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Arbutin (p-hydroxyphenyl-β-D-glucopyranoside), a well-known tyrosinase inhibitor, has been widely used as a cosmetic whitening agent. Although its natural role is to scavenge free radicals within cells, it has also exhibited useful activities for the treatment of diuresis, bacterial infections and cancer, as well as anti-inflammatory and anti-tussive activities. Because function of free radical scavenge is also related to antioxidant and the effects of arbutin on longevity and stress resistance in animals have not yet been confirmed, here the effects of arbutin on *Caenorhabditis elegans* were investigated. The results demonstrated that optimal doses of arbutin can extend lifespan and enhance resistance to oxidative stress. The underlying molecular mechanism for these effects involves decreased level of reactive oxygen species (ROS), improvement of daf-16 nuclear localization, and upregulation of expression of daf-16 and its downstream targets, including sod-3 and hsp16.2. In this work the roles of arbutin in lifespan and health are studied and the results support the use of arbutin as an antioxidant for maintaining overall health.
Arbutin increases *Caenorhabditis elegans* longevity and stress resistance

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Abstract

Arbutin (\(p\)-hydroxyphenyl-\(\beta\)-D-glucopyranoside), a well-known tyrosinase inhibitor, has been widely used as a cosmetic whitening agent. Although its natural role is to scavenge free radicals within cells, it has also exhibited useful activities for the treatment of diuresis, bacterial infections and cancer, as well as anti-inflammatory and anti-tussive activities. Because function of free radical scavenge is also related to antioxidant and the effects of arbutin on longevity and stress resistance in animals have not yet been confirmed, here the effects of arbutin on *Caenorhabditis elegans* were investigated. The results demonstrated that optimal doses of arbutin can extend lifespan and enhance resistance to oxidative stress. The underlying molecular mechanism for these effects involves decreased level of reactive oxygen species (ROS), improvement of *daf-16* nuclear localization, and upregulation of expression of *daf-16* and its downstream targets, including *sod-3*.
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**Introduction**

Tannins, also known as plant polyphenols, comprise the most common category of secondary metabolites and are present in all vegetative organs of flowering plants (Scalbert et al. 2005). Studies have shown that plant polyphenols mainly participate in plant chemical defenses by interfering with normal functions of various macromolecules (Spencer et al. 2008) and also exhibit antioxidant, anti-inflammatory, antibacterial, antitumor and other biological activities (Balandrin et al. 1985).

Arbutin (C_{12}H_{16}O_{7}), a plant polyphenol with one of the least complex molecular structures, is widely distributed in animals, plants and microbes. It exhibits an acicular crystal habit and can be processed into a white or grey powder. Arbutin dissolves in methyl alcohol, ethyl alcohol, acetonitrile and tetrahydrofuran, but is insoluble in solvents such as cyclohexane, diethyl ether, chloroform, petroleum and DMSO. It is unstable and is easily hydrolyzed in an acid environment, but has been successfully isolated using plant extraction techniques, biological transformation, organic synthesis and enzymatic synthesis methods (Seo et al. 2012). Arbutin possesses two functional groups, a hydrophilic anhydroglucose group and a melanin synthase inhibitory phenolic group. The latter group inhibits
melanin synthase to lighten hair and is the reason arbutin was widely used in the cosmetic hairdressing industry. Furthermore, arbutin has recently been shown to exhibit antioxidant, diuretic, antibacterial, anti-inflammatory, anti-tussive, anticancer and other biological activities (Mustapha et al. 2016).

As an animal model for human biomedical research, *Caenorhabditis elegans* possesses many advantages, such as ease of culture and rapid reproduction with short generation times. Moreover, the organism is easy to visualize, due to its translucent body that allows fluorescence labeled organs to be easily visualized. Furthermore, numerous recombinant strains of this organism exist that incorporate GFP reporter genes for many important cellular pathways, including aging, oxidative stress tolerance and many diseases (Abbas & Wink 2010; Henderson & Johnson 2001; Hsu et al. 2003; Link et al. 2015). Finally, *C. elegans* is a suitable tool for the study of human health conditions and diseases because its homologues have been identified for 60-80% of human genes (Kaletta & Hengartner 2006).

