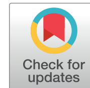

BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 26(6), 568-575 (2018)

Longevity and Stress Resistant Property of 6-Gingerol from *Zingiber officinale* Roscoe in *Caenorhabditis elegans*

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Abstract

In order to discover lifespan-extending compounds made from natural resources, activity-guided fractionation of *Zingiber officinale* Roscoe (Zingiberaceae) ethanol extract was performed using the *Caenorhabditis elegans* (*C. elegans*) model system. The compound 6-gingerol was isolated from the most active ethyl acetate soluble fraction, and showed potent longevity-promoting activity. It also elevated the survival rate of worms against stressful environment including thermal, osmotic, and oxidative conditions. Additionally, 6-gingerol elevated the antioxidant enzyme activities of *C. elegans*, and showed a dose-depend reduction of intracellular reactive oxygen species (ROS) accumulation in worms. Further studies demonstrated that the increased stress tolerance of 6-gingerol-mediated worms could result from the promotion of stress resistance proteins such as heat shock protein (HSP-16.2) and superoxide dismutase (SOD-3). The lipofuscin levels in 6-gingerol treated intestinal worms were decreased in comparison to the control group. No significant 6-gingerol-related changes, including growth, food intake, reproduction, and movement were noted. These results suggest that 6-gingerol exerted longevity-promoting activities independently of these factors and could extend the human lifespan.

Key Words: *Zingiber officinale* Roscoe, 6-Gingerol, *Caenorhabditis elegans*, Longevity, Stress tolerance

INTRODUCTION

Free radicals including reactive oxygen species (ROS) and nitrogen species are highly reactive by-products of many human cell oxidative biochemical reactions. Naturally occurring antioxidants such as catalase, glutathione, and superoxide dismutase normally clear free radicals from the body, however, free radicals accumulate in the body as organisms age (Pelicano *et al*., 2004; Zhao *et al*., 2008; Pinazo-Durán *et al*., 2014; Singh *et al*., 2015). Oxidative damage caused by free radicals can play a key role in the pathogenesis of neurodegenerative disease, cardiovascular disease, and cancer (Na and Bae, 2011; Singh *et al*., 2015). Aging may be caused by various processes, of which the free radical theory of aging appears to be the most important (Sayed, 2011; Baranov and Baranova, 2017).

Aging is often the pathological result of free radicals reactions; these processes often lead to gene mutations and cell death, both of which are related to the overproduction of ROS (Berlett and Stadtman, 1997; Mekheimer *et al*., 2012). Over time, accumulation of lipid, protein, and DNA damage due to ROS induces a loss of normal physiological function, resulting in a shortened lifespan (Beckman and Ames, 1998). Thus, humans need antioxidants that can inhibit toxic ROS by scavenging free radicals (Beckman and Ames, 1998; Na and Bae, 2011). Natural products have played a role in treating and preventing various diseases (Wang *et al*., 2016). Polyphenolic compounds are especially preferred as natural agents for the treatment of diseases because of their antioxidant ability and lower incidence of side effects (Rastogi *et al*., 2014).

In searching for lifespan-extending compounds, we used an ethanol extract of *Zingiber officinale* Roscoe (*Z. officinale*, Zingiberaceae) to measure the lifespan in *Caenorhabditis el-*

Open Access https://doi.org/10.4062/biomolther.2017.215

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Received Oct 20, 2017 **Revised** Dec 22, 2017 **Accepted** Jan 19, 2018
Published Online Mar 14, 2018

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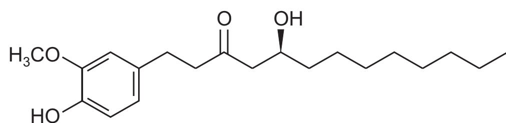

Fig. 1. Structure of 6-gingerol.

egans (*C. elegans*). Ethanol extract from *Z. officinale*, known commonly as a medicinal herb, has been potentially indicate longevity. *Z. officinale* is a spice and one of the most famous dietary plants in the world (Kim *et al*., 2005; Zick *et al*., 2008; Park *et al*., 2016). As an herbal medicine, *Z. officinale* is mainly used as a therapy for diarrhea, digestive disorders, gastritis, nausea and vomiting (Zick *et al*., 2008). Several recent studies have demonstrated anti-inflammatory, antioxidative, antimicrobial, and anti-carcinogenic properties of *Z. officinale* (Weng *et al*., 2010; Al-Nahain *et al*., 2014; Park *et al*., 2016).

Subsequent activity-guided chromatography of *Z. officinale* ethanol extract led to the isolation of 6-gingerol (Fig. 1). In order to study the possible lifespan-extending and stress tolerance activities of this compound, a *C. elegans* model system was adopted. The antioxidant ability of 6-gingerol was analyzed by measuring antioxidant enzyme activities and intracellular ROS level of worms. In order to validate the possible mechanism of 6-gingerol, our study evaluated the expression of oxidative stress resistance proteins (SOD-3), heat-shock stress resistance proteins (HSP-16.2), accumulation of lipofuscin, and aging-related factors.

