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Research Article

Nuciferine Promotes Longevity and Fitness in *Caenorhabditis elegans* through the Regulation of the Insulin/IGF-1 Signaling Pathway

Yan Xu, Yuanxin Miao, and Rong Li

College of Bioengineering, Jingchu University of Technology, Jingmen, China

Correspondence should be addressed to Rong Li; lirong2022@jcut.edu.cn

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Nuciferine, as one of the most abundant plant-derived alkaloids, has multiple bioactivities including anti-inflammatory, antitumor, and lipid-lowering effects. Nevertheless, the antiaging effects and related mechanisms of nuciferine are rarely reported. In this study, we found that nuciferine significantly prolonged the mean lifespan of *Caenorhabditis elegans* (*C. elegans*) by 14.86% at a dose of 100 μM. Moreover, nuciferine promoted the health of *C. elegans* by increasing the body bending and pharyngeal pumping rates and reducing the lipofuscin accumulation level. Meanwhile, nuciferine enhanced stress tolerance by inducing the expression of stress-related genes or proteins. The molecular mechanism behind the antiaging effect of nuciferine occurred by downregulating the insulin/IGF-1 signaling (IIS) pathway. Our findings shed new light on the application of nuciferine for longevity promotion and human health.

1. Introduction

Aging is a series of irreversible degenerative changes that occurs in organisms over time, including damage accumulation, functional decline, difficulty in environmental adaptation, and increased incidence rate [1–3]. Aging can lead to a decrease in normal organic metabolism, damage repair ability, and resistance to external stress, ultimately affecting physical health. Therefore, aging is also the dominant cause of various age-related chronic diseases, especially neurodegenerative diseases, including Parkinson's disease, Alzheimer's disease, and Huntington's disease, posing a huge threat to human health [4]. With the increasing aging population, countries around the world have invested a lot of energy in treating age-related diseases in recent years. Aging has become a global challenge and one of the main factors causing social public health and economic burden [5]. However, currently, there is no medication or therapy that can cure age-related diseases, and dietary intervention has become a safe and effective strategy to delay aging and improve quality of life.

Lotus leaves are the dried leaves of the water lily family plant lotus, with a long medicinal history [6]. Lotus leaves have a wide range of clinical applications, mainly including inducing medicine to ascend, promoting yang and dispersing wind, clearing heat, resolving phlegm and stopping diarrhea, promoting dampness and detoxification, cooling blood, and nourishing the body [7]. Modern research has shown that alkaloids, as the main characteristic pharmacological component of lotus leaves and represented by nuciferine, have received widespread attention from pharmaceutical workers [8]. Pharmacological research reports have shown that nuciferine also has new pharmacological effects such as anticerebral ischemia, inhibiting bone loss, and antibacterial, antiviral, and antimelanin production. Many pharmacological effects have been patented, initiating the industrialization process of nuciferine [9]. However, whether nuciferine has a direct influence on the aging process *in vivo* remains to be determined.

Compared with traditional animal models, *C. elegans* has a smaller size, shorter lifespan, faster passage, stronger reproductive ability, and lower experimental costs, and the use

of nematodes in research does not require approval from the Animal Ethics Review Committee. Besides, the body of nematodes is transparent, which not only facilitates the observation of their growth, development, and activity, but also allows for the observation of the position and fluorescence intensity of fluorescent label proteins within the cells. Last but not least, the genetic background of nematodes is clear and the signaling pathways regulating growth, development, and aging are conserved between nematodes and mammals. These advantages make *C. elegans* a very popular and convenient animal model [10]. The signaling pathways regulating the lifespan in *C. elegans* including IIS, dietary restriction, reproductive signaling, and mitochondrial function-related signaling pathways are evolutionarily conserved [11, 12]. The IIS pathway is regulated by insulin-like peptide ligands that bind to the insulin/IGF-1 transmembrane receptor (IGFR) ortholog DAF-2, which mediates the activity of the AGE-1/phosphoinositide 3-kinase (PI3K), the serine/threonine kinases (SGK-1), and AKT-1/2, ultimately modulating the activity of DAF-16 and SKN-1 [13–15]. In addition, key transcription factors DAF-16 and SKN-1, as intersections of multiple signaling regulatory pathways, play important roles in regulating lifespan and stress resistance [16, 17]. At present, many natural compounds with antiaging activities have been screened using the model of *C. elegans*, and the classical signaling pathways mentioned above have been proved to play an important role in the antiaging effect of these substances.

In this study, *C. elegans* was utilized to explore the antiaging effects of nuciferine. Fitness-related phenotypes of *C. elegans* including body bending rate, pharyngeal pumping rate, body size, and lipofuscin accumulation were also evaluated following the lifespan determination. Furthermore, the underlying molecular mechanisms were illustrated through a series of genetic analyses.

2. Materials and Methods

2.1. Chemicals and Reagents. Nuciferine (≥98%) was purchased from Yuan Ye Biological Technology Co. Ltd (Shanghai, China), while dimethyl sulfoxide (DMSO) and hydrogen peroxide (>30%, w/w) were sourced from Ding-Guo Co. Ltd (Shanghai, China). 5-fluoro-2-deoxyuridine (FUdR) was ordered from Sigma-Aldrich (St. Louis, MO, U.S.A.). All the other chemicals and reagents were of analytical grade.

2.2. Nematode Strains and Maintenance. In this study the following *C. elegans* strains utilized were purchased from the *Caenorhabditis* Genetics Center (University of Minnesota, USA) including N2 (wild-type, Bristol), EU1 *skn-1 (zu67)IV*, TJ1052 *age-1 (hx546)II*, VC345 *sgk-1(ok538)X*, CB1370 *daf-2 (e1370)III*, PS3551 *hsf-1(sy441)I*, CF1038 *daf-16 (mu86)I*, LD1 (ldIs7 (*skn-1b/c*::*GFP* + *rol-6 (su1006)*)), TJ356 (zIs356IV (*daf-16p*::*daf-16a/b*::*GFP* + *rol-6 (su1006)*)), CL2070 (dvIs70 (*hsp-16.2p*::GFP + *rol-6(su1006)*)), CF1553 (muIs84 ((pAD76)*sod-3p*::GFP + rol-6(su1006))), and CL2166 (dvIs19 ((pAF15)*gst-4p*::GFP::NLS)). All strains were maintained at 20°C on nematode growth medium (NGM) plates seeded with live *Escherichia coli* OP50 (*E. coli* OP50) [13].

2.3. Lifespan Assay. The lifespan assays were performed according to the methods as previously described [18, 19]. In short, age-synchronized L4 larvae were transferred to a new 96-well plate (liquid S-completed medium added) and treated with 0.6% DMSO (control) or nuciferine (50, 100, and 200 μM). FUdR (150 μM) was also added to inhibit the reproduction of progeny. The viability of nematodes was then evaluated every other day until the last worm died.

