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Motherwort (*Leonurus japonicus* Houtt.) extends the lifespan and healthspan of *Caenorhabditis elegans* via sir-2.1 and daf-16 activation

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ABSTRACT

Introduction: As a traditional medicine, the aerial parts of *Leonurus japonicus* Houtt. (Lamiaceae), aka motherwort, have been extensively used to treat gynecological diseases. The current study was designed to investigate the longevity properties of the methanolic extract of *L. japonicus* (MLJ) using *Caenorhabditis elegans* model system.

Methods: The longevity effect of MLJ was determined by lifespan assay. Lipofuscin accumulation, thermotolerance, and body movement were measured to test the effects on the healthspan. The antioxidant capacity of MLJ was investigated by analyzing antioxidant enzyme activities, intracellular reactive oxygen species (ROS) levels, and the survival rate against oxidative stress conditions. Pharyngeal pumping rate and body length were observed to determine the effect of MLJ on aging-related factors. Transcriptional activity of daf-16 was observed under fluorescence microscopy using a transgenic mutant carrying DAF-16::GFP transgene.

Results: MLJ could significantly prolong the median and maximum lifespan of worms. In addition, MLJ reduced the accumulation of lipofuscin in aged worms and delayed the age-dependent decrease in locomotion and thermotolerance suggesting its beneficial role in the healthspan. Also, MLJ increased the stress resistance of worms against oxidative stress and decreased intracellular ROS generation by up-regulating the activities of antioxidant enzymes. Additional genetic studies showed that MLJ failed to prolong the lifespan of worms lacking *daf-2, age-1*, *daf-16*, and *sir-2.1* genes. Moreover, in the presence of MLJ, the nuclear translocation of daf-16 was significantly increased.

Conclusion: Collectively, our results demonstrate that the anti-aging properties of MLJ might be attributed to sir-2.1 and insulin/IGF signaling-dependent daf-16/FOXO activation.

Implication for health policy/practice/research/medical education:
Motherwort was found to prolong the lifespan and healthspan of worms through activating longevity-related signals such as sir-2.1 and daf-16, and thereby could be suggested as a candidate for an anti-aging agent.

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Introduction

Leonurus japonicus Houtt. (Lamiaceae), also known as Chinese Motherwort, is distributed throughout Asia, including Korea, China, Thailand, Malaysia, and Cambodia. In Korea and China, the aerial parts of this plant have been extensively used as a traditional medicine for thousands of years to cure multiple health problems, especially gynecological diseases such as menoxenia, dysmenorrhea, and amenorrhea. In compliance with its high significance in traditional Chinese medicine, many researchers have focused their efforts on understanding the phytochemical composition and pharmacological potential of *L. japonicus*. Currently, over 300 chemical compounds have been identified in *L. japonicus*, most notably alkaloids and terpenoids (1). Findings from pharmacological studies suggest that *L. japonicus* and its isolated compounds have a wide range of therapeutic actions including antioxidative,

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cytotoxic, cardioprotective, analgesic, anti-inflammatory, neuroprotective, and antibacterial properties, as well as effect on the uterus (2).

Along with ethnomedical records, the protective effects of *L. japonicus* on oxidative stress, cardiovascular system, and neuronal system strongly suggest its potential for application in aging and age-associated disorders. Previously, our group found that leonurine, a main active compound of *L. japonicus*, and its synthetic derivatives prolong the lifespan of *Caenorhabditis elegans* (3). Intriguingly, it has also been reported that leonurine improves the age-associated mental and motor impairments of mice in the D-galactose-induced aging model (4). However, to date, the role of *L. japonicus* in aging process remains purely speculative. Herein, we investigated the anti-aging effects of the methanolic extract of *L. japonicus* (MLJ) using *C. elegans* model system. The effects of MLJ on prolongation of lifespan and healthspan of worms and the role of sir-2.1 and daf-16 regulation in these effects were also investigated.

