chloride (6.82 g), ethyl acetate (7.32 g), and *n*-butanol (8.15 g) in the given order. After vacuum concentration, the containers of the extract and each fraction were sealed and stored in the dark for use in subsequent analyses.

Analysis of polyphenol and flavonoid contents

About 100 μL of extract or fraction sample was mixed with 100 μL of Folin & Ciocalteu's phenol reagent solution and subsequently with 800 μL of 0.1 M Na_2CO_3 solution to measure the polyphenol content in the COM extract and fractions. The mixture was left to react for 20 min in a 40°C water bath. After 10-min cooling, the absorbance was measured at 700 nm, and the calibration curve was drawn using tannic acid as the reference. The polyphenol content was expressed as tannic acid equivalents per gram of sample (mg TAE/g) (Cicco et al., 2009). To measure the flavonoid content, 1 mL of extract or fraction sample was mixed with 30 μL of 5% $NaNO_2$ solution. After reacting for 5 min, 30 μL of 10% $AlCl_3$ and 200 μL of 1 M NaOH were added. The absorbance was measured at 510 nm, and the calibration curve was drawn using quercetin as the reference. The flavonoid content was expressed as quercetin equivalents per gram of sample (mg QE/g) (Lee et al., 2012).

DPPH and ABTS radical scavenging activities

Ethanol was used as the solvent to prepare varying sample concentrations to measure the DPPH radical scavenging activity in the COM extract and fractions. A 50-μL sample of a specific concentration and 200 μL of 0.2 mM DPPH ethanol solution were left to react in the dark at room temperature for 30 min. The absorbance was measured at 517 nm using a microplate reader (Yoshida et al., 1989). Equal amounts of 7.4 mM ABTS solution and 2.6 mM $K_2S_2O_8$ solution were mixed to measure the ABTS radical scavenging activity in the COM extract and fractions. To induce radical formation, the samples were left to react in the dark at room temperature for 24 h. The resulting solution was diluted using phosphate buffered saline (pH 7.4) until the absorbance range of 0.7± 0.03 was obtained. Varying sample concentrations were prepared using ethanol, and a mixture of 10 μL of sample and 190 μL of ABTS solution was left to react in the dark at room temperature for 10 min. The absorbance was measured at 732 nm, and triplicate measurements were performed using L-ascorbic acid as the control (Re et al., 1999).

Culture of *C. elegans*

C. elegans was cultured on a nematode growth medium (NGM) agar plate with *Escherichia coli* (20°C). A bleaching solution (NaClO, 5 M KOH) was applied to *C. elegans* after washing with M9 buffer to collect the eggs of *C. elegans*. The sample was added with dimethyl sulfoxide (DMSO) as the solvent in a form of stock solution to a sterile NGM plate (50°C). The final DMSO concentration was maintained at 0.1% (v/v) (Brenner, 1974).

Activities of antioxidant enzymes in *C. elegans*

The samples of COM ethyl acetate fraction at different concentrations (250 and 500 μg/mL) were added to the plate to measure the activities of antioxidant enzymes (SOD and catalase) in *C. elegans*, and *C. elegans* was cultured at identical growth stages. When *C. elegans* reached adulthood, the M9 buffer was added on the second day to wash the skin surface, and the ground sample was used (homogenization buffer: 10 mM Tris-HCl, 150 mM NaCl, 0.1 mM EDTA, pH 7.5) to measure the activities of antioxidant enzymes. The SOD activity was measured by mixing 10 μL of sample at a specific concentration and a reaction mixture (1.6 mM xanthine and 0.48 mM nitroblue tetrazolium) prepared in a solvent of 10 mM phosphate buffer (pH 8.0), followed by 5-min pre-incubation at 20°C and 20-min subsequent reaction at 37°C with the addition of 100 μL of xanthine oxidase (0.05 U/mL). The reaction was stopped using 60 mM sodium dodecyl sulfate, and the absorbance was measured at 570 nm (Kim et al., 2015). The catalase activity was measured by adding 25 mM H_2O_2 to 50 μL of sample at each con-

centration for a 3-min reaction. Thereafter, the absorbance was measured at 240 nm (Aebi, 1984).

Analysis of ROS in *C. elegans*

ROS production was induced in *C. elegans* treated with samples of COM ethyl acetate fraction, and H_2DCF-DA was used in the analysis of the antioxidative effects. *C. elegans* was cultured at identical growth stages on the plate with the added COM samples. On the fourth day of adulthood, *C. elegans* was transferred to a 96-well plate containing the M9 buffer with juglone (100 μM) for 2 h to induce ROS production at 20°C. Afterward, *C. elegans* was transferred to a 96-well plate containing 50 μL of M9 buffer. Next, 50 μL of 50 μM H_2DCF-DA was added, and the fluorescence intensities at an excitation wavelength of 485 nm and emission wavelength of 535 nm were measured for 2 h at 30-min intervals (Kim et al., 2014).

