

The Longevity Properties of 1,2,3,4,6-Penta-O-Galloyl- β -D-Glucose from *Curcuma longa* in *Caenorhabditis elegans*

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Abstract

Here in this study, we isolated 1,2,3,4,6-penta-O-galloyl- β -D-glucose (PGG) from *Curcuma longa* L. and elucidated the lifespan-extending effect of PGG using *Caenorhabditis elegans* model system. In the present study, PGG demonstrated potent lifespan extension of worms under normal culture condition. Then, we determined the protective effects of PGG on the stress conditions such as thermal and oxidative stress. In the case of heat stress, PGG-treated worms exhibited enhanced survival rate, compared to control worms. In addition, PGG-fed worms lived longer than control worms under oxidative stress induced by paraquat. To verify the possible mechanism of PGG-mediated increased lifespan and stress resistance of worms, we investigated whether PGG might alter superoxide dismutase (SOD) activities and intracellular ROS levels. Our results showed that PGG was able to elevate SOD activities of worms and reduce intracellular ROS accumulation in a dose-dependent manner.

Key Words: 1,2,3,4,6-Penta-O-galloyl- β -D-glucose, Anti-aging, Lifespan extension, Stress tolerance, *Caenorhabditis elegans*

INTRODUCTION

Curcuma longa L. (Zingiberaceae) is a perennial herb that measures up to 1 m high with a short stem and tufted leaves. The extensive reports in the literature on the rhizomes of *C. longa* have revealed that three major curcuminoids such as curcumin, demethoxycurcumin, and bisdemethoxycurcumin have a wide range of pharmacological activities, including anti-inflammatory, anti-parasitic, anti-mutagenic, anti-proliferative, and antimicrobial activities (Kitabatake *et al.*, 2013). Thus, in this plant species, the parts used are the rhizomes and aerial parts are thrown out. However, available publications on the biological properties of aerial part of *C. longa* have indicated that the turmeric leaves might also be beneficial for health. Phytochemical studies on the leaves of *C. longa* have resulted in the isolation of several phenolic compounds, labda-8(17)-12-diene-15,16-dial (Liu and Nair, 2012), while *C. longa* leaves are known to contain very little curcuminoids, the major bioactive compounds present in rhizomes. In addition, many previous reports have been represented that the antifungal,

mosquitocidal, antioxidant, anti-inflammatory, and anticancer action of leaf constituents of *C. longa* (Roth *et al.*, 1998; Liu and Nair, 2012).

Aging is the accumulation of changes over time which is associated with the increasing susceptibility to different diseases and death. Although we do not know the exact mechanism of aging, many evidences led to the general acceptance of the oxidative stress theory that the accumulation of molecular damage caused by reactive oxygen species (ROS) is a major factor in aging (Finkel and Holbrook, 2000; Bokov *et al.*, 2004). Oxidative stress induced by ROS results in oxidation of biomolecules such as protein, lipid, and DNA which is suggested to be the central cause factor promoting aging process (Oliveira *et al.*, 2010). Therefore, antioxidants which can prevent oxidative stress by scavenging radicals or interfering radical generation may delay aging and prolong the lifespan. Indeed, many previous studies on the correlation between antioxidants and aging support this notion (Harrington and Harley, 1988; Adachi and Ishii, 2000; Wu *et al.*, 2002; Ishii *et al.*, 2004).

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Previously, we isolated 1,2,3,4,6-penta-O-galloyl- β -D-glucose (PGG, Fig. 1) and gallic acid from the leaves of *C. longa* and demonstrated their strong radical scavenging capacities (Ahn *et al.*, 2012). Since plant-derived polyphenols have been shown to have beneficial role in aging-associated changes (Uysal *et al.*, 2013), we hypothesized that the phenolic compounds from *C. longa* leaves might have lifespan-extending ability. Recently, Saul's group has noted that gallic acid can extend the lifespan of *C. elegans* (Saul *et al.*, 2011). In this work, we analyzed the possible longevity activities of PGG using *C. elegans* model system. We also determine the action of PGG on the survival rate of nematodes under heat and oxidative stress conditions. In addition, antioxidant capacity of this compound was analyzed by measuring superoxide dismutase activities and intracellular ROS level of worms.