Materials and Methods

Sample preparation

The plant material was obtained from the herbal market in Yeongcheon, Gyeongsangbuk-do, Republic of Korea. *L. japonicus* was identified by a botanist, Dr. Yong Deok Jeon, at the College of Pharmacy, Woosuk University, Republic of Korea. A voucher specimen (WH009) was deposited at the herbarium of the Department of Oriental Pharmacy, Woosuk University. The dried aerial parts of the plant (3000 g) were extracted by sonication with 100% MeOH for 2 hours. The resultant methanolic extract was concentrated into 15.13 g (Yield: 7.56%) using a rotary evaporator, and lyophilized it to a powder. To make the stock solution, the drug powder was dissolved in distilled water.

Caenorhabditis elegans maintenance and sample treatment

Caenorhabditis elegans strains, including Bristol N2 (wild-type), DR1572 (e1368), GR1307 (mgDf50), TJ1052 (hx546), VC199 (ok434), EU1 (zu67), and TJ356 (zIs356) were obtained from the Caenorhabditis Genetic Center (CGC; University of Minnesota, Minneapolis, MN). Worms were grown on the nematode growth medium (NGM) agar plate with bacterial food (*Escherichia coli* OP50, OD600 = 0.7) and 20 μM of 5-fluoro-2′-deoxyuridine (FUDR) to prevent progeny. The drug plates were prepared by adding MLJ stock solution to autoclaved NGM plates (at 50°C). After embryo isolation, age-synchronized L1 stage worms were transferred to NGM plates and incubated in the absence or presence of MLJ (100, 200, and 300 μg/mL) at 20°C.

Stress tolerance and lifespan assays

Lifespan assay was done using a previously described method (5). For the thermotolerance assay, the worms were incubated at 36°C for 1 hour and their survival rates were scored every hour on the 1st, 3rd, 5th, 9th, and 11th days of adulthood, respectively. To measure the resistance to oxidative stress, worms were incubated on the NGM agar plate containing 200 mM paraquat and their survival rate was recorded every hour on the 4th day of adulthood.

Measurement of antioxidant enzyme activity and intracellular ROS

Intracellular ROS was evaluated using molecular probe H_2DCF-DA (6). To evaluate the effect of MLJ on the superoxide dismutase (SOD) and catalase (CAT) activities, the homogenates of worms were prepared on the 4th day of adulthood. The SOD activity was evaluated spectrophotometrically by analyzing the decolorization of formazan employing the enzymatic reaction between xanthine and xanthine oxidase. To determine the CAT activity, the prepared homogenates were mixed with 25 mM hydrogen peroxide (H_2O_2) and incubated for 5 minutes. Then, the absorbance was measured at 240 nm.

Measurement of pharyngeal pumping, growth, and movement

On the 4th day of adulthood, single worms were transferred to a fresh NGM plate and their pharynx contractions were counted under a dissecting microscope for 1 minute. Employing an upright microscope (Eclipse Ci, Nikon, Japan), the growth of the worms were evaluated by capturing their body images on the 4th day of adulthood and calculating the length of each worm by the Nikon NIS-Elements Imaging Software (Nikon, Japan). To check the body movement, each worm was transferred to a fresh NGM plate and its motility was recorded using a dissecting microscope for 1 minute on the 1st, 3rd, 5th, 9th, and 11th days of adulthood, respectively. The traveling distance of the worms were evaluated by the Nikon imaging software.

Fluorescence microscopy

The lipofuscin level of wild-type worms and the GFP fluorescence of transgenic worms were observed as described previously (5). Briefly, the test worms were anesthetized with 2% sodium azide before microscopy observation, and fluorescence images were captured. The fluorescent signals were quantified by measuring average pixel intensity.

Statistical analysis

The data from the stress resistance lifespan assays were plotted using Kaplan–Meier analysis and the significances were calculated by log-rank test. Other data were presented as mean ± standard deviation. The statistical significance of differences was analyzed by one-way analysis of variance (ANOVA) following the Tukey test.

Results

Effects of MLJ on lifespan and healthspan of worms

First, we performed the lifespan assay to test the longevity potential of MLJ. Our results showed that MLJ treatment prolonged the lifespan of the worms in a dose-dependent manner. The mean lifespan of MLJ-treated worms was respectively extended by 13.2%, 21.1%, and 27.2% at 100, 200, and 300 μg/mL concentrations, compared to that of vehicle-treated worms (Figures 1A and 1B). The maximum lifespan was also extended to 6 days at 300 μg/mL, compared to that of vehicle treatment.

