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).

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

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.
Longevity Property of 6-Gingerol in *C. elegans*

**Fig. 1.** Structure of 6-gingerol.

**MATERIALS AND METHODS**

**General**

NMR spectra were determined on a JEOL JNM-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% H2SO4 in ethanol followed by heat treatment. Selected peptide and yeast extracts were obtained from BD bioscience USA). Agar, catalase, juglone, 2′,7′-dichlorodihydrofluorescein 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 H2O 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)**

1H-NMR (400 MHz, CD3OD) \(\delta : 0.84 \) (3H, t, H-10'), 1.20-1.31 (8H, m, -(CH3)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, OCH3), 3.87 (1H, m, H-4'), 4.07 (1H, s, H-10'), 4.94 (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). 13C-NMR (100 MHz, CD3OD) \(\delta : 134.3 \) (C-1), 113.2 (C-2), 149.0 (C-3), 145.8 (C-4), 163.3 (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).

**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.
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    | 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***   |

*p 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.

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 (H₂DCF-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 H₂DCF-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 antioxidant 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.
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 formaldehyde 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 nitroblue tetrazolium (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 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.

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**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* | 13.5 ± 0.9 | 20 | 7.6 | p<0.05* |
|                   | 12.5 | 14.2 ± 0.9 | 23 | 13.0 | p<0.01** |
|                   | 25 | 15.6 ± 0.1 | 25 | 24.8 | p<0.001*** |
| 1 mM juglone      | Control | 14.9 ± 0.2 | 25 | - | - |
|                   | 4-HBA 12.5* | 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*** |

*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.
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-gingerol-fed worms and control worms on the reproduction rate, food intake, and body length (Fig. 7A, 7B, 7C).

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).
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.
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. 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. 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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