MATERIALS AND METHODS

Chemicals

The isolation of 1,2,3,4,6-penta-O-galloyl- β -D-glucose (PGG) from *C. longa* (Zingiberaceae) has been detailed in a previous report (Ahn *et al.*, 2012). Selected peptone and yeast extracts were obtained from BD bioscience (USA). Agar, 2',7'-dichlorodihydrofluorescein diacetate, methyl viologen dichloride hydrate (paraquat), catalase, xanthine, xanthine oxidase, and nitrobluetetrazolium were purchased from Sigma (St. Louis, MO, USA).

C. elegans strains and maintenance

Bristol N2 (wild-type) was kindly provided by Dr. Myon-Hee Lee (East Carolina University, NC, USA). The worms were grown at 20°C on nematode growth medium (NGM) agar plate with *E. coli* OP50 as described previously (Brenner, 1974). To prepare plates supplemented with PGG, the stock solution in DMSO was inserted into autoclaved NGM plates (at 50°C). A final DMSO concentration of 0.2% (v/v) was maintained under all conditions.

Lifespan assay

The lifespan assays were performed at 20°C using wild-type N2 worms at least 3 times independently. To obtain age-

synchronized worms, eggs were transferred to NGM plate in the absence or presence of PGG (50 μ M, 100 μ M) after embryo isolation. Test worms were considered dead when they failed to respond to prodding with the tip of a platinum wire (Lithgow *et al.*, 1995). The worms were transferred to fresh NGM plate every 2 days.

Assessment of stress resistance

The age-synchronized N2 worms were bred on NGM agar plates with or without various concentrations of PGG. For the heat tolerance assay worms were transferred to fresh plates on the 4th day of adulthood and then incubated at 36°C. The viability was scored over 16 h as previously described (Lee *et al.*, 2005). Oxidative stress tolerance was assessed as described previously with minor modification (Mekheimer *et al.*, 2012). Briefly, on the 7th day of adulthood, worms were subjected to containing 60 mM paraquat liquid culture and then survivals were recorded over 67 h.

Measurement of antioxidant enzyme activities

To assess enzymatic activity, the worm homogenates were prepared. Briefly, worms (5th day of adulthood) were harvested from plate with M9 buffer and washed 3 times. Then, the collected worms were resuspended 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 analysing the decolorization of formazan using enzymatic reaction between xanthine and xanthine oxidase. The reaction mixture contained 20 μ l of worm homogenates and 480 μ l of 1.6 mM xanthine, 0.48 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 1 ml of xanthine oxidase (0.05 U/ml) and incubation at 37°C for 20 min. The reaction was stopped by adding 500 μ l of 69 mM SDS, and the absorbance at 570 nm was measured. SOD activity was expressed as a percentage of the scavenged amount per control.

Analysis of intracellular ROS

Intracellular ROS in the nematodes was measured using molecular probe 2',7'-dichlorodihydrofluorescein diacetate (H_2 -DCF-DA). Equal number of wild-type worms was incubated in the absence or presence of PGG. On the 4th day of adulthood, animals were exposed to 96-well plate containing 60 mM paraquat for 3 h. Subsequently, 5 worms were transferred into the wells of a 96-well plate containing 50 μ l of M9 buffer. Immediately after addition of 50 μ l of 25 μ M H_2 DCF-DA solution resulting in a final concentration 12.5 μ M, basal fluorescence was quantified in a microplate fluorescence reader at excitation 485 nm and emission 535 nm.

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. Other data were presented as mean \pm standard error of the mean. Statistical significance of differences between the control and treated groups were analyzed by one-way analysis of variance (ANOVA).

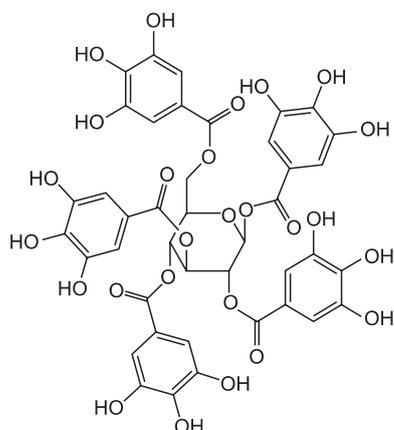


Fig. 1. Structure of 1,2,3,4,6-penta-O-galloyl- β -D-glucose (PGG) isolated from the aerial part of *Curcuma longa*.