2.4. Phenotypic Assays. The *C. elegans* were cultured as described above. On the 3rd, 6th, and 9th day of adulthood, the body bending rates, pharyngeal pumping, and body size of worms were determined using an inverted microscope according to our previous protocols [20]. For the lipofuscin accumulation assay, worms were harvested on the 10th day of adulthood and anesthetized with 2% sodium azide before photographing with a fluorescence microscope (Olympus, Japan). The body size and fluorescence intensity of worms were quantified by Image J.

2.5. Stress Resistance Assessment. For the oxidative stress and heat tolerance assays, the worms cultured in the lifespan assay were collected on the 7th day of adulthood and then treated with 1 mM hydrogen peroxide at 20°C or incubated at 37°C, respectively [21]. The survivors were scored every 2 h until all the worms died.

2.6. Measurement of Superoxide Dismutase (SOD) and Catalase (CAT) Activities, and Glutathione (GSH) Content. The wild-type worms were harvested and homogenized on ice to obtain the supernatant liquid on the 3rd day of adulthood [22]. The SOD and CAT activities, and GSH levels were determined using the commercially available kits (Nanjing Jiancheng Bioengineering Inst., Nanjing, China) based on the manufacturer's recommendations. Then, the results were normalized to the protein content using a BCA kit (Nanjing Jiancheng Bioengineering Inst., Nanjing, China) [13, 23].

2.7. Subcellular Localization of DAF-16::GFP and SKN-1::GFP. The synchronized L4-stage nematodes of transgenic strains LD1 (SKN-1::GFP reporter) and TJ356 (DAF-16::GFP reporter) were treated with 100 μM nuciferine or DMSO for 2 h, and then loaded on slides for photographing [24]. For the specific analysis methods for subcellular localization, our previous reports can be referred to [18, 23].

2.8. Fluorescence Measurement of GFP Proteins. The synchronized L1 larvae of the transgenic strains carrying HSP-16.2::GFP reporter (CL2070), SOD-3::GFP reporter (CF1553), and GST-4::GFP reporter (CL2166) were incubated with 100 μM nuciferine or DMSO for 72 h and then photographed. Before microscopy observation, the young

adults of CL2070 strains were exposed to heat shock at 37°C for 2 h and then allowed to recover at 20°C for 4 h [25]. The protein expression levels were analyzed using Image J.

2.9. Gene Expression Assays by qRT-PCR. According to the standard protocols (Tiangen Biotech, China), the wild-type adults were collected and homogenized for extracting total RNA after a 3-day treatment. Afterwards, cDNAs were synthesized by reverse transcription with HiScript II Q RT SuperMix for qPCR (+gDNA wiper) (Vazyme Biotech, China). Then, qRT-PCR was performed using SuperReal PreMix Plus (SYBR Green) (Tiangen Biotech, China) on a QuantStudio 3.0 PCR system (ABI, USA). The PCR reactions were performed according to the methods previously described [22, 23]. In addition, the expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method for each sample. For qRT-PCR, the *actin-1* gene was used as the internal control. The primers used in this study are listed in Table S1.

2.10. Statistical Analysis. The lifespan curves were analyzed by the log-rank (Kaplan–Meier) test using SPSS software 19.0 (SPSS Inc., Chicago, USA). Statistical analyses were conducted using GraphPad Prism 9.0 (GraphPad Software, Inc., San Diego, CA, U.S.A.). One-way or two-way analysis of variance (ANOVA) with Tukey's multiple comparisons test or *t*-test was applied for comparing different groups, and $p < 0.05$ ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$) was considered statistically significant.

3. Results

3.1. Nuciferine Prolonged the Lifespan of C. elegans. We investigated the longevity-promotion effect of nuciferine by exposing wild-type worms to a serial concentration of nuciferine at 20°C. Compared to the control, nuciferine at 50, 100, and 200 μM significantly increased the mean lifespan of wild-type worms by 8.06%, 14.86%, and 11.89%, respectively, with the maximum mean lifespan observed at 100 μM ($p < 0.001$ for all, Figure 1(b) and Table 1).

3.2. Nuciferine Promoted the Healthspan of C. elegans. The body bending rate and pharyngeal pumping rate of worms are two important parameters to describe the aging process [25]. Although the body bending and pharyngeal pumping rates of worms declined with increasing age from the 3rd day to the 9th day, nuciferine-treated worms displayed a higher level than the control group at each stage measured (Figures 2(a) and 2(b), $p < 0.05$). In worms, lipofuscin is a product of intracellular lysosome digestion, which is deposited in the gut of worms and accumulates more with age [26]. It is an important biological marker of worms' aging. The results showed that nuciferine notably decreased the lipofuscin accumulation level of worms by 10.42%, 19.93%, and 22.21% at concentrations of 50, 100, and 200 μM, respectively (Figures 2(c) and 2(d), $p < 0.0001$). Besides, the growth and development indicators of nematodes, body length and body width, are commonly used to evaluate the safety of drugs [27, 28]. However, no significant differences were found in body length and body width between nuciferine-treated worms and control worms (Figures 2(e) and 2(f), $p > 0.05$). The results suggested that nuciferine is nontoxic at this concentration and has no adverse effects on the growth and development of worms. Collectively, these results indicated that nuciferine could promote the fitness in addition to prolonging the lifespan.

3.3. Nuciferine Enhanced Stress Tolerance and Antioxidant Capacities in C. elegans. Currently, several reports have proven a strong correlation between lifespan extension and stress resistance [29, 30]. We tested the stress tolerance of nuciferine-treated worms to evaluate this possibility. Under oxidative stress induced by 1 mM hydrogen peroxide, the mean lifespan of worms treated with 50, 100, and 200 μM nuciferine considerably increased by 11.38%, 15.62%, and 10.85%, respectively (Figure 3(a) and Table S2, $p < 0.0001$). In the case of thermal stress assay, nuciferine markedly extended the mean lifespan of worms by 10.01%, 15.23%, and 10.58% at doses of 50, 100, and 200 μM, respectively (Figure 3(b) and Table S2, $p < 0.0001$). To reveal the mechanisms of nuciferine in enhancing the stress resistance and longevity, the effect of nuciferine on antioxidant enzyme activities was further examined. In comparison to the control, the SOD and CAT activities and GSH contents of worms treated with nuciferine were significantly increased (Figures 3(c)–3(e), $p < 0.05$). Taken together, it is conceivable that enhanced stress resistance and antioxidant ability might contribute to the longevity-promotion effect of nuciferine. In addition, due to its optimal effect on lifespan extension, we chose the dose of 100 μM nuciferine for the subsequent experiments.