Evaluation of oxidative and thermal stress resistance

C. elegans was cultured in a plate with different concentrations (250 and 500 μg/mL) of samples of COM ethyl acetate fraction to evaluate the survival of *C. elegans* under oxidative stress. On the seventh day of adulthood, 25 individuals of *C. elegans* cultured on the plate with different COM concentrations were transferred to a plate containing the M9 buffer with juglone (1 mM). The survival rate was measured over time until all individuals had died. The death of *C. elegans* was defined as a state of unresponsiveness upon stimulation using a platinum wire tip. *C. elegans* was cultured at identical growth stages in an NGM plate containing COM ethyl acetate fractions to determine resistance against thermal stress. On the fourth day, 25 individuals of *C. elegans* were transferred to a fresh NGM plate. The hourly survival rate was measured during culture at 36°C, a high temperature condition. Death was defined as described above (Horikawa and Sakamoto, 2009).

Expression of SOD-3::GFP and accumulation of aging pigments or HSP

Culture media with different concentrations of COM samples (250 and 500 μg/mL) were used in the culture of *C. elegans* at identical growth stages to examine the changes of the expression levels of GFP in CF1553 and SOD-3::GFP and of HSP-16.2 in CL2070. The expression level of SOD-3::GFP was assessed as follows: Surface impurities were removed from *C. elegans* on the third day of adulthood using the M9 buffer. After anesthetization with 4% sodium azide, GFP expression was observed under a fluorescence microscope. The expression level of HSP-16.2 was assessed as follows: After applying thermal stress at 36°C to CL2070 for 2 h followed by a 4-h recovery period at 20°C, HSP-16.2 expression was observed under a fluorescence microscope. Microscopic images were taken for a quantitative analysis of the expression intensity using the ImageJ software (National Institutes of Health) (Liu et al., 2013; Qi et al., 2021).

Evaluation of longevity effects

To determine the effects of COM ethyl acetate fractions on the longevity of *C. elegans*, the eggs of *C. elegans* at identical growth stages were isolated from the NGM plate and then transferred to a plate containing the COM extract and fractions for culture. To ensure accurate monitoring of survival and prevent contamination of the NGM plate, a fresh NGM plate was used for culture at 1-day intervals while the survival of *C. elegans* was monitored. The death of *C. elegans* was defined as the lack of response upon careful stimulation using a platinum wire tip (Lithgow et al., 1995).

Statistical analysis

All variables are expressed as the mean±standard error. Student's *t*-test was used for comparisons between groups. One-way analysis of variance followed by the Tukey-Kramer test was used to test the statistical significance of the mean difference between the control and treatment groups. The log-rank test was used to analyze the survival rate of *C. elegans*. The level of significance was set at $P<0.05$, $P<0.01$, and $P<0.001$.

RESULTS AND DISCUSSION

Analysis of polyphenol and flavonoid contents

Polyphenols and flavonoids significantly contribute to antioxidant activity and exhibit antiaging and whitening (Jung et al., 2020), anti-inflammatory (Heim et al., 2002), and anticancer effects (Williams et al., 2004). The polyphenol and flavonoid contents in the COM extract and fractions were as follows: The highest polyphenol content was found in the ethyl acetate fraction (483.48±0.09 μg TAE/mL), followed by the methylene chloride fraction (387.68±0.16 μg TAE/mL), *n*-butanol fraction (278.35±0.05 μg TAE/mL), *n*-hexane fraction (242.19±0.05 μg TAE/mL), and ethanol extract (163.16±0.08 μg TAE/mL). The highest flavonoid content was found in the ethyl acetate fraction (1,844.00±0.05 μg QE/mL), followed by the methylene chloride fraction (594.26±0.04 μg QE/mL), *n*-hexane fraction (488.62±0.02 μg QE/mL), *n*-butanol fraction (474.17±0.23 μg QE/mL), and ethanol extract (283.94±0.30 μg QE/mL) (Table 1). Lee et al. (2021) reported the polyphenol and flavonoid contents (372.77±1.88 μg TAE/mL and 283.48±14.07 μg CE/mL, respectively) in COM ethyl acetate fractions, which supported previous studies reporting that the antioxidant activity tended to increase as the polyphenol and flavo-

Table 1. Total polyphenol contents of *Cnidium officinale* Makino extract and fraction

Extract and fraction	Total polyphenol (μg TAE/mL)[1)]	Total flavonoids (μg QE/mL)[2)]
Ethanol extract	163.16±0.08[d]	283.94±0.30[c]
n-hexane fraction	242.19±0.05[c]	488.62±0.01[b]
Methylene chloride fraction	387.68±0.16[b]	594.26±0.04[b]
Ethyl acetate fraction	483.48±0.09[a]	1,844.00±0.05[a]
n-butanol fraction	278.35±0.05[c]	474.17±0.23[b]

Values are presented as mean±standard error.
Different letters (a-d) represent significant differences at $P<0.05$, as determined by Duncan's multiple range test.
[1)]Total polyphenol content analyzed as tannic acid equivalent (TAE) μg/mL of extract and fraction.
[2)]Total flavonoid content analyzed as quercetin equivalent (QE) μg/mL of extract and fraction.

noid contents increased (Imai et al., 1994; Halliwell et al., 1995). The ethyl acetate fraction had the highest polyphenol and flavonoid contents probably because of the midlevel polarity of phenolic compounds in plants.