In this study, we investigated the effect of arbutin on *C. elegans* longevity and stress resistance and evaluated the signaling pathways involved.

**Materials and methods**

**Chemicals and reagents**

Juglone and DCFH-DA and sodium azide were purchased from TransGen Biotech Co., Ltd. (Beijing, China). All other chemicals were of analytical or reagent grade.
**C. elegans strains and maintenance**

The following strains in this study were obtained from the Caenorhabditis Genetics Center: wild type N2, CF1038 [daf-16(mu86)I], TJ356 zIs356 [daf-16p::daf-16a/b::GFP + rol-6(su1006)]. Worms were maintained at 20 °C on agar plates containing nematode growth medium (NGM, 1.7% agar, 25 mM potassium phosphate, pH 6.0, 50 mM NaCl, 2.5 µg/ml peptone, 5 µg/ml cholesterol, 1 mM MgSO$_4$, 1 mM CaCl$_2$) seeded with *Escherichia coli* OP50. Arbutin (Wuhan Fude Chemical Co., Ltd., China; > 98% pure, HPLC grade) was dissolved in ultrapure water to a concentration of 50 mM and added directly to *E. coli* OP50 cultured in LB liquid medium supplement present to a final concentration of 0.5, 2.5, 5, 10 and 20 mM.

**Lifespan assay**

The lifespan assay of *C. elegans* was investigated as previously described (Schlotterer et al. 2009). The pre-fertile period of adulthood was used as time zero (t = 0). The nematodes were maintained on NGM plates containing either various concentrations of arbutin or vehicle (control) form birth and transferred to new plates every day. Worms were recorded as dead if they did not move after repeated stimuli; if they crawled away from the plate, they were excluded. The assay was run until all animals were dead. Experiments were performed in at least triplicate with 120 nematodes each at 20 °C. All subsequent assays were conducted using the concentration that was deemed most effective in inducing lifespan extension.
Stress resistance assays

Twenty gravid adult nematodes (N2 and CF1038) were placed on NGM plates seeded with *E. coli* strain OP50 with no arbutin (control) or with 5 mM arbutin (experimental). Worms were allowed to lay eggs at 20 °C for approximately 2 h to obtain a synchronous population. After 2 h, the nematodes were removed and the plates were placed back at 20 °C until the progeny reached young adulthood (about 72 h). Worms were transferred every other day to fresh control plates or arbutin plates. On the fifth day (at about 120 h), animals were submitted to various kinds of stressors. Experiments were performed in at least triplicate, with 60 nematodes each.

In the heat shock stress assay, animals were transferred from 20 °C to a 35 °C incubator and numbers of surviving worms were recorded every hour until all worms died. In the juglone stress assay, worms were placed in 96-well plates containing 200 μl S medium (100 mM NaCl, 0.01 mM cholesterol and 50 mM potassium phosphate, pH 6.0) with 200 μM juglone in each well (Waterston & Brenner 1978). Worms were observed at 20 °C every hour until no worms remained alive. In the UV-irradiation stress assay, worms were treated with 254 nm UVC for 8 min for 40 s with an irradiation dose of 1000 J/m². Worms were counted every hour during incubation at 20 °C until they died.
Measurement of general ROS levels

Five-day-old worms without arbutin or with treatment of 5 mM arbutin from birth to 5 days of age were transferred into 100 μl M9 buffer (22 mM KH$_2$PO$_4$, 42 mM Na$_2$HPO$_4$, 85 mM NaCl, 1 mM MgSO$_4$) with 200 μM DCFH-DA (Beyotime, China). After 2 h incubation with DCFH-DA, animals were washed with M9 buffer to remove residual DCFH-DA and were then added to 96-well plates containing 200 μl M9 buffer per well. Ten worms were added to each well and five parallel experiments were performed for each group. ROS-associated fluorescence levels were measured using a fluorescence microplate reader (Infinite F200 PRO, Tecan, Switzerland) at 485 nm excitation and 535 nm emission settings. Arbutin-treated group relative fluorescence unit (RFU) were compared with control group of which mean value RFU was set as 1 to reflect relative ROS level. Experiments were performed in at least triplicate, with 20 nematodes each.