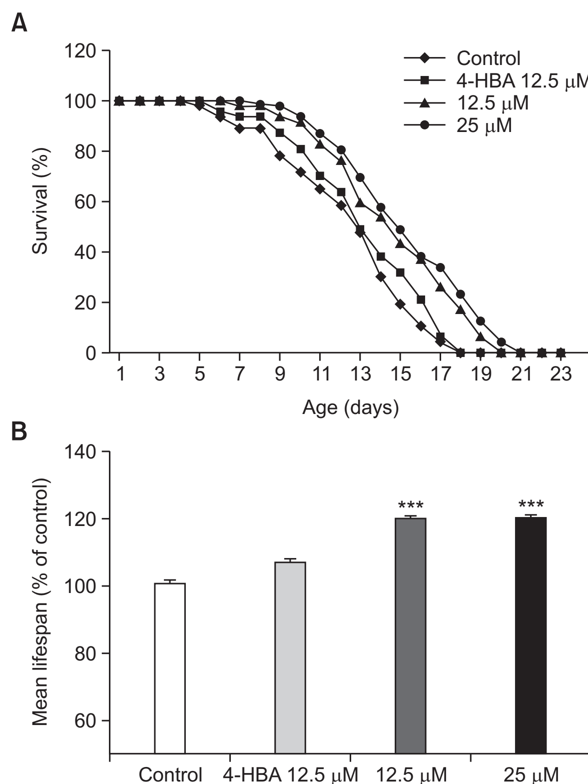

Fig. 2. Effects of 6-gingerol on the lifespan of wild-type N2 nematodes. Worms were grown on an NGM agar plate at 20°C in the absence or presence of 6-gingerol. The number of worms used per each lifespan assay experiment was 40-43 and three independent experiments were repeated (N=3). (A) The mortality of each group was determined by daily counting of the live and dead animals. (B) The mean lifespan of the worms was calculated from the survival curves. Statistical difference between the curves was analyzed by log-rank test. Error bars represent the standard error of mean (SEM). Differences compared to the control were considered significant at $^{***}p<0.001$ by one-way ANOVA. 4-HBA (4-hydroxybenzoic acid): positive control.

MATERIALS AND METHODS

General

NMR spectra were determined on a JEOL JMN-EX 400 spectrometer (Jeol, Tokyo, Japan). HPLC was performed using a JAI-GS310 column (20×500 mm, Jai, Tokyo, Japan). The absorbance was examined using a microplate reader (ELISA, Sunrise, Grödig, Austria). Sephadex LH-20 (GE Healthcare, Uppsala, Sweden), and Kiesel gel 60 (230-400 mesh, Merck, Darmstadt, Germany) were used for column chromatography. TLC was carried out on Merck precoated silica gel F_{254} plates. Spots were detected under UV and by spraying with 10% H_2SO_4 in ethanol followed by heat treatment. Selected peptone and yeast extracts were obtained from BD bioscience (Sparks, USA). Agar, catalase, juglone, 2′,7′-dichlorodihydrofluoroscein diacetate, xanthine, xanthine oxidase, and nitroblue tetrazolium were purchased from Sigma (St. Louis, MO, USA).

Plant materials, extraction and isolation

The dried *Z. officinale* was purchased from an oriental drug store, Bohwadang (Jeonju, Korea), and identified by one of the authors (Kim, D. K.). A voucher specimen was deposited in the herbarium of the College of Pharmacy, Woosuk University (WSU-15-012). The air dried plant material (600 g) was extracted four times with ethanol at 50°C, and then the extracts were combined and evaporated *in vacuo* at 50°C. The extract (95 g) was successively fractionated as methylene chloride (11.2 g), ethyl acetate (30.5 g), *n*-butanol (25.9 g) and H_2O soluble fractions. Each fraction was tested for its lifespan extending effect using the *C. elegans* model system. The ethyl acetate fraction showed the most potent longevity property (data are not shown). Sephadex LH-20 column chromatography of ethyl acetate soluble fraction gave six subfractions (EA1-EA6) using methanol as a mobile phase. Subfraction EA5 (160 mg) was purified by JAI-GS310 column (MeOH) to give compound 1 (19 mg).

6-Gingerol (1)

^{1}H-NMR (400 MHz, CD_3OD) δ : 0.84 (3H, t, H-10’), 1.20-1.31 (8H, m, -$(CH_2)_2$ H-6’~9’), 2.43 (2H, dd, *J*=8.3, 2.0 Hz, H-4’), 2.69 (4H, s, H-1’, 2’), 3.74 (3H, s, OCH_3), 3.87 (1H, m, H-5’), 6.52 (1H, dd, *J*=8.3, 2.0 Hz, H-6), 6.59 (1H , d, *J*=8.3 Hz, H-5), 6.68 (1H, d, *J*=2.0 Hz, H-2). ^{13}C-NMR (100 MHz, CD_3OD) δ : 134.3 (C-1), 113.2 (C-2), 149.0 (C-3), 145.8 (C-4), 116.3 (C-5), 121.8 (C-6), 30.5 (C-1’), 46.6 (C-2’), 212.0 (C-3’), 51.5 (C-4’), 69.1 (C-5’), 38.6 (C-6’), 33.1 (C-7’), 26.6 (C-8’), 23.9 (C-9’), 14.8 (C-10’), 56.6 (C-OMe). Structure characterization of 6-gingerol was carried out by interpretation of its spectral data compared with data reported in the literature (Shoji *et al*.,1982).