DPPH and ABTS radical scavenging activities

The method of estimating the DPPH radical scavenging activity involves evaluating the color change that occurs upon the response of the DPPH radical to the antioxidant (Dudonné et al., 2009). The DPPH radical scavenging activities in the COM extract and fractions were as follows: ethanol extract (IC_{50} value, 241.5 μg/mL), *n*-hexane fraction (IC_{50} value, 155.7 μg/mL), methylene chloride fraction (IC_{50} value, 162.9 μg/mL), ethyl acetate fraction (IC_{50} value, 69.1 μg/mL), and *n*-butanol fraction (IC_{50} value, 312.5 μg/mL). This finding indicated that the scavenging activity in the COM ethyl acetate fraction was the most pronounced and increased in a concentration-dependent manner (Fig. 1A). The method of estimating the ABTS radical scavenging activity involves observing the discoloration upon the removal of the radical generated in the reaction between ABTS and potassium persulfate (Re et al., 1999). The ABTS radical scavenging activities in the COM extract and fractions were as follows: ethanol extract (IC_{50} value, 69.7 μg/mL), *n*-hexane fraction (IC_{50} value, 358.6 μg/mL), methylene chloride fraction (IC_{50} value, 87.2 μg/mL), ethyl acetate fraction (IC_{50} value, 17.6 μg/mL), and *n*-butanol fraction (IC_{50} value, 42.1 μg/mL). This finding indicated that the scavenging activity in the ethyl acetate fraction exceeded that of vitamin C (IC_{50} value, 20.4 μg/mL) (Fig. 1B). Ku et al. (2020) reported that the DPPH radical scavenging activity was high (65%) in the presence of COM dissolved in distilled water (500 μg/mL). Based on the DPPH and ABTS radical scavenging activities at IC_{50} 6.79 μg/mL and IC_{50} 7.33 μg/mL, respectively, Jeong et al. (2009a) reported that the antioxidant activity in COM extracts obtained using a steam distillation apparatus (80°C, 4 h) was high. Furthermore, Lee et al. (2021) reported that the COM ethyl acetate fraction obtained through extraction using 70% methanol exhibited outstanding DPPH and ABTS radical scavenging activities. Lee et al. (2002) reported that terpenes were among the main components of COM. Li and Liu (2009) found that terpene-based constituents have high antioxidant activity. The results of this study and previous studies suggest that the COM ethyl acetate fraction has excellent radical scavenging activities, which are partly attributed to terpene compounds and polyphenol and flavonoid contents.

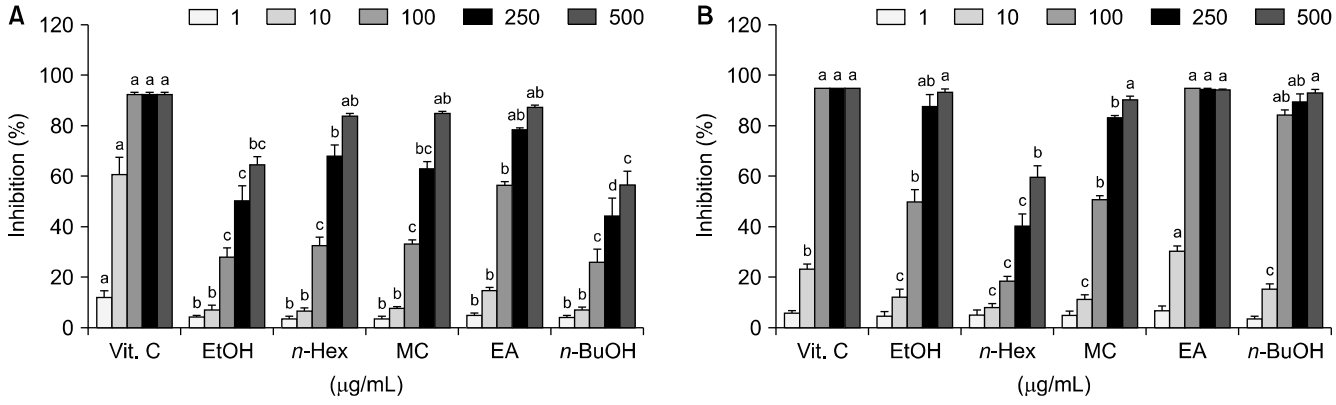

Fig. 1. (A) DPPH radical scavenging effects of the ethanol extract, and its fractions from the *Cnidium officinale* Makino. (B) ABTS radical scavenging effects of the ethanol extract, and its fractions from the *Cnidium officinale* Makino. Different letters (a-d) represent significant differences at $P<0.05$, as determined by Duncan's multiple range test. DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; Vit. C, vitamin C; EtOH, ethanol; *n*-Hex, *n*-hexane; MC, methylene chloride; EA, ethyl acetate; *n*-BuOH, *n*-butanol.