Fluorescence quantification of DAF-16::GFP

Transgenic strains TJ356 worms harboring the DAF-16::GFP reporter gene were subjected to arbutin treatments from birth to 5 days of age. Then, they were treated with in 35 °C heat shock for 30 min. Later, animals were narcotized with 10 μM sodium azide and fixed to glass slides containing 5% agar for observation under a fluorescence microscope. Green fluorescence intensity corresponded to DAF-16 expression. Experiments were performed in at least triplicate, with 10 nematodes each.
Quantitative real-time PCR

After birth followed by 5 days of incubation either with or without 5 mM arbutin, worms were collected and washed with M9 buffer to remove residual *E. coli* OP50 on their skin. Trizol reagent (Thermo Fisher Scientific, USA) was used for total RNA extraction. The primers used in RT-PCR were as follows:

- **daf-16**, 5'-TTTCCGTCCCCCGAACTCAA-3' and 5'-ATTCGCCAACCCATGATGG-3';
- **sod-3**, 5'-AGCATCATGCCACCTACGTGA-3' and 5'-CACCACCATTGAATTTCAGCG-3';
- **hsp-16.2**, 5'-CTGCAGAATCTCTCCATCTGAGTC-3' and 5'-AGATTCGAAGCAACTGCACC-3';
- **ama-1**, 5'-CTGACCCAAAGAACACGGTGA-3' and 5'-TCCAATTCGATCCGAAGAAGC-3'.

The gene **ama-1** was used as an internal reference gene and the AB 7500 RT-PCR (Applied Biosystems, Foster City, CA, USA) detection system was used to visualize RT-PCR products. RT-PCR data were analyzed using the comparative 2^-ΔΔCt_ method (Livak & Schmittgen 2001). Experiments were performed in at least triplicate, with 200 nematodes per group.

Brood size

Animals synchronized at the first larval stage were grown on NGM/OP50 plates with or without 5 mM arbutin. On the second day, animals were transferred
to relevant plates (at one animal per plate for N = 10 animals per group) and then were transferred every 24 h to fresh control or 5 mM arbutin plates until cessation of egg production. The total number of progeny from each animal was counted and the numbers of progeny for each group were averaged (Li et al. 2008). Experiments were performed in triplicate, with 10 nematodes each.

**Statistical analysis**

Statistical analysis was performed using Prism 4 software (GraphPad Software, Inc., La Jolla, CA, USA). Results were expressed as the mean ± standard deviation of three or four independent experiments. The lifespan of nematodes cultured in the absence or presence of arbutin was compared between groups using the two-tailed, unpaired Student’s *t*-test. *P*-values < 0.05 were taken as statistically significant. (0.01 ≤ *p* < 0.05, 0.001 ≤ **p** < 0.01, ***p** < 0.001)

**Results**

**Arbutin extends lifespan of *C. elegans* under normal culture conditions**

Low concentration of arbutin showed dose-related lifespan-extending effects, with the maximum effect observed at 5 mM (extending worm lifespan by 11.89%, *p* < 0.01), whereas 10 mM and 20 mM arbutin exhibited toxicity. It is certain that arbutin significantly extended nematode lifespan, but the effect was not very large. (Fig. 1A-1B) Therefore, we performed additional experiments using 5 mM arbutin to further test its protective effect on worms under various types of stress.
**Arbutin improves worm survival under stress**

a. **Heat stress**

Results demonstrated worms’ resistance to heat stress after pretreatment with 5 mM arbutin from birth to 120 h after birth, with increased survival rate after heat shock at 35 °C. The average survival time significantly increased, reaching 11.71% using 5 mM arbutin (p < 0.05) relative to the control. (Fig. 2A-2B)