Table 1. Effects of 6-gingerol on the lifespan of *C. elegans*

Treatment (μM)	Mean Lifespan (day)	Maximum lifespan (day)	Change in mean lifespan (%)	Log-rank test
Control	12.5 ± 0.4	18	-	-
4-HBA 12.5[a]	13.3 ± 0.4	18	6.0	-
12.5	15.0 ± 0.4	20	20.0	p<0.001***
25	15.1 ± 0.5	21	20.9	p<0.001***

[a]positive control: 4-hydroxybenzoic acid. Mean lifespan presented as mean ± SEM data. Change in mean lifespan compared with control group (%). Statistical significance of the difference between survival curves was determined by log-rank test using the Kaplan-Meier survival analysis. Differences compared to the control were considered significant at ***p<0.001.

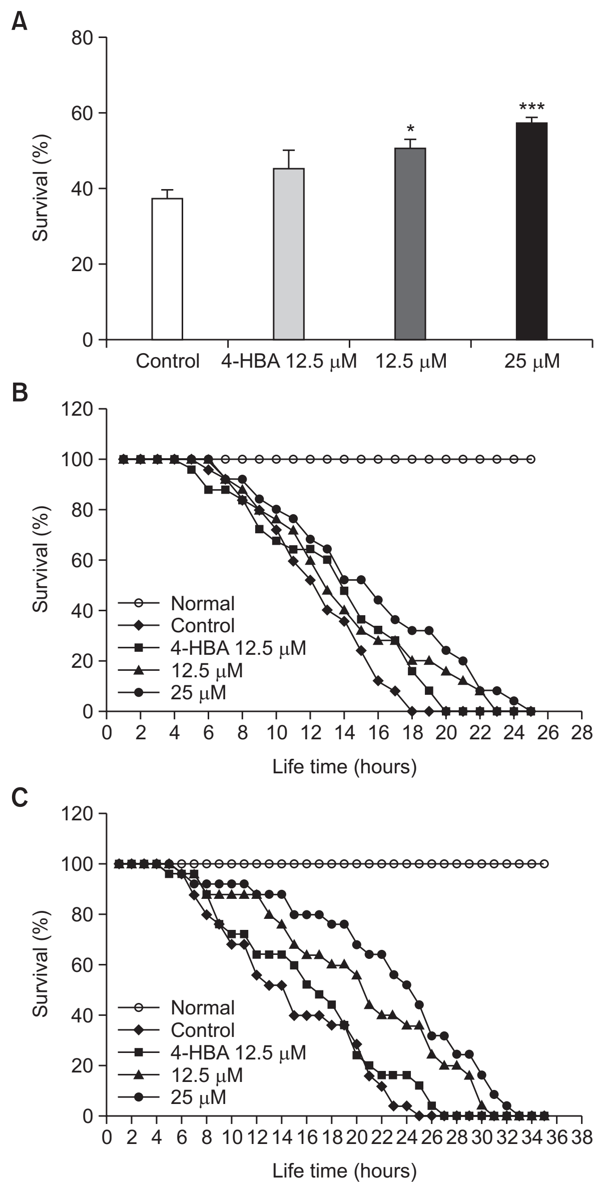

Fig. 3. Effects of 6-gingerol on the stress tolerance of wild-type N2 nematodes. (A) Resistance to osmotic stress was measured by placing worms on an NGM agar plate and the survival rate was calculated after 12 h incubation. (B) To assess thermal tolerance, worms were incubated at 36°C, and then their viability was scored. (C) For the oxidative stress assays, worms were transferred to 96-well plates containing 1 mM of juglone liquid culture, and then their viability was scored. Statistical differences between the curves were analyzed by log-rank test. All experiments were done in triplicates. Differences compared to the control were considered significant at *p<0.05 and ***p<0.001. 4-HBA (4-hydroxybenzoic acid): positive control.

C. elegans strains and maintenance

Bristol N2 and *E. coli* OP50 were kindly provided by prof. Dong Seok Cha (Woosuk University, Korea). The worms were grown at 20°C on nematode growth medium (NGM) agar plate with *E. coli* as described previously (Brenner 1974). To prepare plates supplemented with 6-gingerol, the stock solution in DMSO was inserted into autoclaved NGM plates at 50°C. A final DMSO concentration was 0.1% (v/v).

Lifespan assay

The lifespan assays were performed using wild-type *C. elegans* at least 3 independent times at 20°C. To obtain age-synchronized worms, eggs were transferred to an NGM plate in the absence or presence of sample after embryo isolation. To test the worms whether they were dead or alive, a platinum wire was used. Worms were considered dead when they did not respond to prodding with the tip (Lithgow *et al*., 1995). The worms were transferred to a fresh NGM plate every 2 days.