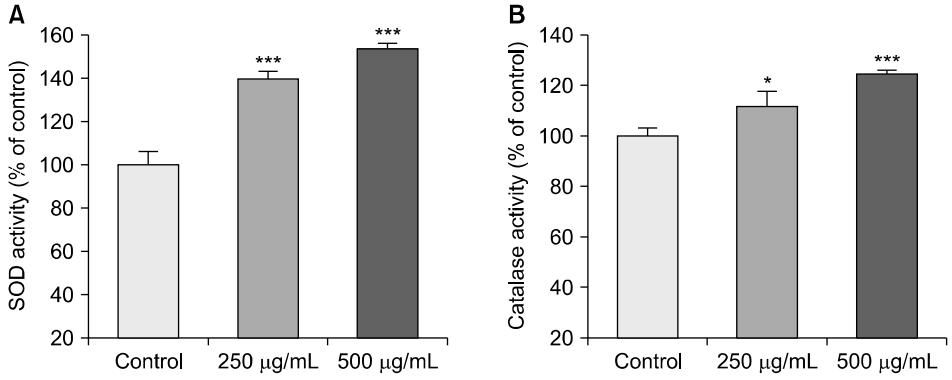

Fig. 2. Effects of the ethyl acetate fraction of *Cnidium officinale* Makino on the antioxidant enzyme activity of wild-type N2 nematode. (A) SOD activity as a percentage of superoxide scavenged per control. (B) Average catalase activity of each group, calculated from the residual H_2O_2 concentration (determined spectrophotometrically). Differences compared with the control group were considered significant at $^{*}P<0.05$ and $^{***}P<0.001$.

Activities of antioxidant enzymes in *C. elegans*

SOD is the first enzyme to respond against ROS-induced oxidative damage (Halliwell and Gutteridge, 1989). Previous studies reported that SOD reduces the effects of ROS generated in ischemia and reperfusion (Feller et al., 1989) and prevents lipid peroxidation during an inflammatory reaction (Zhang et al., 1994). In the present study, the SOD activity in the body of *C. elegans* was measured using superoxide anions produced during an enzymatic reaction of xanthine oxidase with xanthine as the substrate. The SOD activity in *C. elegans* treated with the COM ethyl acetate fraction was 39.7% in the 250 μg/mL group and 53.7% in the 500 μg/mL group (Fig. 2), indicating increased activity compared with that of the control group. Catalase helps defend against oxidative damage by removing the reduced H_2O_2 created by SOD (Halliwell and Gutteridge, 1990). The catalase activity in *C. elegans* treated with the COM ethyl acetate fraction was 11.8% in the 250 μg/mL group and 24.5% in the 500 μg/mL group, indicating increased activity compared with that of the control group (Fig. 2). In the study of Jeong et al. (2019), the SOD-like activity for the COM extract was examined before and after fermentation using *Lactobacillus plantarum* BHN-LAB 33; the activity increased in a concentration-dependent manner for COM samples before and after fermentation. Ramalingam and Park (2010) reported that the SOD activity in COM extracts obtained using methanol exceeded that in ascorbic acid. Heo and Ha (2011) also reported that the SOD and catalase activities in mice fed a high-fat diet increased by 72.51% and 51.69% with the administration of COM ethanol extract, respectively, compared with those in mice without COM administration. The results of this study and previous studies collectively suggest that the COM ethyl acetate fraction could increase the SOD and catalase activities and help protect against ROS-induced oxidative damage.

Analysis of ROS in *C. elegans*

The deacetylation of H_2DCF-DA to non-fluorescent DCFH because of oxidative hydrolysis and the subsequent reaction between DCFH and ROS that produces fluorescent DCF allow the changes in intracellular ROS to be readily examined (Rosenkranz et al., 1992; Rota et al., 1999). In the present study, the fluorescence produced by the reaction between H_2DCF-DA and ROS in the body of *C. elegans* was evaluated. Compared with that in the control group, the level of reduced fluorescence in the COM ethyl acetate fraction group was 7.4% in the 250 μg/mL group and 8.5% in the 500 μg/mL group up to 120 min and 7.1% in the 250 μg/mL group and 8.7% in the 500 μg/mL group at 120 min, suggesting inhibitory effects on ROS