RESULTS

PGG extends lifespan of *C. elegans* in normal conditions

To determine the lifespan extension properties of PGG, lifespan assays were performed with wild-type N2 worms. As shown in Fig. 2A, a concentration-dependent effect of PGG on longevity was observed. In addition, there was a significant increase (15.7% at 100 μ M of PGG, $p < 0.001$) in the estimated mean life of the PGG treated worms compared to control worms (Fig. 2B). The mean life duration was 16.0 ± 0.3 days for control worms and 19.6 ± 0.7 for the worms fed 100 μ M of PGG (Fig. 2B).

PGG enhances stress tolerance of *C. elegans*

Then, we determined the effect of PGG on several stress conditions including thermal and oxidative stress using wild-type N2 worms. As can be seen in Fig. 3A, we could observe

that the PGG exposure induced significant increase in thermo-tolerance, and consequently elevated survival rate of worms ($p < 0.001$). Further, the PGG exposure prolonged the maximum lifespan of worms by 23.0%. Moreover, we also found that PGG-fed worms showed a concentration-dependent increase in survival time under oxidative stress condition induced by 60 mM paraquat ($p < 0.001$, Fig. 3B).

PGG increases SOD activities and decreases intracellular ROS levels of *C. elegans*

To verify the mechanism of PGG on enhancing lifespan and stress resistance of worms, SOD activities and intracellular ROS levels were investigated. The SOD enzymatic activities were measured spectrophotometrically using prepared worm homogenates. Our results showed that PGG was able to elevate SOD activity of worms significantly ($p < 0.05$, Fig. 4A). Next, we further quantified intracellular ROS levels of PGG treated worms compared to untreated controls. Fig. 4B shows that PGG-fed (100 μ M) worms effectively reduced the production of ROS ($p < 0.05$) compared with solvent-treated control

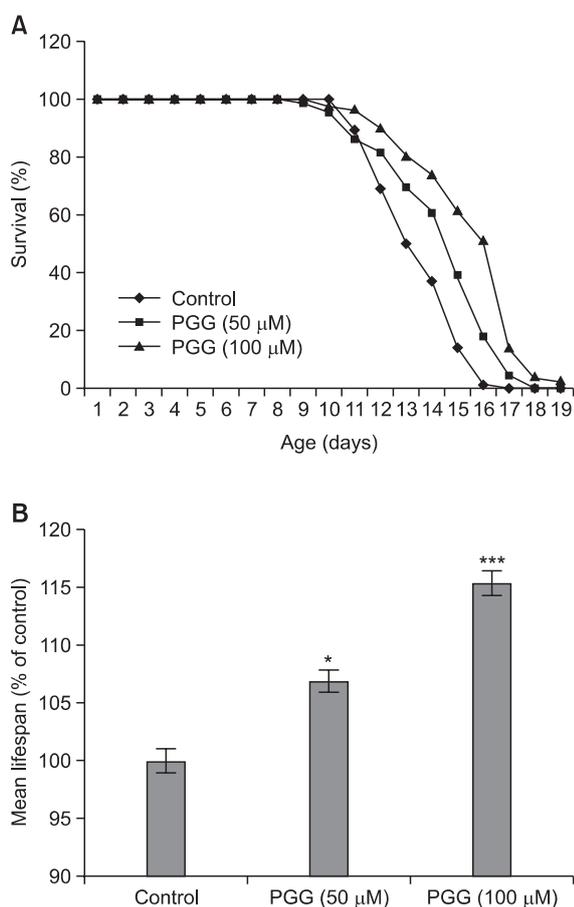


Fig. 2. Effects of PGG on the lifespan of wild-type N2 nematodes. Worms were grown in the NGM agar plate at 20°C in the absence or presence of PGG. The number of worms used per each lifespan assay experiment was 27-41 and three independent experiments were repeated (N=3). (A) The mortality of each group was determined by daily counting of surviving and dead animals. (B) The mean lifespan of the N2 worms was calculated from the survival curves in (A). Statistical difference between the curves was analyzed by log-rank test. Error bars represent the standard error of mean (S.E.M.). Differences compared to the control were considered significant at $p < 0.05$ and $***p < 0.001$.