3.4. Nuciferine Prolonged the Lifespan by Activating DAF-16/FOXO. DAF-16, the mammalian ortholog of the FOXO transcription factor in *C. elegans*, serves as a key transcription factor in the IIS pathway [14, 31]. After entering the nucleus, DAF-16 can activate the expression of downstream genes related to lifespan extension and stress resistance, thus playing an important role in lifespan regulation [31]. To determine whether the nuciferine-mediated lifespan-extension effect was dependent on DAF-16 activation, we examined the effect of nuciferine on the nuclear localization of DAF-16 in the TJ356 strain. The results showed that the nuclear proportion of DAF-16 was increased from 15.27% to 48.81% ($p < 0.0001$), whereas the cytosolic fraction was decreased from 50.76% to 20.89% ($p < 0.0001$) when treated with nuciferine (Figures 4(a) and 4(b)), indicating that nuciferine promoted the nuclear localization of DAF-16. Furthermore, the mRNA levels of *daf-16*, as well as its downstream targeted genes, *sod-3*, *sod-2* (superoxide dismutase), *hsp-16.2* (heat shock protein), and *ctl-1* (catalase), were significantly upregulated by nuciferine treatment (Figure 4(c), $p < 0.05$). Consistently, compared to the control group, the expression levels of SOD-3::GFP and HSP-16.2::GFP in nuciferine-treated group were significantly raised by 19.82% and 17.38%, respectively

Table 1: Effect of nuciferine on the lifespan of wild-type (N2) worms at 20°C.

Group	Mean lifespan (days ± SEM)	Percentage change	Number of worms	*P* value
Control	17.85 ± 0.38^{a}	—	158	—
Nuciferine 50 μM	19.29 ± 0.43^{b}	8.06%	164	0.0002
Nuciferine 100 μM	20.50 ± 0.57^{c}	14.86%	152	<0.0001
Nuciferine 200 μM	19.97 ± 0.54^{bc}	11.89%	161	<0.0001

Different letters (a, b, and c) indicated a significant difference between the two groups. p values were determined by the log-rank test using the Kaplan–Meier survival analysis ($p < 0.001$). Mean lifespan values were calculated by the log-rank test and presented as mean ± SEM by SPSS.

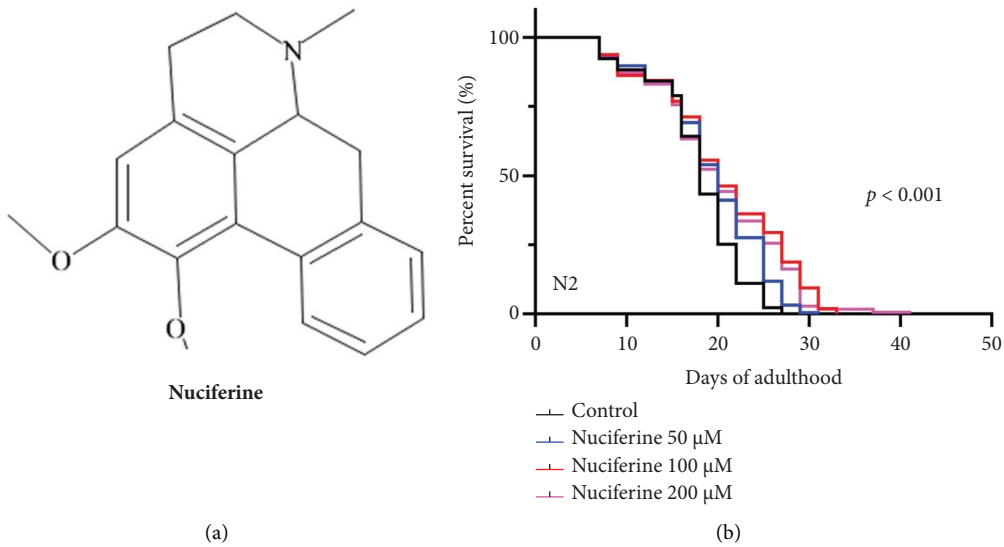

Figure 1: Effects of nuciferine on the lifespan of *C. elegans*. (a) Chemical structure of nuciferine. (b) Survival curves of N2 worms exposed to either nuciferine (50, 100, and 200 μM) or vehicle (DMSO). The log-rank (Kaplan–Meier) test was employed for statistical analysis by SPSS ($p < 0.001$).

(Figures 4(d) and 4(e), $p < 0.0001$). The abovementioned results indicated that nuciferine treatment can promote the nuclear localization of DAF-16 and activate the expression of downstream genes and proteins, which contributed to the lifespan extension of *C. elegans*.

3.5. Nuciferine Extended the Lifespan by Activating SKN-1/Nrf2. In addition to DAF-16/FOXO, SKN-1, the Nrf2 ortholog of *C. elegans,* has been reported to regulate the lifespan mainly through oxidative stress response [32, 33]. LD1 is a transgenic nematode strain with GFP-labeled protein, which can be used to observe the nuclear localization of SKN-1. The previous results indicated that nuciferine remarkably enhanced oxidative stress tolerance and antioxidant enzyme activity, so we further investigated the role of SKN-1/Nrf2 in nuciferine-induced longevity-promotion effect. The results revealed that nuciferine treatment remarkably increased the nuclear fraction of SKN-1 from 19.06% to 52.42% ($p < 0.001$), whereas the cytosolic fraction was decreased from 46.43% to 20.76% ($p < 0.01$) in the LD1 strain treated with nuciferine (Figures 5(a) and 5(b)). In addition, the mRNA levels of *skn-1*, as well as its target genes *gst-4* (glutathione S-transferase) and *gcs-1* (γ-glutamyl cysteine synthetase), were significantly upregulated by nuciferine treatment (Figure 5(c), $p < 0.05$). Meanwhile, compared with the control group, the expression of GST-4::GFP in the nuciferine-treated group increased by 17.61% (Figure 5(d), $p < 0.0001$), which was consistent with the results of *gst-4* expression. Taken together, these data illustrated that nuciferine indeed activated SKN-1 and induced oxidative defense response, and the lifespan-extension effect of nuciferine also involved the activation of SKN-1/Nrf2.