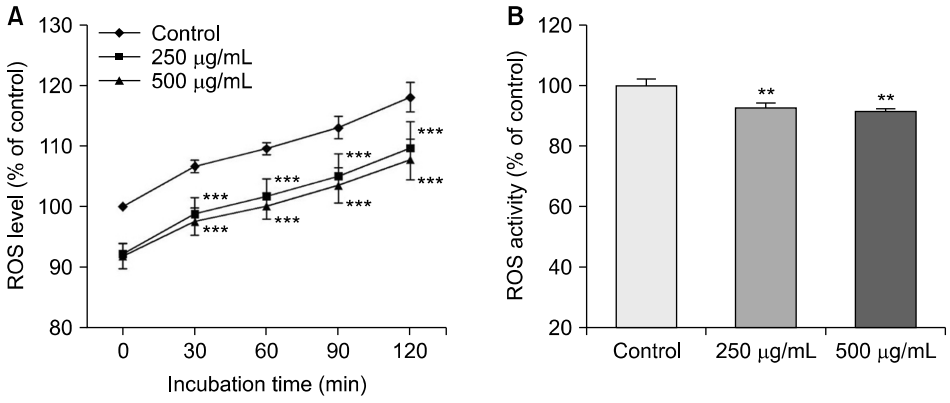

Fig. 3. Effects of the ethyl acetate fraction of *Cnidium officinale* Makino on intracellular ROS accumulation of wild-type N2 nematodes. (A) Intracellular ROS accumulation spectrophotometrically quantified at an excitation wavelength of 485 nm and emission wavelength of 535 nm, recorded every 30 min for 120 min. (B) Average percentages of intracellular ROS levels. Differences compared with the control group were considered significant at $^{**}P<0.01$ and $^{***}P<0.001$.

generation (Fig. 3). In the study of Jeong et al. (2009b), HFF-1 cells treated with H_2O_2 showed significantly reduced ROS generation upon treatment with the COM methanol extract. ROS such as superoxide anion, singlet oxygen, and peroxide cause oxidative damage in cells, which may lead to diseases (Sreedhar et al., 2020). The COM ethyl acetate fraction may help prevent diseases by suppressing ROS production.

Evaluation of oxidative and thermal stress resistance

Juglone is a natural toxin produced by walnut trees (genus *Juglans*) (Paulsen and Ljungman, 2005). It can induce oxidative stress as semiquinone radicals are formed to reduce oxygen to peroxides (Willis, 2000). It can also induce cell membrane damage, necrosis, and apoptosis (Aithal et al., 2009). In the present study, *C. elegans* was exposed to juglone-induced oxidative stress, and the resistance against oxidative stress was examined on the basis of the hourly survival rate. The survival of *C. elegans* treated with the COM ethyl acetate fraction was as follows: The mean and maximal survival hours were 17.1±0.7 and 26 in the control group and 21.0±0.6 and 28 in the 250 μg/mL group, respectively, indicating a 23.0% increase in survival rate (P<0.001). Meanwhile, the corresponding values were 23.6±0.7 and 32 in the 500 μg/mL group, respectively, indicating a 38.1% increase in survival rate (P<0.001) (Fig. 4, Table 2). Oxidative stress may affect longevity, which can be increased using pharmacological treatment (Melov et al., 2000). Thermal stress induces intestinal dysfunction, which may increase mortality risk and induce other negative effects on physiological function and health (Lara and Rostagno, 2013). The life cycle of *C. elegans* is closely associated with

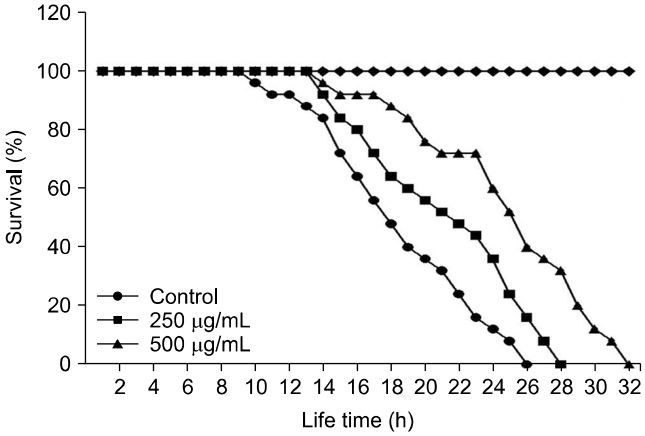

Fig. 4. Effects of the ethyl acetate fraction of *Cnidium officinale* Makino on the stress tolerance of wild-type N2 nematodes. For oxidative stress assays, the worms were transferred to a 96-well plate containing 1 mM juglone liquid culture, and then their viability was scored. The statistical difference between the curves was analyzed using the log-rank test.

Table 2. Effects of ethyl acetate fraction from *Cnidium officinale* Makino on the oxidative stress tolerance of *Caenorhabditis elegans*

Stress condition	Treatment	Mean lifespan (h)	Maximum lifespan (h)	Change in mean lifespan (%)	Log-rank test
1 mM juglone	Control	17.1±0.7	26	-	-
	250 μg/mL	21.0±0.6	28	23.0	P<0.001
	500 μg/mL	23.6±0.7	32	38.1	P<0.001

The mean lifespan is presented as the mean±standard error of the mean of three independent experiments. The statistical significance of the difference between survival curves was determined by log-rank test using the Kaplan-Meier survival analysis. Differences compared with the control were considered significant at P<0.001.
-, not available.