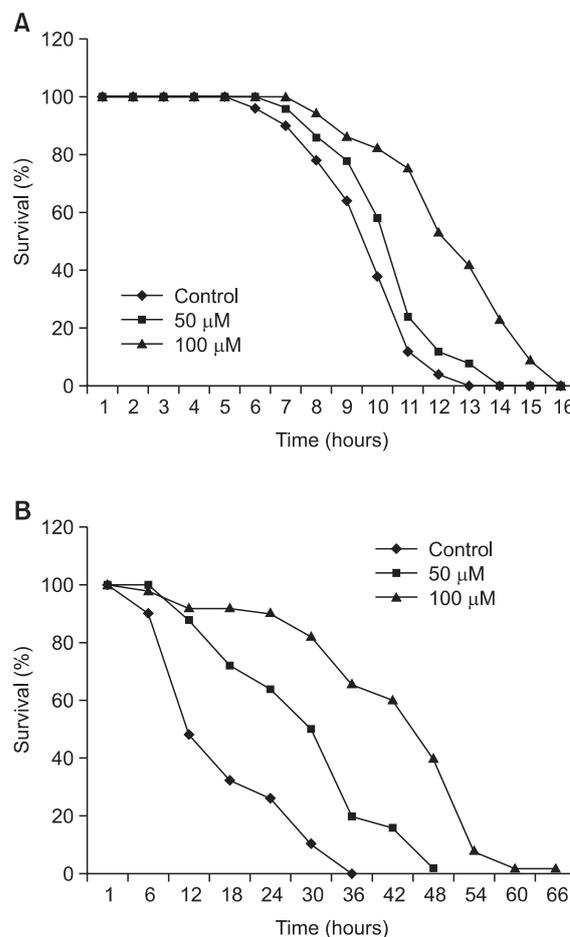


Fig. 3. Effects of PGG on the stress tolerance of wild-type N2 nematodes. (A) To assess thermal tolerance, worms were incubated at 36°C and then their viability was scored. (B) For the oxidative stress assays, worms were transferred to 96-well plate containing 60 mM of paraquat, and then their viability was scored. Statistical difference between the curves was analyzed by log-rank test.

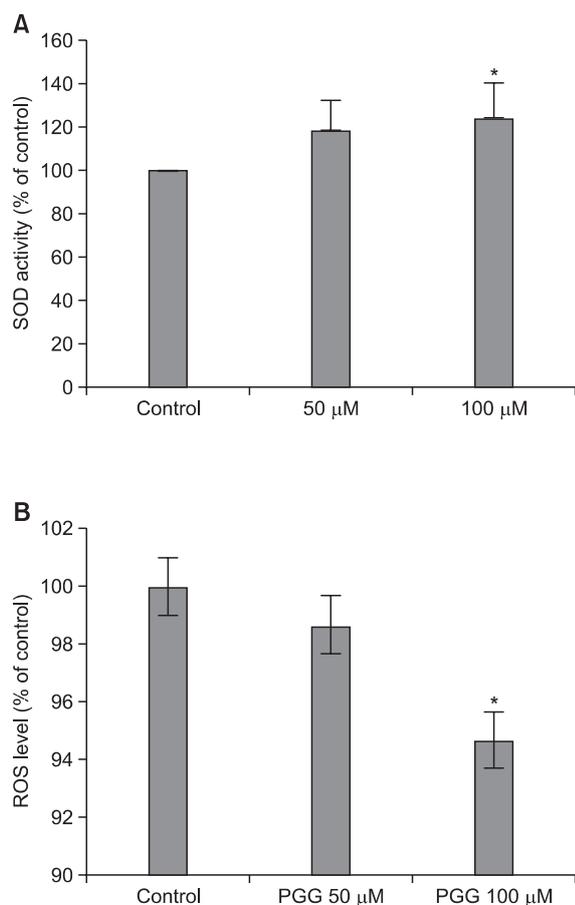


Fig. 4. Effects of PGG on the antioxidant enzyme activity and intracellular ROS levels of wild-type N2 nematodes. (A) The enzymatic reaction of xanthine with xanthine oxidase was used to generate $\cdot\text{O}_2^-$ and the SOD activity was estimated spectrophotometrically through formazan formation by NBT reduction. SOD activity was expressed as a percentage of the scavenged amount per control. (B) Intracellular ROS accumulation was quantified spectrometrically at excitation 485 nm and emission 535 nm. Data are expressed as the mean \pm S.E.M. of three independent experiments (N=3). Differences compared to the control were considered significant at $*p < 0.05$ by one-way ANOVA.

worms. Since PGG is one of phenolic compound which has strong antioxidant capacity, we assume that PGG-mediated radical scavenging and up-regulation of antioxidant enzyme activities were likely associated with the attenuation of intracellular ROS level.