3.6. Nuciferine Prolonged the Lifespan via Downregulating the IIS Pathway. In *C. elegans*, the IIS pathway is one of the most thoroughly studied and classic signaling pathways, regulating the growth and development, stress resistance, aging, metabolism, and other aspects of nematodes [14]. Our previous results confirmed that nuciferine can activate DAF-16/FOXO and SKN-1/Nrf2, which are two key transcription factors of the IIS pathway. Therefore, we further investigated whether nuciferine prolonged the lifespan through the IIS pathway. As displayed in Figures 6(a)–6(f) and Table 2, nuciferine failed to extend the lifespan of *daf-2(e1370)III*, *age-1(hx546)II*, *sgk-1(ok538)X*, *daf-16(mu86)I*, *skn-1(zu67)IV*, and *hsf-1(sy441)I* null mutants, which were primary components of the IIS pathway, suggesting that the IIS pathway was involved in nuciferine-induced longevity. Furthermore, nuciferine significantly downregulated the expressions of *daf-2*, *age-1*, and *sgk-1* (Figure 4(c), $p < 0.001$), which were upstream targets of IIS pathway. Overall, these results illustrated that nuciferine extended the lifespan of *C. elegans* by inhibiting the IIS pathway.

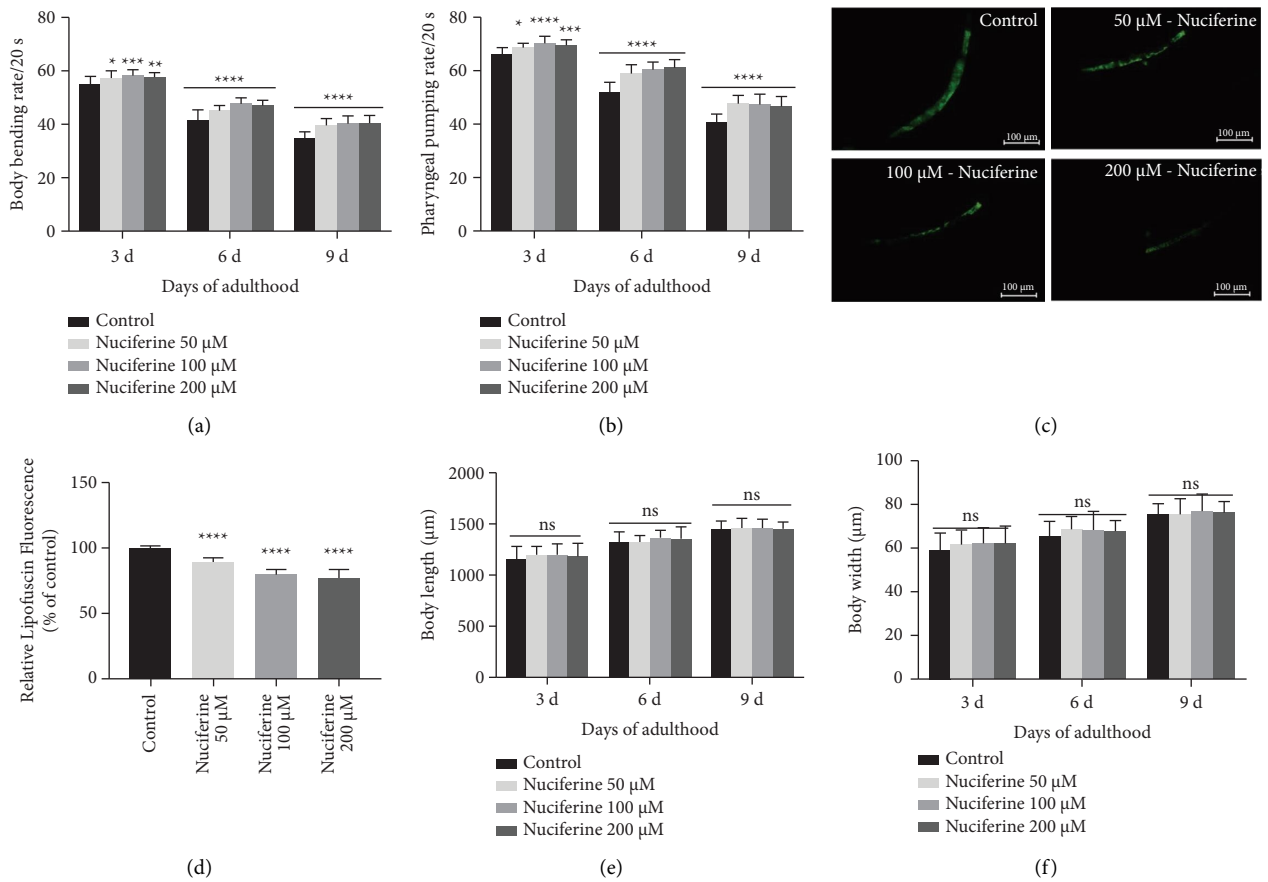

Figure 2: Nuciferine promoted the healthspan in N2 worms. Effects of nuciferine on (a) body bending rates, (b) pharyngeal pumping rates, (c) fluorescent images of lipofuscin in N2 worms on the 10th day of adulthood, (d) relative fluorescence intensity of lipofuscin, (e) body length, and (f) body width on days 3, 6, and 9 of adulthood in nematodes. Data were represented as mean ± SD, $n = 3$. Statistical significance was obtained at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ by ANOVA with Tukey's multiple comparisons test. ns: not significant.

4. Discussion

Nuciferine, an alkaloid found in lotus heart leaves, has been widely used in food and drug formulations in China [34]. Although nuciferine has a wide range of biological activities, there are currently no reports regarding their antiaging activity. This study established a genetic system of *C. elegans* to evaluate the longevity effect and molecular mechanism of nuciferine.

C. elegans is a commonly used model organism in the field of antiaging research. Lifespan is a basic aging indicator of *C. elegans*, which can intuitively evaluate the antiaging activity of drugs [35]. We found that 100 μM nuciferine can significantly prolong the average lifespan of nematodes. Similar to human aging, *C. elegans* also exhibit similar aging-related phenotypes. For example, in aging *C. elegans*, muscle cells throughout their body gradually lose vitality, leading to a decrease in body movement and pharyngeal muscle activity speed [36]. As a biological marker of aging, lipofuscin accumulates continuously with age in both *C. elegans* and humans, reflecting physiological aging status [26]. Therefore, in order to comprehensively evaluate the antiaging activity of nuciferine, we measured the effects of nuciferine on the healthy phenotypes of *C. elegans*. The results showed that nuciferine increased the body bending and pharyngeal pumping rates of *C. elegans*, reduced the accumulation level of lipofuscin, and slowed down the decline of body movement ability and pharyngeal muscle vitality of *C. elegans*, thereby prolonging the healthspan of *C. elegans*. In addition, nuciferine did not have harmful effects on the body size of *C. elegans*, indicating that their effect on prolonging lifespan is not at the cost of affecting the normal growth and development of *C. elegans*. This study confirmed the benefits of nuciferine on the healthspan of *C. elegans*, which helps to enrich people's understanding of the antiaging effects of these compounds.