Assessment of stress resistance

The age-synchronized *C. elegans* were bred on NGM agar plates with or without various concentrations of 6-gingerol. For the heat shock tolerance assay, on the 4th day of adulthood, worms were transferred to a fresh plate and then incubated at 36°C. The survival rate was scored over 25 h as previously described (Lee *et al*., 2005). Oxidative stress tolerance was measured as described previously with minor modification (Mekheimer *et al*., 2012). In brief, on the 7th day of adulthood, worms were transferred to a 96-well plate containing 1 mM of juglone, and then survivals were recorded over 35 h. To observe the osmotic effect, the 5th days of worms were transferred in an NGM agar plate containing 500 mM NaCl at 20°C, and determined the survival rates against the osmotic stress following 12 h (Pujol *et al*., 2008).

Analysis of intracellular ROS

Intracellular ROS in *C. elegans* was analyzed using molecular probe 2′,7′-dichlorodihydrofluoroscein diacetate (H_2DCF-DA). An equal number of worms was incubated in the absence or presence of 6-gingerol. On the 4th day of adulthood, animals were exposed to 96-well plate containing 50 μM juglone liquid culture for 2 h. Subsequently, five worms were transferred into the wells of a 96-well plate containing 50 μL of M9 buffer. Immediately after addition of 50 μL of 25 μM H_2DCF-DA solution resulting in a final concentration 12.5 μM, basal fluorescence was quantified in a microplate fluorescence reader at excitation 485 nm and emission 535 nm (Seo *et al*., 2015).

Measurement of antioxdant enzyme activities

The worm homogenates were prepared to evaluate enzymatic activity. Briefly, on the 5th day of adulthood, worms were harvested from plate with M9 buffer and washed three times.

Table 2. Effects of 6-gingerol on the stress tolerance of *C. elegans*

Stress condition	Treatment (μM)	Mean lifespan (h)	Maximum lifespan (h)	Change in mean lifespan (%)	Log-rank test
36°C thermal tolerance	Control	12.5 ± 0.6	18	-	-
	4-HBA 12.5[a]	13.5 ± 0.9	20	7.6	-
	12.5	14.2 ± 0.9	23	13.0	$p<0.05$*
	25	15.6 ± 0.1	25	24.8	$p<0.01$**
1 mM juglone	Control	14.9 ± 0.2	25	-	-
	4-HBA 12.5[a]	16.3 ± 0.3	27	9.6	-
	12.5	20.4 ± 0.5	31	36.7	$p<0.01$**
	25	22.9 ± 0.5	33	53.8	$p<0.001$***

[a]positive control: 4-hydroxybenzoic acid. Mean lifespan presented as mean ± SEM data. Change in mean lifespan compared with control group (%). Statistical significance of the difference between survival curves was determined by log-rank test using the Kaplan-Meier survival analysis. Differences compared to the control were considered significant at *$p<0.05$, **$p<0.05$ and ***$p<0.001$.

Then, the collected worms were suspended in homogenization buffer (10 mM Tris-HCl, 150 mM NaCl, 0.1 mM EDTA, pH 7.5) and homogenized on ice. SOD activity was measured spectrophotometrically analyzing the decolorization of formazan using enzymatic reaction between xanthine and xanthine oxidase. The reaction mixture contained 5 μL of worm homogenates and 120 μL of 0.57 mM xanthine, 0.24 mM nitrobluetetrazolium (NBT) in 10 mM phosphate buffer (pH 8.0). After pre-incubation at room temperature for 5 minutes, the reaction was initiated by adding 100 μL of xanthine oxidase (0.05 U/mL) and incubation at 37°C for 20 min. The reaction was stopped by adding 275 μL of 69 mM SDS, and the absorbance at 570 nm was measured. Catalase activity was calculated by spectrophotometry as previously described (Aebi, 1984). Briefly, the prepared homogenates were mixed with the 25 mM H_2O_2 and after 3 min incubation, absorbance was determined at 240 nm. The enzyme activities were expressed as a percentage of the scavenged amount per control.

Fluorescence microscopy and visualization

The age-synchronized transgenic nematodes including CF1553 containing a SOD-3::GFP reporter and CL2070 containing HSP-16.2::GFP reporter were maintained in the presence or absence of 6-gingerol. Prior to microscopy observation, CL2070 mutants were received heat shock at 36°C for 2 h and allowed to recover at 20°C for 4 h. On the 3rd day of adulthood, both transgenic worms were anesthetized with sodium azide (4%) and mounted on 2% agarose pad. The GFP fluorescence of GFP-expressing populations was directly observed under a fluorescence microscope (Olympus, Tokyo, Japan). To determine the protein expression levels, photographs of the transgenic worms were taken and assayed using Image J software (Seo *et al*., 2015). All experiments were done in triplicate.

Lipofuscin accumulation

C. elegans was cultivated from embryo to use the lifespan assay. The worms were anesthetized with sodium azide to determine the intestinal lipofuscin level, and fluorescence photographs were taken using a fluorescence microscope (Olympus) on the 8th day of adulthood. Fluorescence intensity was measured by determining pixel intensity in worm's intestines using Image J software (National Institutes of Health, Bethesda, MD, USA).

Measurement of aging-related factors and locomotion

The age-synchronized N2 worms were bred on NGM agar plates with or without 6-gingerol. The reproduction assay involved raising N2 worms from embryos, as in the lifespan assay. L4 larvae were individually transferred to the fresh plate every day to distinguish the parent from the progeny. The progeny was counted at the L2 or L3 stage. On the 4th and 8th days of adulthood, a single worm was transferred to a fresh plate, then pharynx contraction and body movement of the animal was counted under an inverted microscope for 20 sec. For the growth alteration assay, the worms were photographed, and the body length of each animal was measured by Cellsense dimension (Olympus). Each test was performed at least three times.