DISCUSSION

PGG, a prototypical gallotannin, is highly enriched in many medicinal plants. This compound has been shown to exert attractive pharmacological properties including anti-proliferative, anti-diabetic, antioxidant, anti-mutagenic, anti-inflammatory, antibacterial, and cardiovascular protective activities (Zhang *et al.*, 2009). However, as far as we know, PGG's effects on the lifespan have never before been reported. Here in this study, we tested the longevity properties of PGG isolated from

C. longa leaves, using *C. elegans* model system. Recently, this model has become a popular tool for aging research with multiple merits, including short lifespan, rapid generation, and ease of handling (Guarente and Kenyon, 2000). Moreover, worms and mammals share many specific features of aging (Finkel and Holbrook, 2000; Herndon *et al.*, 2002; Glenn *et al.*, 2004), and thus, this model provides excellent environment for identifying drugs which can prolong the human lifespan (Collins *et al.*, 2006; Petrascheck *et al.*, 2007).

To determine the effects of PGG on the longevity, we carried out lifespan assay with wild-type N2 worms under normal culture condition. We found that PGG treatment significantly prolonged the lifespan of worms in a concentration-dependent manner. In addition, the mean lifespan was also elevated by PGG exposure. Since there is a considerable correlation between longevity and the enhanced stress tolerance (Kenyon, 2010), we tested whether PGG increases stress resistance of worms under stress conditions. In the current study, PGG-fed worms exhibited significant increase in survival rate under heat stress condition, compared to control worms, indicating that the PGG treatment enhanced thermotolerance. Moreover, the results of paraquat-induced oxidative stress assay showed that PGG-fed worms also survived longer than the controls. These results strongly suggest that the PGG's lifespan extension activity is possibly associated with increased stress tolerance.

Since ROS-induced oxidative stress causes accelerating aging process and limiting lifespan across a variety of organisms from worm to human (Kampkotter *et al.*, 2007), we evaluated PGG's antioxidant potential in *C. elegans*. Herein, we showed that the PGG was able to elevate SOD activities of worms in a dose dependent manner. Moreover, we found that the intracellular ROS accumulation was also decreased by PGG exposure. Indeed, early studies have demonstrated that PGG can protect the cells from oxidative stress via heme oxygenase-1 induction, indicating that the PGG also can act as an indirect antioxidant (Choi *et al.*, 2002). Furthermore, our results are consistent with previous findings that the PGG can reduce intracellular hydrogen peroxide results in inhibition of mutagenesis of cancer cells. As noted above, PGG is one of phenolic compound which has strong antioxidant capacity, we assume that PGG's direct radical scavenging and up-regulation of antioxidant enzyme activities result in the attenuation of intracellular ROS level might give rise to enhanced resistance under oxidative stress condition and prolonged lifespan. In addition, we speculate that PGG's antioxidant potential might be also involved in increased survival rate under thermal stress condition. Because ROS production has been shown to increase under heat stress in isolated mitochondria and antioxidants can affect heat stress-mediated gene expression (Abele *et al.*, 2002; Bowler *et al.*, 2002).

Although, mitochondria are essential for many biological functions including ATP production, the oxidative stress theory of aging proposes that mitochondria play a pivotal role in aging by generating ROS. Thus, inhibition of mitochondrial function can actually increase lifespan and delay aging. Indeed, extension of lifespan by down-regulation of mitochondrial respiration has been shown to be evolutionarily conserved (Hwang *et al.*, 2012). Interestingly, previous studies on PGG have provided evidence that the PGG is a potential mitochondria respiration inhibitor with multiple targets. PGG is known to suppress the mitochondrial respiration through not only direct inhibition

of electron transport system, but induction of damage of the mitochondrial membrane (Adachi *et al.*, 1989). Taken together, we can possibly estimate that the PGG's inhibitory effects on the mitochondrial respiration may be also responsible for the enhanced resistance and prolonged lifespan by PGG treatment in *C. elegans*.

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