The aging free radical theory has suggested that endogenous oxygen free radicals produced by cells can attack biological macromolecules such as proteins, lipids, and nucleic acids, leading to cumulative damage to the body and inducing aging [37]. Therefore, clearing free radicals is beneficial for delaying aging. The results of this study showed that the SOD and CAT enzyme activities and reduced glutathione levels in *C. elegans* treated with nuciferine were significantly increased, which play an important role in clearing free radicals and maintaining redox homeostasis of

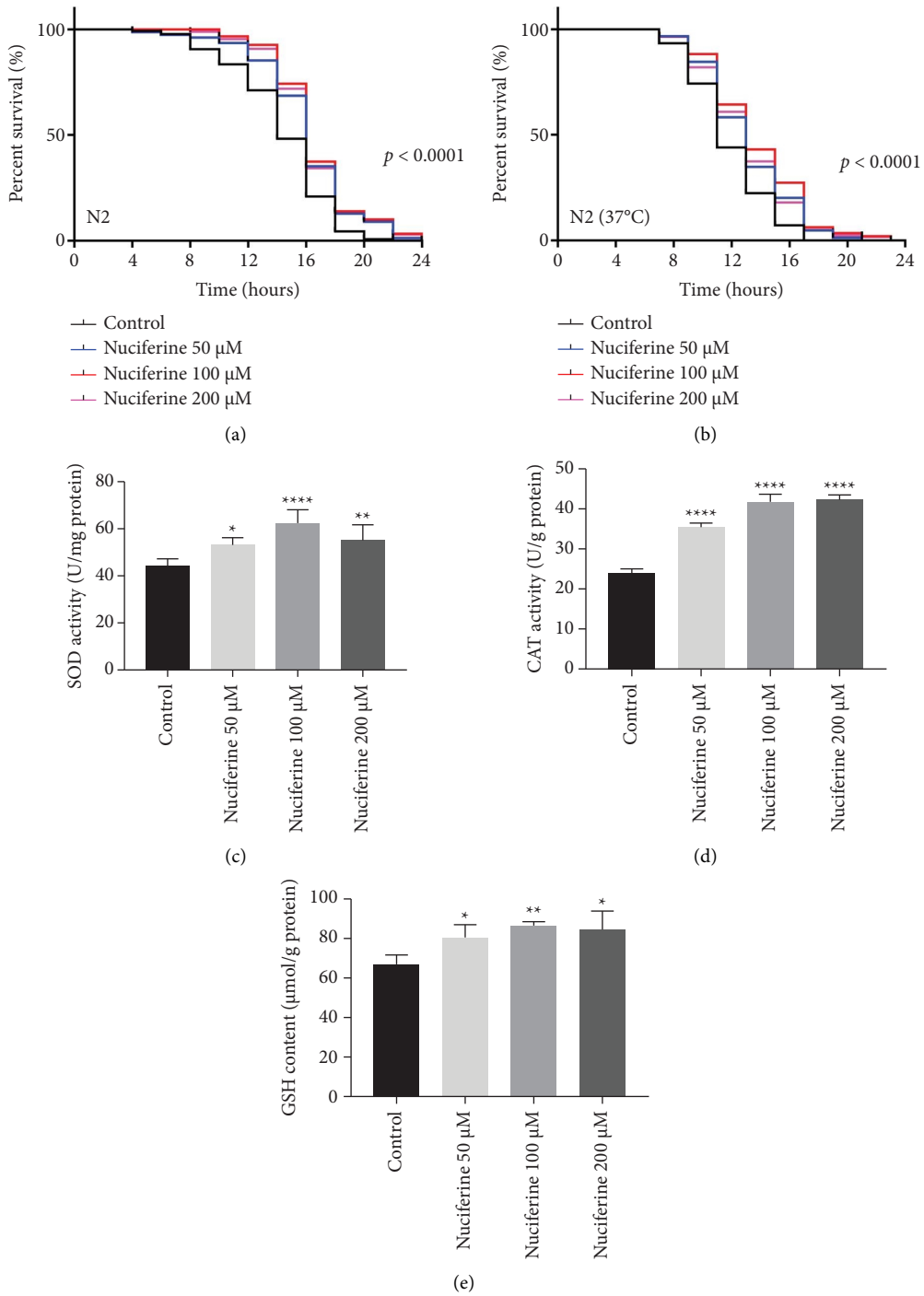

Figure 3: Effects of nuciferine on the stress resistance and antioxidant abilities in the wild-type worms. (a) Survival curves of the N2 nematodes under oxidative stress. The log-rank (Kaplan–Meier) test by SPSS was employed for statistical analysis ($^{***}p < 0.001$ and $^{****}p < 0.0001$). (b) Survival curves of the N2 nematodes under thermal stress. Survival rates were analyzed by the log-rank (Kaplan–Meier) test using SPSS and detailed data are summarized in supporting information Table S2. (c) The SOD enzyme activities were quantified. (d) The CAT enzyme activities were quantified. (e) The GSH contents were quantified. Data were represented as mean ± SD, $n = 3$. Statistical significance was obtained at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ by ANOVA with Tukey's multiple comparisons test using GraphPad Prism 9.0.

C. elegans. In addition, the lifespan extension of *C. elegans* is often accompanied by an increase in resistance to external stress [38], which includes oxidative stress resistance and heat stress resistance. Therefore, we further investigated the effect of nuciferine on the oxidative stress resistance of *C. elegans*. The results showed that under 1 mM hydrogen peroxide stimulation, the lifespan of *C. elegans* would sharply decrease, which is consistent with the report of Meng et al. [39]. This is because high concentrations of hydrogen peroxide can cause a sharp increase in ROS levels in