b. **Juglone stress**

Additionally, 5 mM arbutin pretreatment enhanced average survival time by 34.47% as compared to untreated worms under oxidative stress conditions induced by juglone from the second day of adulthood (p < 0.01). (Fig. 2C-2D)

c. **UV stress**

Next, we observed 5 mM arbutin-pretreated worms were more irradiation tolerant than untreated control worms, as 5 mM arbutin pretreatment significantly enhanced the animals’ resistance to UV-irradiation and increased the average survival time by 23.37% relative to the control (p < 0.001). (Fig. 2E-2F)

Apparently, protective effects of a low but suitable arbutin dose were observed under both normal and stress culture conditions, suggesting that low-dose arbutin could promote statistically significant longevity in worms.

**Arbutin reduces the ROS levels**

According to known effects of free radicals, excessive free radical accumulation may harm organisms. Cellular respiration is one of the chief causes
of oxidative damage in aerobic organisms because oxygen, an electron receptor, may promote the formation and accumulation of ROS. Meanwhile, newly generated free radicals, such as superoxide anions, hydrogen peroxide and hydroxyl radicals, are immediately eliminated by antioxidant enzyme systems (Harman 1956). Therefore, we studied whether the longevity effect of arbutin under normal culture conditions or during exposure to a variety of stresses to detect any associated ROS scavenging ability. Compared to controls, ROS level was significantly decreased in worms treated with arbutin, while levels were usually higher during a stress response. We therefore suggest that arbutin may act as an antioxidant by scavenging free radicals, ultimately extending *C. elegans* lifespan and improving resistance to environment stress. (Fig. 3, p < 0.001)

**Arbutin has no effect on the resistance to stresses of daf-16 mutant worms**

DAF-16 is a major transcription factor which modulates longevity and stress resistance in *C. elegans* (Murphy et al. 2003). We examined the lifespan of *daf-16* mutants CF1038 under different kinds of stresses. We found that there was no different with or without treatment with 5 mM arbutin on mutant CF1038 lifespan under three kinds of stresses (heat shock, juglone and UV-irradiation), indicating that arbutin acting on animals was related to *daf-16*. (Fig. 4A-4F, p > 0.05)

**Arbutin improves DAF-16::GFP nuclear localization in TJ356**
The nuclear localization of DAF-16 is essential for its transcriptional activity. Therefore, we conjectured that arbutin could also positively regulate DAF-16 expression in nuclear. Here, we employed a transgenic strain TJ356 strain expressing an DAF-16::GFP (Lee et al. 2001) reporter to quantitatively visualize DAF-16 expression using a fluorescence microscope. After 35 °C heat shock for 30 min, 5 mM arbutin-treated worms displayed a fluorescence intensity that was significantly upregulated relative to the control group. These results suggest that arbutin may also activate antioxidant-related signaling pathways that ultimately lead to upregulation of DAF-16 nuclear localization for increased resistance to oxidative stress. (Fig. 5A-5D, p < 0.001)

Arbutin upregulates relative mRNA levels of daf-16 and its downstream genes sod-3 and hsp-16.2

To further investigate whether arbutin’s mechanism of action is regulated by DAF-16 and its target proteins, we measured mRNA levels of daf-16, sod-3 and hsp-16.2 in N2 worms treated with or without 5 mM arbutin. It is known that protein activities which are dependent on DAF-16-mediated transcriptional control are generally recognized as important in stress responses and longevity (Heidler et al. 2010). As expected, daf-16 (P < 0.001) and its downstream targets sod-3 (P < 0.05) and hsp-16.2 (P < 0.001) were all upregulated with arbutin treatment relative to the control. Therefore, the results demonstrate that DAF-16 is important to arbutin’s
positive effect on lifespan and stress resistance in *C. elegans*. (Fig. 6, p < 0.05)