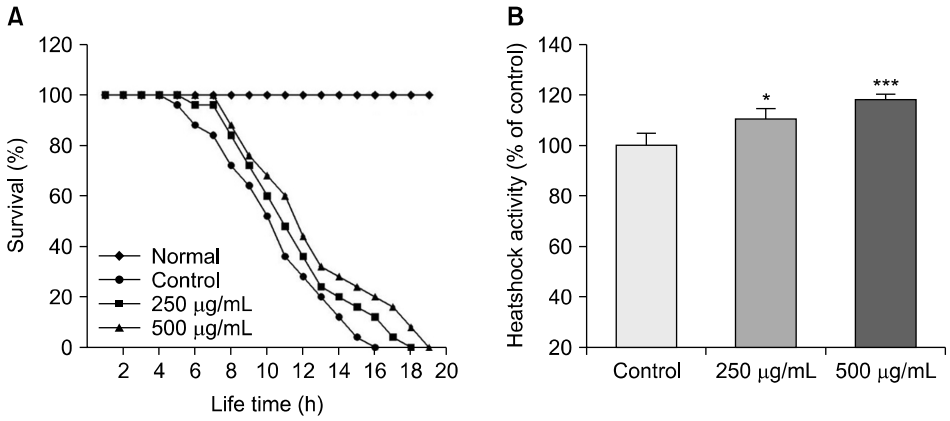

Fig. 5. Effects of the ethyl acetate-soluble fraction of *Cnidium officinale* Makino on the thermal stress tolerance of wild-type N2 nematodes. To assess thermal tolerance, the worms were incubated at 36°C, and then their viability was scored. The statistical difference between the curves was analyzed using the log-rank test. All experiments were performed in triplicates. Differences compared with the control group were considered significant at *P<0.05 and ***P<0.001.

stress (Lithgow et al., 1995). The optimal growth temperature of *C. elegans* is 20°C. Thus, it does not lay eggs at temperatures above 35°C, at which it ceases to migrate and dies within a short time (Lithgow and Walker, 2002). The mean and maximum survival times of *C. elegans* treated with COM ethyl acetate fraction under heat stress were 10.5±0.3 and 16 in the control group and 11.6±0.4 and 18 in the 250 μg/mL group, respectively, indicating a 10.4% increase in survival rate (P<0.05). The corresponding values in the 500 μg/mL group were 12.4±0.4 and 19, respectively, indicating a 18.3% increase in the survival rate (P<0.001; Fig. 5, Table 3). These values suggest that the use of COM ethyl acetate fraction in *C. elegans* may increase resistance to oxidative and thermal stress, thereby increasing its longevity.

Expression of SOD-3::GFP and HSP-16.2::GFP

SOD-3::GFP is an antioxidant enzyme induced in response to oxidative stress; it is found in CF1553, a transformed *C. elegans* strain (Darr and Fridovich, 1995; Tawe et al., 1998). Analysis of the expression level of SOD-3::GFP in CF1553 showed that the expression level was increased by 4.0% in the 250 μg/mL group and 9.6% in the 500 μg/mL group after treatment with the COM ethyl acetate fraction compared with that in the control group (Fig. 6A and 6C). Studies on the aging process have examined HSP-16.2::GFP, which may be a predictor of *C. elegans* longevity (Schaffitzel and Hertweck, 2006; Swindell, 2009). After treatment with the COM ethyl acetate fraction, the expression level of HSP-16.2 in CL2070 increased by 7.9% in the 250 μg/mL group and 13.2% in the 500 μg/mL group compared with that in the control group (Fig. 6B and 6D). This finding suggests that the COM ethyl acetate fraction may have increased oxidative stress resistance by increasing the expression level of SOD-3::GFP in CF1553 and increased thermal stress resistance by increasing the expression level of HSP-16.2::GFP in CL2070.

Evaluation of longevity effects

The mean and maximal survival days of *C. elegans* treated with COM were 10.4±0.3 and 16 in the control group, respectively. Meanwhile, the mean and maximal survival days in the ethanol extract group were 11.5±0.3 and 17, respectively, indicating a 10.6% increase in longevity compared with that in the control group (P<0.01). The corresponding values in the groups treated with the *n*-hexane fraction, methylene chloride fraction, ethyl acetate fraction, and *n*-butanol fraction were 11.2±0.3 and 17, 11.9±0.3 and 19, 12.7±0.3 and 19, and 11.3±0.2 and