Next, we investigated the effect of MLJ on the healthspan of worms by measuring age-associated parameters such as locomotion, thermotolerance, and lipofuscin accumulation. As can be seen in Figure 1C, the MLJ-treated worms showed better performance in locomotion than untreated worms in all tested time points. We also found that the resistance of worms to heat stress sharply declined with age, while MLJ treatment significantly improved the survival of worms under heat stress conditions, suggesting the beneficial role of MLJ in maintaining thermotolerance (Figure 1D). Then, we tested the effect of MLJ on the accumulation of age pigment lipofuscin, which negatively correlated with healthspan (7). Our data showed that MLJ treatment significantly decreased the lipofuscin deposition by 31.4% at 300 μg/mL, compared with the control worms (Figure 1E). Taken together, these findings suggest that MLJ boosts the healthy longevity of worms.

Effects of MLJ on aging-related factors

Given the strong link between aging and oxidative stress, the antioxidant effects of MLJ were investigated *in vivo*. We found that MLJ treatment could increase the survival of worms against paraquat-induced oxidative stress conditions (Figure 2A). Additional studies on the activities of antioxidant enzymes revealed that MLJ could up-regulate the enzymatic activities of both SOD and CAT at all designated concentrations (Figures 2B and 2C). Consequently, worms fed with MLJ showed a significant decrease in intracellular ROS production, suggesting a possible contribution of MLJ's antioxidant capacity to its longevity effect (Figure 2D). However, MLJ treatment altered neither pharyngeal pumping rate nor body length of worms (Figures 2E and 2F). Considering the antimicrobial activity of *L. japonicus* (8), we further tested the effect of MLJ on the growth of bacterial food (*E. coli* OP50) by measuring the inhibition zone diameter, because the loss of bacterial food by the drug could