**Arbutin does not affect *C. elegans* brood size**

An increase in lifespan is often correlated with a decrease in fecundity (Wang et al. 2017). To test whether arbutin adversely affected fecundity, we measured brood sizes for ten N2 animals in each group treated with either 0 mM (control) or 5 mM arbutin. The results demonstrated that there were no significant differences between treatment group and control. (Fig. 7, p = 0.74)

**Discussion**

Arbutin (p-hydroxyphenyl-β-D-glucopyranoside) is a well-known tyrosinase inhibitor that has been widely used as a cosmetic whitening agent. It also plays an important role in the scavenging of free radicals and has served as a diuretic, as well as an anti-bacterial, anti-phlogistic, anti-tussive and anticancer agent (Mustapha et al. 2016). In this work, nematodes served as a model to test our hypothesis that arbutin could play a positive role in health and survival. Our work demonstrates that a suitable dose of arbutin could extend the lifespan of *C. elegans* in a dose-dependent manner and that the effect is sensitive to various stressors. Meanwhile, arbutin was observed to decrease ROS levels. Furthermore, *daf-16* played a role in the effects of arbutin on worms. Arbutin had no influenced with stress resistance in *daf-16* mutant. It not only increased *daf-16* transcriptional activity to nuclear but also enhanced mRNA expression of *daf-16* and its downstream targets *sod-3* and *hsp-16.2* relative to controls. Therefore, the final results demonstrated that arbutin
could improve lifespan and health of *C. elegans* which was related to *daf-16*.

We selected a series of doses of arbutin dissolved in deionized water to explore arbutin’s effects. Although arbutin was an anti-bacterial agent in *E. coil* OP50, worms were all treated with excess food, thus arbutin could not cause food deprivation. When treated with 0.5 mM, 2.5 mM and 5 mM arbutin, animals exhibited longer lifespans than control animals only fed *E. coil* OP50 alone in a dose-dependent manner. Conversely, worms treated with 10 mM and 20 mM arbutin died earlier than controls, indicating that 10 mM and 20 mM arbutin exhibited a toxic effect on the worms. Thus, within a specific range of suitable concentration, arbutin could extend the lifespan of *C. elegans*. By comparison, we chose 5 mM arbutin for subsequent experiments. At the same time, 5 mM arbutin had no change of brood size of N2 worms, indicating that 5 mM also did no harm to animals reproduction and development.

To further confirm the influence of arbutin on lifespan, we treated worms with or without 5 mM arbutin, which had previously been demonstrated to be the most effective dose for prolonging *C. elegans* lifespan. Worms were next exposed to a variety of stress circumstances. Regardless of the mode of stress applied, arbutin-treated animals exhibited greater resistance to stressors, including heat shock, juglone and UV-irradiation. These results demonstrated that arbutin could extend *C. elegans* lifespan in its natural environment under routine stress conditions and also could enhance resistance to stressors.
After arbutin effects on *C. elegans* phenotypes were determined, we investigated arbutin’s mechanism of action. Previous research had established that oxidative damage correlates with functional and metabolic decline during aging (Sena & Chandel 2012). Moreover, while physiological ROS levels alter a cell’s redox state and play a role in mediation of cell signaling, pathological ROS levels can operate in concert with intracellular oxidative damage and activate several cell death pathways (Dai et al. 2014). Therefore, first we measured ROS levels in worms pretreated with or without 5 mM arbutin and demonstrated that arbutin could reduce ROS levels effectively.

DAF-16, the orthologue of FOXO in *C. elegans*, is the crucial transcription factor which is associated with stress resistance. We further investigated lifespan of *daf-16* mutant CF1038 under a variety of stresses. We found that the effect of arbutin on stress resistance of N2 worms disappeared in CF1038 worms in all condition of stresses. Meanwhile, we employed transgenic strain TJ356 (DAF-16::GFP) to test DAF-16 expression level in *C. elegans* and demonstrated that arbutin could increase DAF-16 nuclear localization. Since moderate oxidative stress was reported to promote the expression of DAF-16 target genes downstream of *daf-16* (Chiang et al. 2012), including *sod-3* and *hsp-16.2*, we designed primers for *daf-16*, *sod-3* and *hsp-16.2* and measured their relative mRNA levels. Subsequently, arbutin upregulated *daf-16* mRNA levels, as well as levels of its downstream transcription factors, as expected.
Thus, we concluded that suitable doses of arbutin can benefit nematodes through its antioxidant effects in nematodes including lifespan extension and stressors resistance.