Data analysis

The data from the lifespan assay and stress resistance assays were plotted using Kaplan-Meier analysis, and statistical significance was analyzed by log-rank test. The other data are presented as the mean ± standard deviation or standard error of the mean (SEM), as indicated. Statistical significance of differences between the control and treated groups were analyzed by one-way analysis of variance (ANOVA).

RESULTS

Effects of 6-gingerol on the lifespan of *C. elegans*

6-Gingerol was isolated from the ethyl acetate fraction that showed the most potent longevity property among several fractions of the dried *Z. officinale*. The lifespan extension properties of 6-gingerol were performed with wild-type N2 worms. As shown Fig. 2A, 6-gingerol revealed a concentration-dependent effect on longevity. In addition, there was a significant increase (20.0% at 12.5 μM of 6-gingerol, $p<0.001$) in the estimated mean life of 6-gingerol-treated worms compared to control worms (Fig. 2B, Table 1). The mean lifespan was 12.5 ± 0.4 days for control worms, 15.0 ± 0.4 days for the worms fed 12.5 μM 6-gingerol (Table 1).

Effects of 6-gingerol on the stress tolerance of *C. elegans*

The effects of 6-gingerol were determined under osmotic, thermal, and oxidative stress conditions using wild-type N2 worms. In the hypertonic stress assay, 6-gingerol-treated worms exhibited increased resistance to osmotic stress (Fig.

3A). In addition, as can be seen in Fig. 3B, thermal tolerance was improved as a result of 6-gingerol treatment, which consequently increased survival rate. Treatment with 6-gingerol prolonged the mean and maximum lifespan of worms by 13.0% and 24.8% at 12.5 μM (p<0.05) and 25 μM (p<0.01), respectively (Fig. 3B, Table 2). Moreover, the 6-gingerol-treated worms lived longer than the control worms under an oxidative stress condition induced by 1 mM juglone (Fig. 3C, Table 2). The maximum survival time for the control group was 25 h, while for the 6-gingerol-treated group, the time increased to 31 h (36.7%) and 33 h (53.8%) at 12.5 μM and 25 μM, respectively.

Effects of 6-gingerol on the antioxidant enzyme activities and intracellular ROS levels

The mechanism responsible for the increased lifespan and stress resistance of *C. elegans* by 6-gingerol, was investigated by determining the effect of 6-gingerol on the intracellular antioxidant enzyme activity. The superoxide dismutase (SOD) and catalase enzymatic activities were measured spectrophotometrically using prepared worm homogenates. The results revealed that 6-gingerol elevated SOD and catalase activities of worms significantly by 21.8% (p<0.001) and 28.3% (p<0.001) at 25 μM, respectively (Fig. 4A, 4B). The intracellular ROS levels of the 6-gingerol-treated worms were quantified and compared to the untreated control. Fig. 4C shows that 6-gingerol-fed worms decreased ROS production by 18.5% (25 μM, p<0.001), compared to the control.

Effects of 6-gingerol on the SOD-3 and HSP-16.2 expressions in transgenic nematodes

To investigate whether 6-gingerol-mediated increased stress tolerance was due to regulation of stress-response genes, SOD-3 and HSP-16.2 expressions were quantified using transgenic strains including CF1553 and CL2070, respectively. 6-Gingerol-treated CF1553 worms showed significantly higher SOD-3::GFP intensity (17.5% at 25 μM, p<0.01), compared to the untreated control worms (Fig. 5A, 5C). The CL2070 worms containing HSP-16.2::GFP reporter gene were treated heat shock at 36°C for 2 h and then, were recovered at 20°C for 4 h, before the quantifying of fluorescence intensity. The heat shock-induced HSP-16.2::GFP expression level was further enhanced by 25 μM of 6-gingerol approximately 28.5% (p<0.001, Fig. 5B, 5D).

Effects of 6-gingerol on the lipofuscin accumulation

The autofluorescence level of lipofuscin was measured by fluorescence microscope. 6-Gingerol-treated worms showed significant decrease of fluorescence intensity from intestinal lipofuscin by 12.5% at 25 μM, compared to the control (p<0.01, Fig. 6).

Effects of 6-gingerol on the aging-related factors of *C. elegans*

6-Gingerol-induced changes in parameters of aging-related factors, including progeny, pharyngeal pumping, and body length were examined to verify the possible mechanism of 6-gingerol on the lifespan of *C. elegans*. There were no significant statistical changes between 6-gingero-fed worms and control worms on the reproduction rate, food intake, and body length (Fig. 7A, 7B, 7C).