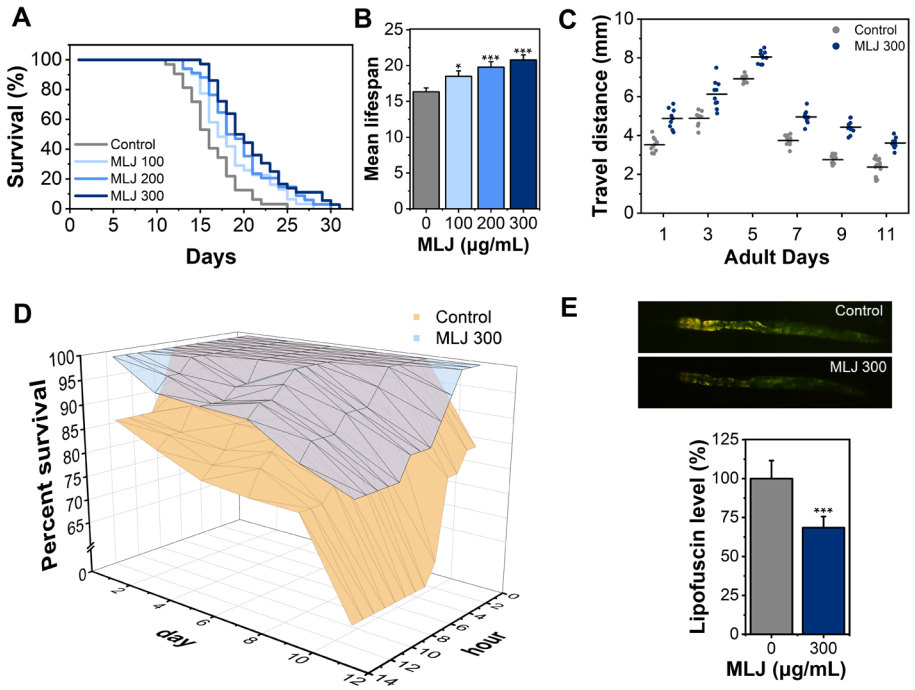

Figure 1. Effects of methanolic extract of *Leonurus japonicus* (MLJ) on lifespan and healthspan of worms. (A) Lifespan of N2 wild-type worms treated with or without MLJ (100, 200, 300 μg/mL). The survival rate was plotted using Kaplan-Meier analysis and the statistical difference between the curves was analyzed by log-rank test. (B) The mean lifespan of wild-type nematodes was calculated from the survival curves in (A). (C) The travel distance of worms on solid media plates was plotted as a time course (1st, 3rd, 5th, 9th, and 11th days of adulthood). (D) Survival of worms exposed to 35°C heat stress for 1 h. The graph represents changes in the percentage survival of the worms on the 1st, 3rd, 5th, 9th, and 11th days of adulthood, respectively. Approximately 30-45 age-synchronized worms were used in each replication. The percentage survival was calculated for each time point. (E) Fluorescence of lipofuscin in worms on the 11th day of adulthood. Ten to fifteen worms from each group were photographed (n = 45), and the fluorescence intensity was quantified using Image J software. The graph represents the average intensity of lipofuscin autofluorescence. Values are mean ± SD. Differences between groups were statistically analyzed by one-way ANOVA. * $P < 0.05$, *** $P < 0.001$ compared to the control groups. All experiments were repeated three independent times (n = 3).

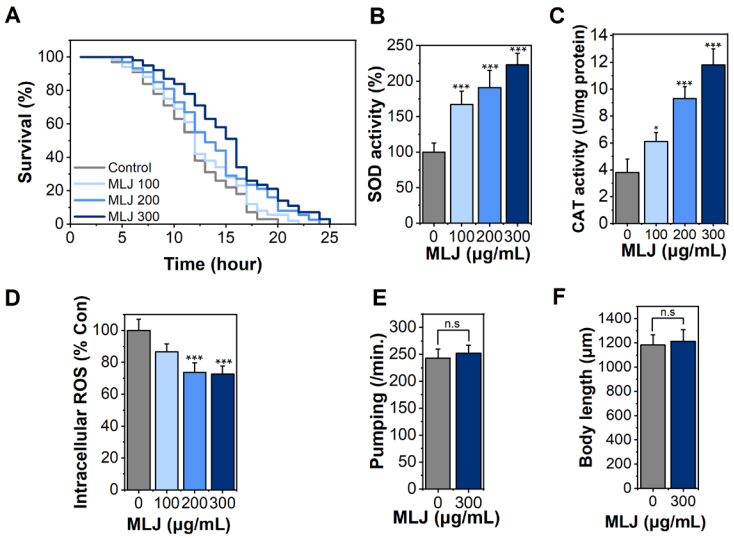

Figure 2. Effects of methanolic extract of *Leonurus japonicus* (MLJ) on aging-related factors. (A) Survival rate of nematodes exposed to oxidative stress (200 mM paraquat) on the 4th day of adulthood. The graph represents the changes in the percentage survival of the worms. Antioxidant enzymes, including (B) superoxide dismutase (SOD) and (C) catalase (CAT) activities were determined spectrophotometrically using worm homogenates. (D) Age-synchronized worms treated with or without MLJ were incubated with fluorescence probe H_2DCFDA for the measurement of intracellular ROS levels. On the 4th day of adulthood, (E) pharyngeal pumping rate was counted for a period of 60 s. (F) body length of worms was analyzed under a dissecting microscope. Values are mean ± S.D. Differences between groups were statistically analyzed by one-way ANOVA. ***$P < 0.001$ compared to the control groups. All experiments were repeated three independent times (n = 3).

also induce dietary restriction of worms. We found that the growth of *E. coli* was not affected by MLJ (Data not shown). These results indicate that the longevity potential of MLJ might not be associated with the modulation of food intake and growth.