Conclusions

In conclusion, our experiments demonstrate that arbutin significantly extends lifespan and improves antioxidant resistance of *C. elegans*. More important, its antioxidant activity might be related to DAF-16/FOXO-dependent pathways. Studies are underway to explore the mechanisms by which arbutin may act on anti-aging and other health benefits.

References


Figure 1 (on next page)

Effect of arbutin on the lifespan of *C. elegans*.

Wild type animals (*N* = 120-182 in each group) were treated without (0 mM) or with low (0.5, 2.5 mM), moderate (5 mM, *p* < 0.001) and high (10, 20 mM) doses of arbutin at 20 °C from birth, when survival was monitored. The experiment was repeated multiple times and a representative trial is shown.
Fig. 1

A

![Graph showing percent survival over time with different concentrations of a substance.](image)

- 0 mM
- 0.5 mM
- 2.5 mM
- 5 mM
- 10 mM
- 20 mM

B

![Graph showing time vs. concentration with error bars.](image)

- 0 mM
- 0.5 mM
- 2.5 mM
- 5 mM
- 10 mM
- 20 mM
Effect of pretreatment with arbutin on resistance to stress in *C. elegans*.

Animals were treated with 5 mM arbutin from birth to 120 hours at 20 °C and exposed to a variety of stressors. Worms pretreated with arbutin survived significantly longer after (A-B) 35 °C heat shock (N = 66-84 animals, p < 0.05), (C-D) exposure to 200 μM juglone (N = 90 animals, p < 0.001) or (E-F) UV irradiation at 1000 J/m² (N = 68-96 animals, p < 0.001). Each experiment is representative of three independent trials.
Fig. 2

A

![Graph showing percent survival over time with two conditions: 0 mM and 5 mM.](image1)

B

![Bar graph comparing time between two groups: 0 mM and 5 mM.](image2)
Effect of arbutin on ROS accumulation on *C. elegans*.

Wild type N2 animals treated with 5 mM arbutin accumulated less ROS than wild type N2 control animals. (N = 24-25 times and 20 animals per group. p < 0.001)
Fig. 3
Effect of arbutin on resistance to stress in transgenic strain CF1038 *C. elegans*.

Animals were treated with 5 mM arbutin from birth to 120 hours at 20 °C and exposed to a variety of stressors. There was no survival significance with worms pretreated with or without arbutin after (A-B) 35 °C heat shock (N = 74 animals, p > 0.05), (C-D) exposure to 200 μM juglone (N = 96 animals, p > 0.05) or (E-F) UV irradiation at 1000 J/m² (N = 85-88 animals, p > 0.05). Each experiment is representative of three independent trials.
Effect of arbutin on DAF-16::GFP nuclear localization on transgenic strain TJ356.

Worms treated with 5 mM arbutin (A-B) displayed a fluorescence intensity that was significantly upregulated (E) relative to the control group (C-D). (N = 10 animals per group, p < 0.001)
Fig. 5
A

B
Effect of arbutin on relative mRNA levels of *daf-16*, *sod-3* and *hsp-16.2* of *C. elegans*.

*daf-16* (P < 0.001) and its downstream targets *sod-3* (P < 0.05) and *hsp-16.2* (P < 0.001) were all upregulated with arbutin treatment relative to the control. (N = 3 and 200 animals per group, p < 0.05)
Fig. 6
Effect of arbutin on brood size of *C. elegans*.

There was no significant difference in the number of total progeny between arbutin-treated and untreated animals (N = 10 animals per group, p > 0.05).
Fig. 7