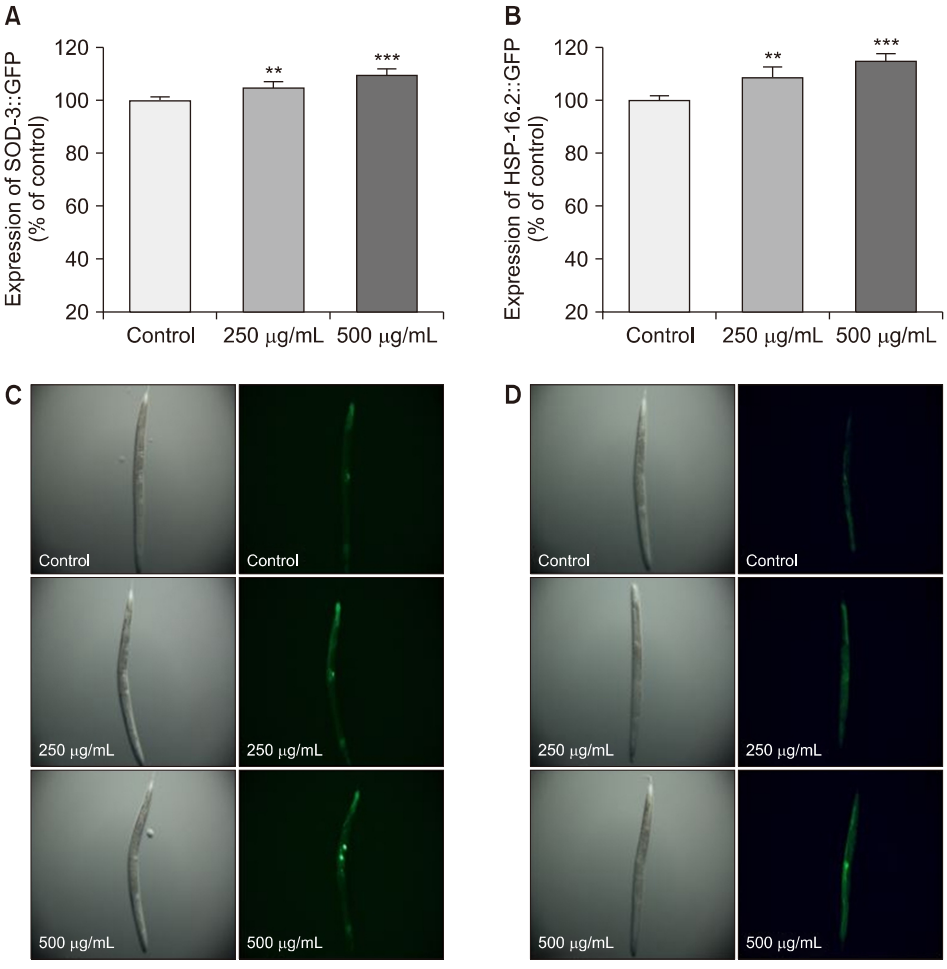

Fig. 6. Effects of the ethyl acetate fraction of *Cnidium officinale* Makino on the expression levels of SOD-3 and HSP-16.2 as determined using transgenic nematodes. The mean GFP intensity of CF1553 (A) and CL2070 (B) mutants is presented as the mean±standard error of the mean using 100 organisms per experiment. The GFP intensity was quantified using ImageJ software by determining the average pixel intensity. Images of SOD-3::GFP (C) and HSP-16.2::GFP (D) expression taken from the corresponding mutants grown with or without *Cnidium officinale* Makino. Data are presented as the mean±standard deviation of three independent experiments (n=3). Differences compared with the control group were considered significant at $^{**}P$<0.01 and $^{***}P$<0.001.

Table 3. Effects of *Cnidium officinale* Makino fractions on the stress tolerance of *Caenorhabditis elegans*

Stress condition	Fraction	Mean lifespan (h)	Maximum lifespan (h)	Change in mean lifespan (%)	Log-rank test
36°C	Control	10.5±0.3	16	-	-
	250 μg/mL	11.6±0.4	18	10.4	P<0.05
	500 μg/mL	12.4±0.4	19	18.3	P<0.001

The mean lifespan is presented as the mean±standard error of the mean. Changes in the mean lifespan were compared with the control group (%). The statistical significance of the difference between survival curves was determined by log-rank test using the Kaplan-Meier survival analysis. Differences compared with the control were considered significant at P<0.05 and P<0.001. -, not available.

18, indicating increases in longevity of 8.1% (P<0.05), 15.2% (P<0.001), 22.7% (P<0.001), and 9.3% (P<0.05), respectively, relative to those in the control group (Fig. 7, Table 4). Thus, the COM ethyl acetate fraction may increase the activities of antioxidant enzymes and suppress ROS accumulation in *C. elegans*, thereby increasing longevity. However, these findings should be validated in future studies.