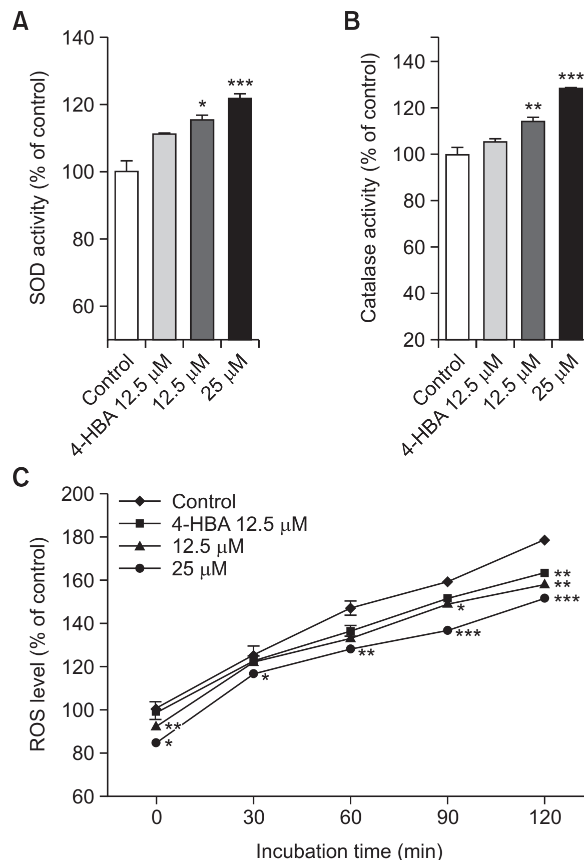

Fig. 4. Effects of 6-gingerol on the stress resistance proteins of wild type N2 nematodes. (A) The enzymatic reaction of xanthine with xanthine oxidase was used to generate $\bullet O_2$- and the SOD activity was estimated spectrophotometrically through formazan formation by NBT reduction. SOD activity was expressed as a percentage of the scavenged amount per control. (B) Catalase activity was calculated from the concentration of residual H_2O_2, as determined by a spectrophotometric method. (C) Intracellular ROS accumulation was quantified spectrometrically at excitation 485 nm and emission 535 nm. Plates were read every 30 min for 2 h. Data are expressed as the mean ± SEM of three independent experiments (N=3). Differences compared to the control were considered significant at *p<0.05, **p<0.01 and ***p<0.001 by one-way ANOVA. 4-HBA (4-hydroxybenzoic acid): positive control.

Effects of 6-gingerol on the locomotory activities of *C. elegans*

The body movement of nematodes was measured to know the effects of 6-gingerol on age-related functional changes in *C. elegans*. As shown Fig. 7D, there were no differences between 6-gingerol-fed worms and control worms on growth rate.

DISCUSSION

The 6-gingerol compound, which is associated with longevity property, was isolated from the ethyl acetate soluble fraction of *Z. officinale*. Several previous studies have reported the anti-bacterial, anti-inflammatory and anti-tumorigenic activities of 6-gingerol (Kim *et al*., 2005; Rastogi *et al*., 2014).