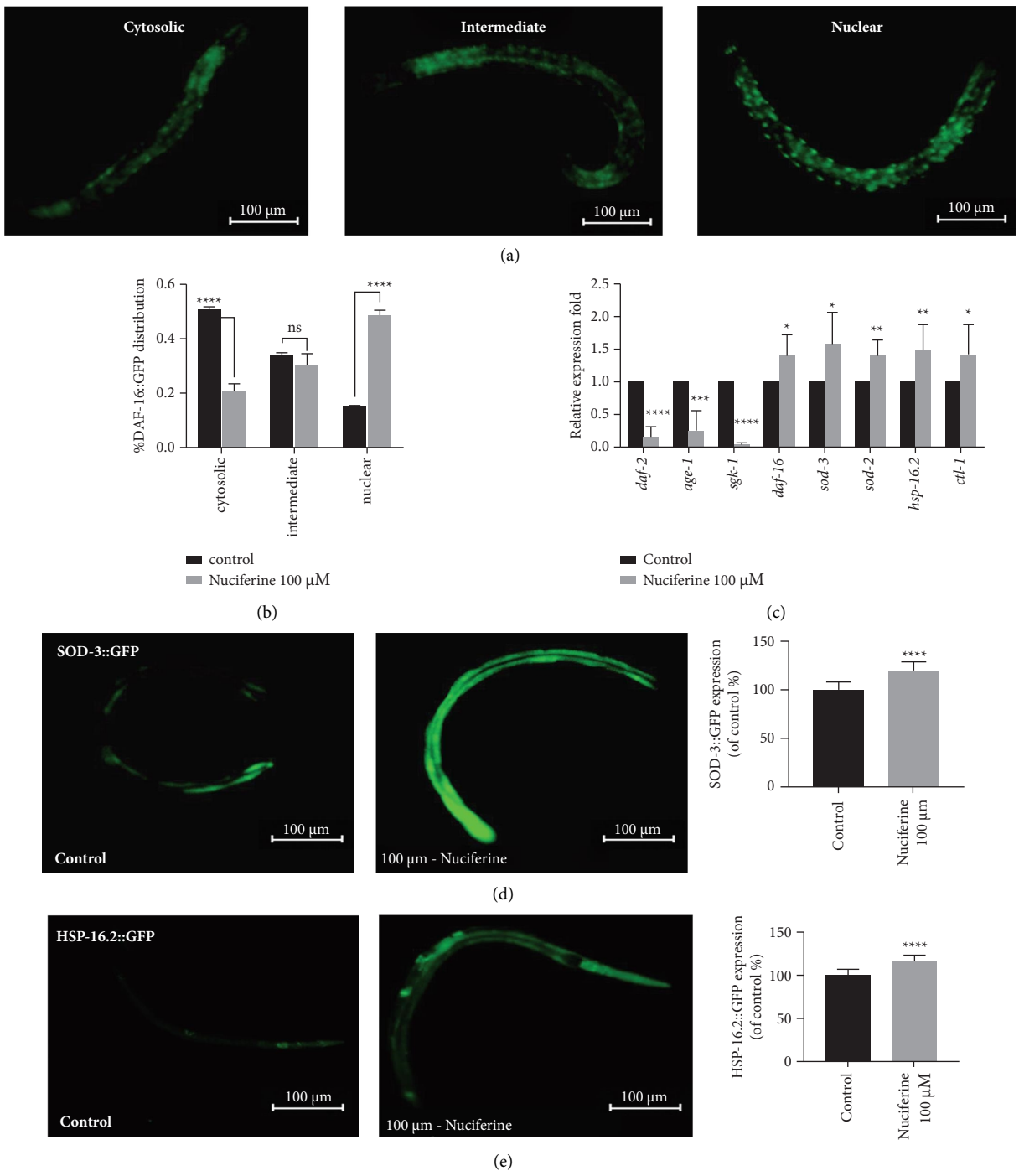

Figure 4: Nuciferine-activated DAF-16/FOXO and its downstream genes and proteins. (a) Typical fluorescence images of DAF-16::GFP distribution in TJ356: cytosolic, intermediate, and nuclear. (b) Nuciferine promoted the nuclear translocation of DAF-16. (c) The mRNA expression of *daf-2*, *age-1*, *sgk-1*, *daf-16,* and its downstream genes. (d) The fluorescent images and quantification of SOD-3::GFP expression in CF1553 strain. (e) The fluorescent images and quantification of HSP-16.2::GFP expression in CL2070 strain. Data were represented as mean ± SD, $n = 3$. Statistical significance was obtained at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ by the *t*-test. ns: not significant.

C. elegans, disrupting the redox homeostasis. However, compared with the control group, nuciferine extended the average lifespan of *C. elegans* under oxidative stress. In addition, heat stress resistance is closely related to longevity [40], and the results indicated that nuciferine prolonged the average lifespan of *C. elegans* under heat stress. Heat shock proteins (HSPs) in *C. elegans* are closely related to heat stress resistance [41], and the results showed that nuciferine significantly upregulated the expression of heat shock protein HSP-16.2.