In conclusion, the COM ethanol extract was fractionated according to polarity. The polyphenol and flavonoid contents were analyzed for the obtained *n*-hexane, methylene chloride, ethyl acetate, and *n*-butanol fractions, and the DPPH and ABTS radical scavenging activities were measured. The ethyl acetate fraction exhibited high polyphenol and flavonoid contents and radical scavenging activities. Thus, it was used to evaluate antioxidant and longevity effects using *C. elegans*. The COM ethyl acetate fraction increased the activities of antioxidant enzymes (SOD and catalase) in a concentration-dependent manner and markedly decreased ROS generation in *C. elegans*.

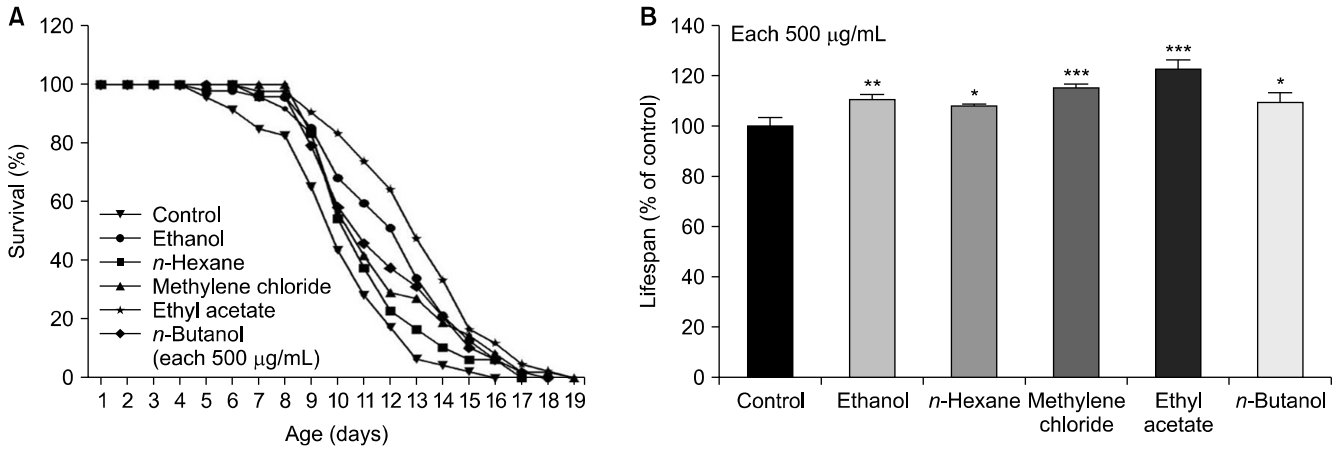

Fig. 7. Effects of *Cnidium officinale* Makino on the lifespan of *Caenorhabditis elegans*. (A) Mortality of each group, which was determined by counting the number of dead organisms daily. (B) Mean lifespan of N2 nematodes, which was calculated from the survival curves. The statistical difference between the curves was analyzed using the log-rank test. The error bars represent the standard error of the mean. Differences compared with the control group were considered significant at *P<0.05, **P<0.01, and ***P<0.001.

Table 4. Effects of *Cnidium officinale* Makino fractions on the lifespan of wild-type N2

Fraction	Mean lifespan (d)	Maximum lifespan (d)	Change in mean lifespan (%)	Log-rank test
Control	10.4±0.3	16	-	-
Ethanol	11.5±0.3	17	10.6	P<0.01
n-Hexane	11.2±0.3	17	8.1	P<0.05
Methylene chloride	11.9±0.3	19	15.2	P<0.001
Ethyl acetate	12.7±0.3	19	22.7	P<0.001
n-Butanol	11.3±0.2	18	9.3	P<0.05

The mean lifespan is presented as the mean±standard error of the mean. Changes in the mean lifespan were compared with the control group (%). The statistical significance of the difference between survival curves was determined by log-rank test using the Kaplan-Meier survival analysis. Differences compared with the control group were considered significant at P<0.05, P<0.01, and P<0.001.
-, not available.

Furthermore, when the survival rate of *C. elegans* was examined after juglone-induced oxidative stress, the survival rate of the COM ethyl acetate fraction group was higher than that of the control group, especially in the group treated with a higher concentration. The survival rate in high temperatures also increased, suggesting increased thermal stress resistance. Moreover, the COM ethyl acetate fraction increased the longevity of *C. elegans*. In the model of GFP-fused transgenic strain of *C. elegans* (CF1553), the expression level of SOD-3::GFP, which reflects oxidative stress resistance, was increased in the COM ethyl acetate fraction group, and the accumulation of lipofuscin in *C. elegans* was simultaneously decreased. Overall, these findings suggest that the COM ethyl acetate fraction may contribute to the development of natural antioxidants.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Concept and design: all authors. Analysis and interpretation: all authors. Data collection: HK, JSM. Writing the article: HK, JSM. Critical revision of the article: JHK, SHO. Final approval of the article: all authors. Statistical analysis: HK, JSM. Overall responsibility: JHK.

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