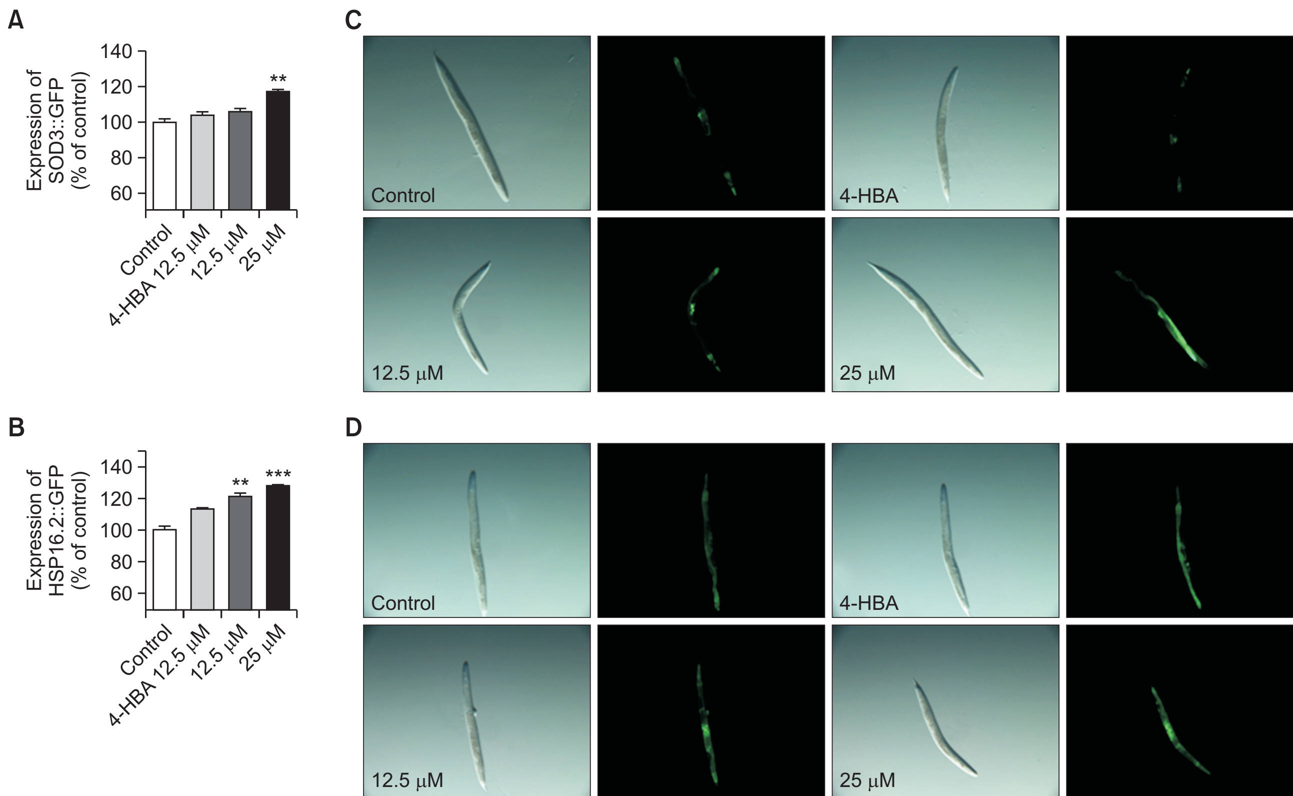

Fig. 5. Effects of 6-gingerol on the expression of SOD-3 and HSP-16.2 were determined using transgenic nematodes. The mean GFP intensity of CF1553 (A) and CL2070 (B) mutants were represented as mean ± SEM of values from 70 to 80 animals per each experiment. The GFP intensity was quantified using Image software by determining average pixel intensity. Images of SOD-3::GFP (C) and HSP-16.2::GFP (D) expressions of CF1553 worms in the presence or absence of 6-gingerol. Data are expressed as the mean ± standard deviation of three independent experiments (N=3). Differences compared to the control were considered significant at **p<0.01 and ***p<0.001 by one-way ANOVA. 4-HBA (4-hydroxybenzoic acid): positive control.

Although 6-gingerol has shown various levels of effective in several studies, its effects on lifespan extension of *C. elegans* remains unknown. In this study, the effect of 6-gingerol on longevity was investigated by a lifespan assay of wild-type N2 nematodes and *C. elegans* under a normal culture condition. The 6-gingerol treatment considerably increased the lifespan of worms in a concentration-dependent manner. Since there is a considerable correlation between increased stress tolerance and longevity, the stress resistance of 6-gingerol-fed worms under several stress conditions was measured (Kenyon, 2010). The 6-gingerol-treated worms exhibited a significant increase in respective survival rates under osmotic and thermal stress conditions as compared to control group worms.

Juglone (5-hydroxy-1,4-naphthoquinone) is an allelochemical produced by a living organism which exerts a detrimental physiological effect on another species (Willis, 2000). The main route of juglone toxicity is the formation of semiquinone radicals, which can reduce oxygen to superoxide, subsequently, superoxide creates oxidative stress. Using this mechanism, juglone causes oxidative stress (Weir *et al*., 2004). In this study, juglone-induced oxidative stress revealed that 6-gingerol-treated worms lived longer than the controls. The results explained that 6-gingerol was capable of increasing survival rate of the worms affected by three types of stress conditions. These results correlated with earlier reports showing that stress resistance and lifespan are usually connected (Wu *et al*., 2002; Kaletsky and Murphy 2010; Surco-Laos *et al*., 2012).

Excessive ROS levels are associated with aging and age-related diseases. Modulating ROS levels and antioxidant defense systems, including SOD and catalase, may contribute to the delay of senescence (Wang *et al*., 2016). In this study, we found that 6-gingerol elevated SOD and catalase activity in the worms in a dose-dependent manner. These results suggest that the radical scavenging and up-regulation of 6-gingerol antioxidant enzyme activities may partly contribute to a prolonged lifespan and increased stress resistance. Our study tested if 6-gingerol affects the gene expressions of SOD-3 and HSP-16.2 using transgenic strains CF1553 and CL2070, respectively. It appeared that 6-gingerol-fed worms had a higher green fluorescent protein (GFP) intensity compared to the control group, indicating that 6-gingerol-treatment increased SOD-3 and HSP-16.2 gene expression. Additionally, our study showed that heat shock proteins are expressed under heat stress conditions (Swindell, 2009), and that higher HSP-16.2 levels predict a longer lifespan. Lipofuscin is a yellow-brown pigment often seen in granular form, composed of lipid-containing remnants of lysosomal digestion, it is known as one of the endogenous markers of cellular damage during aging process and seen in many organisms such as the N2 nematode