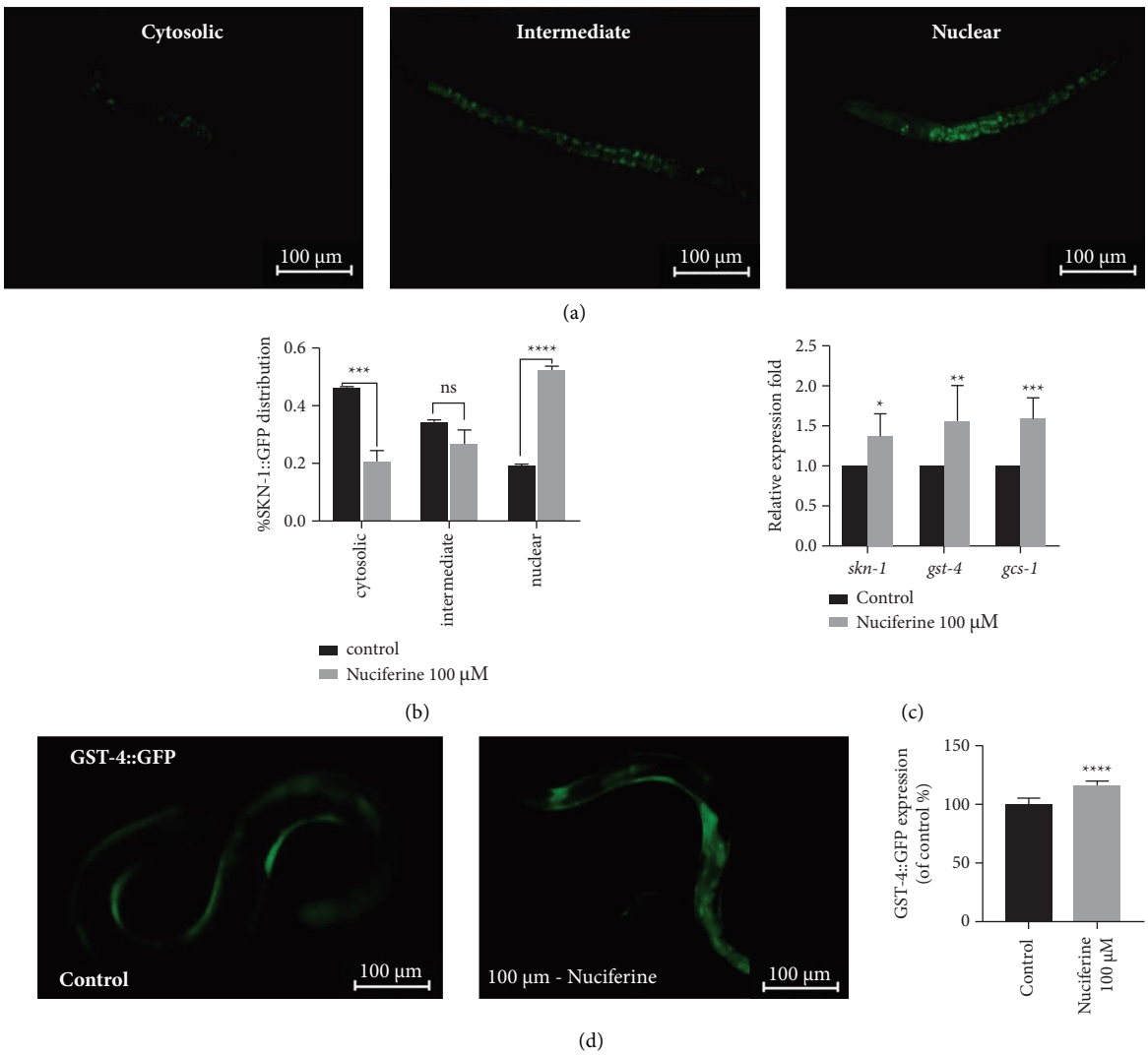

Figure 5: Nuciferine-activated SKN-1/Nrf2 and its downstream genes and proteins. (a) Typical fluorescence images of SKN-1::GFP distribution in LD1: cytosolic, intermediate, and nuclear. (b) Nuciferine promoted the nuclear translocation of SKN-1. (c) The mRNA expression of *skn-1* and its downstream genes. (d) The fluorescent images and quantification of GST-4::GFP expression in CL2166 strain. Data were represented as mean ± SD, $n = 3$. Statistical significance was obtained at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ by the *t*-test.

In *C. elegans*, DAF-16 encodes a direct homolog of mammalian FOXO and regulates growth, development, metabolism, protein stability, and stress response [14]. In addition, DAF-16 is predicted to induce the expression of stress-related genes in its nuclear transfer [14, 42]. Considering that nuciferine can prolong the lifespan of *C. elegans* and enhance their stress resistance, we further investigated the role of DAF-16/FOXO in nuciferine-mediated longevity. The results showed that nuciferine could induce the translocation of DAF-16 protein from the cytoplasm to the nucleus, which indicated that nuciferine was involved in the posttranslational regulation of DAF-16. It was supported by the fact that nuciferine significantly increased the expression of downstream genes *sod-3* (encoding SOD), *sod-2*, *hsp-16.2*, and *ctl-1* (encoding CAT) in DAF-16. In addition, the *C. elegans* treated with nuciferine showed a corresponding increase in SOD-3::GFP and HSP-16.2::GFP expression, confirming that nuciferine prolonged the lifespan and promoted stress tolerance by activating downstream targets of DAF-16/FOXO.

SKN-1 can regulate the lifespan of *C. elegans* from the late embryonic stage by activating the cell's defense response to oxidative stress. Conversely, SKN-1 functional deficient mutants are sensitive to oxidative stress and exhibit a short lifespan phenotype [42, 43]. Meanwhile, the nuclear translocation of SKN-1 enhances the antioxidant stress capacity of *C. elegans* [44]. Given that nuciferine increased the antioxidant activity of *C. elegans*, we further tested whether SKN-1/Nrf2 plays an important role in nuciferine-mediated lifespan extension. As expected, nuciferine significantly promoted the translocation of SKN-1 from the cytoplasm to the nucleus and upregulated the expression of *skn-1*, indicating that nuciferine indeed activated SKN-1. The observed results of increased expression of *gst-4*

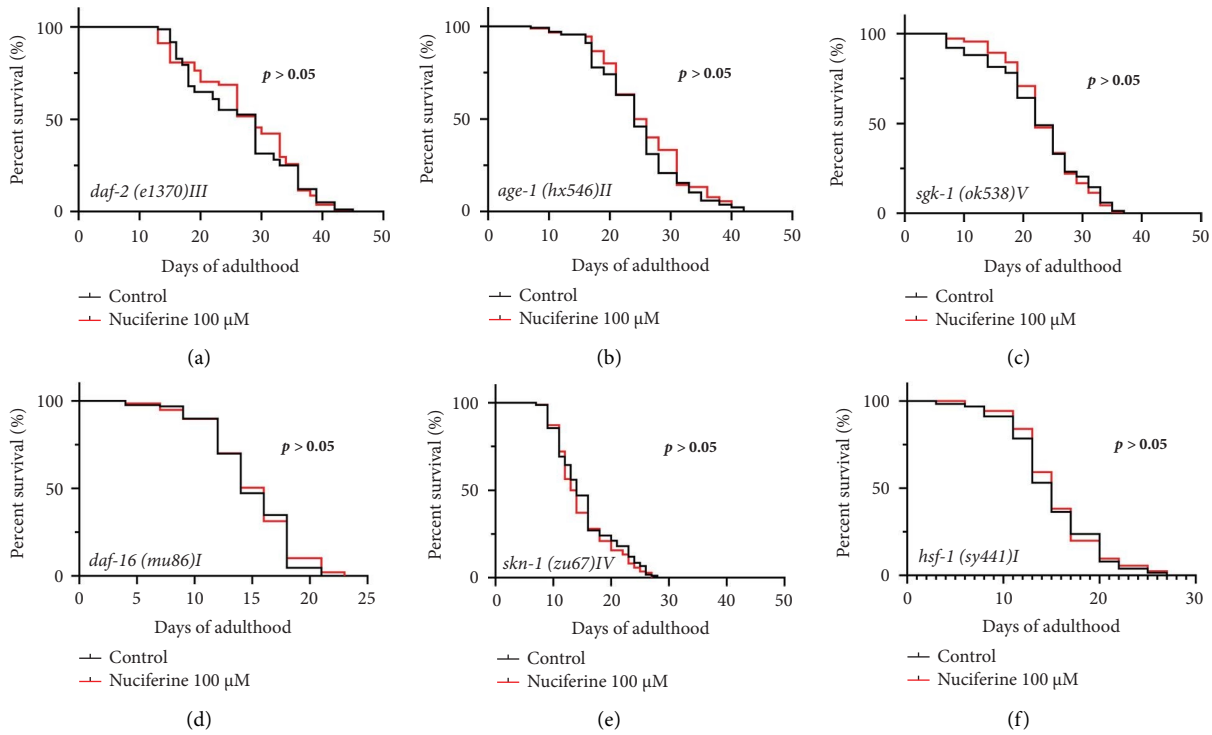

Figure 6: Nuciferine extended the lifespan via the IIS pathway. Survival curves of (a) *daf-2 (e1370)* mutants, (b) *age-1(hx546)* mutants, (c) *sgk-1 (ok538)* mutants, (d) *daf-16 (mu86)* mutants, (e) *skn-1 (zu67)* mutants, and (f) *hsf-1 (sy441)* mutants treated with nuciferine or DMSO. The log-rank (Kaplan–Meier) test was employed for statistical analysis ($p > 0.05$). ns: not significant.