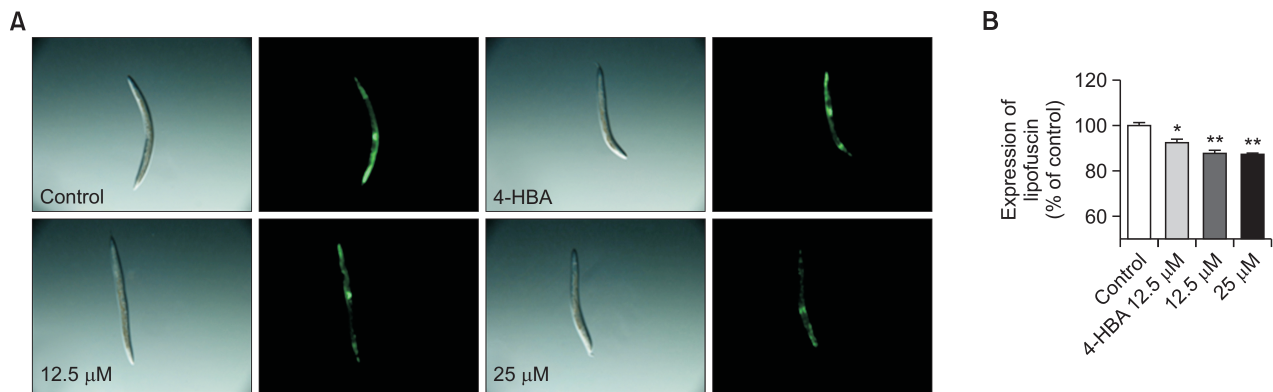

Fig. 6. Effects of 6-gingerol on the lipofuscin accumulation in wild-type N2 nematodes. (A) Fluorescence intensity was measured by determining pixel intensity in worm's intestines using Image J software. (B) Image of intestinal autofluorescence from lipofuscin accumulation of worms on the 8th adult days. Mean fluorescence intensity of lipofuscin accumulation was expressed as mean ± SEM. Differences compared to the control were considered significant at *p<0.05 and **p<0.01 by one-way ANOVA. 4-HBA (4-hydroxybenzoic acid): positive control.

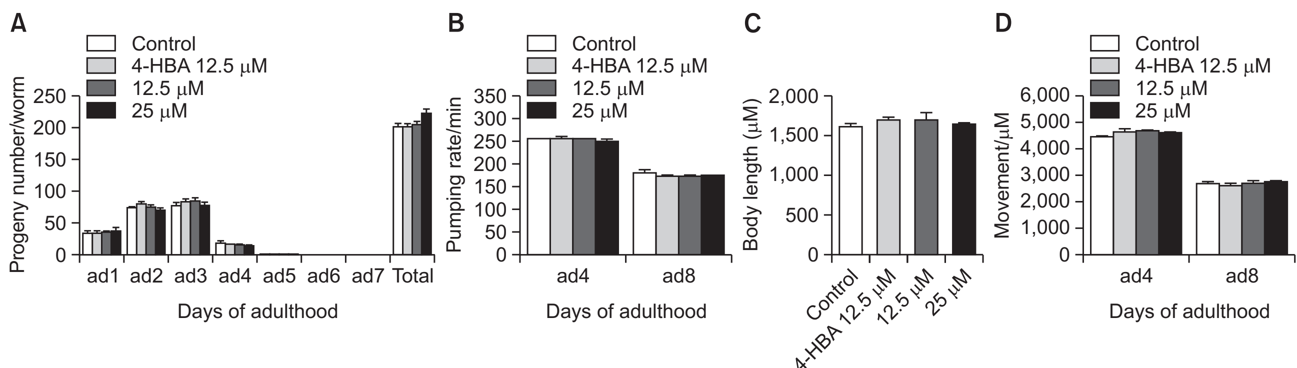

Fig. 7. Effects of 6-gingerol on the various aging-related factors of wild-type N2 nematodes. (A) Daily and total reproductive outputs were counted. The progeny was counted at the L2 or L3 stage. (B) On the 4th and 8th days of adulthood, the pharyngeal pumping rates were measured. (C) For the growth alteration assay, photographs were taken of worms and the body length of each animal was analyzed. (D) The body movements were counted under a dissecting microscope for 20 seconds. Data are expressed as the mean ± SEM of three independent experiments (N=3). 4-HBA (4-hydroxybenzoic acid): positive control.

(Terman and Brunk, 2004). The intestinal lipofuscin levels of worms were significantly decreased by 6-gingerol treatment as compared to the control group.

We further investigated whether 6-gingerol affects aging-related factors such as reproduction, food intake, growth, and locomotion (Partridge *et al*., 2005; Mörck and Pilon 2006). However, there were no significant variation in the number of progeny, pharyngeal pumping, body movement, and body length between 6-gingerol-treated worms and control group worms. These results suggest that these aging-related factors are not responsible for the longevity property of 6-gingerol in *C. elegans*. These results proved that 6-gingerol was able to increase lifespan in nematodes through various antioxidant activities independent of aging-related factors. In this study, at 12.5 and 25 μM, 6-gingerol showed significant effects in a dose-dependent manner. Dried ginger usually contains more than 0.4% of 6-gingerol (Seo *et al*., 2017). This is a relatively high natural content, making it easy to get 6-gingerol even from a small amount of a ginger plant.

Consequently, 6-gingerol prolonged the lifespan of *C. elegans*, it also increased thermal and oxidative stress tolerances, antioxidant enzyme activity, and expression of heat shock and oxidative stress resistance proteins. In addition, 6-gingerol decreased intracellular ROS and lipofuscin accumulation. Thus, 6-gingerol has the potential to be an effective anti-aging compound. To the best of our knowledge, this is the first report on the lifespan effect of this compound. However, the present data are preliminary, and further research is necessary to determine the definite mechanism of 6-gingerol-mediated longevity.

ACKNOWLEDGMENTS

This research was financially supported by the Ministry of Trade, Industry, and Energy (MOTIE), under the "Regional Specialized Industry Development Program" supervised by the Korea Institute for Advancement of Technology (KIAT).

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