Table 2: Effects of nuciferine on the lifespan of mutants related to the IIS pathway.

Strains	Group	Mean lifespan (days ± SEM)	Change (%)	Number of worms	*P* value
daf-2(e1370)III					
	Control	26.51 ± 0.70	—	156	
	Nuciferine 100 μM	27.54 ± 0.65	−3.92%	182	0.622 (ns)
age-1(hx546)II					
	Control	24.56 ± 0.61	—	135	
	Nuciferine 100 μM	25.70 ± 0.78	4.66%	90	0.2287 (ns)
sgk-1(ok538)X					
	Control	22.66 ± 0.64	—	151	
	Nuciferine 100 μM	23.45 ± 0.60	3.48%	113	0.9182 (ns)
daf-16(mu86)I					
	Control	14.74 ± 0.31	—	129	
	Nuciferine 100 μM	14.93 ± 0.32	1.29%	137	0.5786 (ns)
skn-1(zu67)IV					
	Control	15.66 ± 0.43	—	169	
	Nuciferine 100 μM	14.88 ± 0.38	−4.96%	172	0.2270 (ns)
hsf-1(sy441)I					
	Control	15.02 ± 0.40	—	126	
	Nuciferine 100 μM	15.41 ± 0.38	2.61%	125	0.6143 (ns)

P values were calculated by the log-rank test using the Kaplan–Meier survival analysis ($p > 0.05$). ns, not significant. Mean lifespan values were calculated by the log-rank test and presented as mean ± SEM by SPSS.

(encoding glutathione transferase), *gcs-1* (involved in glutathione biosynthesis), and GST-4: GFP in the nuciferine treatment group further supported this point. The antioxidant genes *gst-4* and *gcs-1* are involved in the metabolism and biosynthesis of glutathione, which helps maintain the redox homeostasis in *C. elegans*. These were in line with the increase in GSH levels and oxidative stress resistance in *C. elegans* after treatment with nuciferine, reflecting the positive regulation of the antioxidant signaling pathway by nuciferine.

In *C. elegans*, the insulin-like ligands interact with DAF-2/IIS receptor to activate several kinases, AGE-1/phosphoinositide 3-kinase (PI3K), 3-phosphoinositide-dependent kinase1 (PDK-1), serine/threonine-protein kinase (SGK-1), and

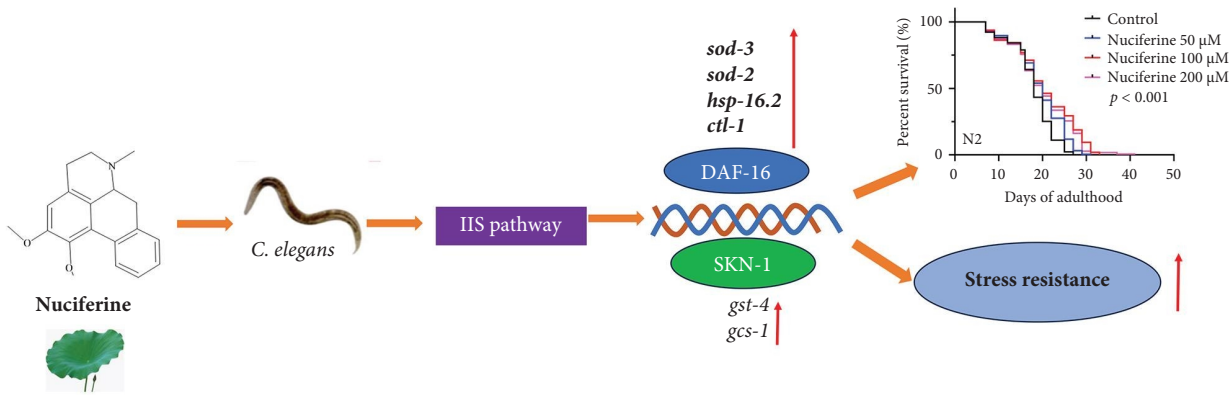

Figure 7: Summary diagram of the mechanism action of nuciferine in *C. elegans*.

AKT-1/2 to phosphorylate and inhibit the DAF-16/FOXO and SKN-1/Nrf2 [12, 42]. Our results showed that the lifespan-extension effects of nuciferine were abolished in *daf-2*, *age-1*, *sgk-1*, *daf-16*, *skn-1*, and *hsf-1* null mutants, indicating that the longevity-promotion effect of nuciferine was dependent on the IIS pathway. In addition, a few studies have reported that reduced insulin/IGF-1 signaling will promote the nuclear localization of DAF-16/FOXO and SKN-1/Nrf2 and induce the expression of genes related to longevity-promotion [12, 14]. In the current study, the expression of *daf-2*, the starting point of the IIS pathway, was remarkably decreased by nuciferine. Combining with the observation that nuciferine significantly downregulated the expressions of *age-1* and *sgk-1*, two downstream genes of *daf-2*, we concluded that nuciferine extended the lifespan by inhibiting the IIS pathway.

5. Conclusion

This study confirmed that nuciferine has a beneficial effect on not only lifespan but also on healthspan in *C. elegans* through the activation of stress-related transcription factors SKN-1, DAF-16, and their downstream target genes by regulating the IIS pathway (Figure 7). Our extensive evidence illustrating the longevity-beneficial effects of nuciferine, warrants its recognition to serve as a novel natural alkaloid for the treatment of aging and age-related diseases.

Abbreviations

C. elegans: *Caenorhabditis elegans*
IIS: Insulin/IGF-1 signaling
DMSO: Dimethyl sulfoxide
SOD: Superoxide dismutase
CAT: Catalase
GSH: Glutathione
qRT-PCR: Quantitative real-time polymerase chain reaction
HSPs: Heat shock proteins.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Additional Points

Practical Applications. In the present study, the protective effects and mechanisms of nuciferine against aging were systemically evaluated in *C. elegans* model. Our findings suggest that nuciferine may serve as a novel nutraceutical for enhancing the health of people.

Disclosure

This manuscript was submitted as a pre-print in the following link: https://www.researchsquare.com/article/rs-3803683/v1.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Yan Xu conceptualised, investigated, and validated the study, developed the methodology and the software, performed the formal analysis, and wrote the original draft. Yuanxin Miao developed the methodology and the software and performed the formal analysis. Rong Li supervised the study and wrote, reviewed, and edited the study.

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Supplementary Materials

Supplementary Table S1: primers used in this research. Supplementary Table S2: the effect of nuciferine on the stress resistance of N2 worms. (*Supplementary Materials*)

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