The underlying mechanism of MLJ-mediated longevity

To clarify the molecular mechanism by which MLJ prolonged healthy aging, we investigated the lifespan of the knockout strain of worms implicated in the aging pathway. As in mammals, insulin/insulin-like growth factor-1 signaling (IIS) is believed to play a key role in controlling lifespan and stress resistance in *C. elegans*. Mutants lacking IIS cascade components, such as *daf-2*/insulin receptor and *age-1*/phosphoinositide 3-kinase (PI3K) worms had an extended lifespan and improved the tolerance to environmental stress conditions via negative regulation of daf-16, a homolog of mammalian Forkhead box O (FOXO) (9). Under stress conditions, the daf-16 transcriptional factor moves into the nucleus, where it affects the expression of genes involved in aging, development, metabolism, and immunity (10). To determine the involvement of IIS and daf-16 in MLJ-mediated longevity, we tested the lifespan of worms lacking *daf-2*, *age-1*, or *daf-16* gene. In this study, MLJ treatment failed to show a significant lifespan extension in these null mutants, which strongly suggests that the inhibition of IIS results in daf-16 activation, which might play an important role in MLJ-mediated longevity (Figure 3A). To confirm the effects of MLJ on the daf-16 modulation, we directly observed the nuclear translocation of daf-16 using mutant worms carrying daf-16-GFP fusion transgene. As expected, MLJ treatment accelerated the movement of daf-16 protein into the nuclear (Figure 3B). Based on the described antioxidant effects of MLJ, we further examined the possible association of *sir-2.1*/sirtuins and *skn-1*/Nrf2, which are known to play critical roles in promoting longevity by regulating the responses of worms to oxidative stress (11,12). It was found that the lifespan of *sir-2.1* worms treated with MLJ was similar to that of vehicle-treated control worms, while MLJ treatment was still able to lengthen the lifespan of *skn-1* null mutants (Figures 3C and 3D). These results demonstrated that sir-2.1 was also required for the longevity effect of MLJ.

Discussion

In this work, we first presented the effect of the methanolic extract of *L. japonicus* (MLJ) on longevity and its underlying mechanism using *C. elegans* model system. MLJ had a beneficial role in extending lifespan and improving health indicators through the activation of daf-16, a homolog of FOXO transcriptional factors. FOXOs play a central role in enhancing stress resistance and promoting longevity across species including *C. elegans*. In mammals, FOXOs also regulate energy metabolism and are thus considered key regulators of diabetes and obesity (13). Previous reports concerning the therapeutic effects of motherwort on metabolic disorders (14,15), raised the intriguing possibility that MLJ might modulate FOXOs in mammals.

Daf-16, a *C. elegans* homolog of FOXO, is negatively regulated by the IIS via its phosphorylation and nuclear

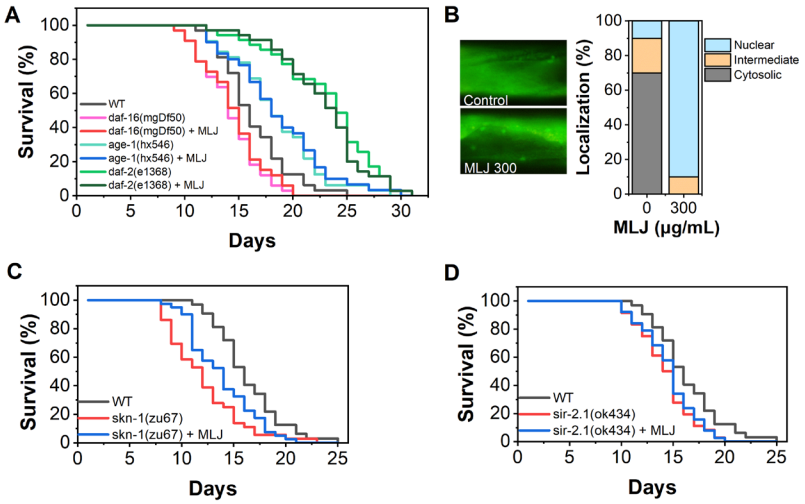

Figure 3. The underlying mechanism of MLJ-mediated longevity. (A) Lifespan analysis of wild-type and *daf-2* (e1368), *age-1* (hx546), and *daf-16* (mgDf50) worms treated with or without MLJ (300 μg/mL). (B) The fluorescence signals of transgenic worms harboring daf-16::GFP were photographed under a fluorescence microscope. Obtained images were manually analyzed and represented as the percentage of worms showing cytosolic, intermediate, and nuclear localization of daf-16. The lifespan of wild-type and knockout worms such as (C) *sir-2.1*(ok434) and (D) *skn-1*(zu67) in the presence or absence of MLJ (300 μg/mL) was plotted as a survival curve using Kaplan-Meier analysis. Statistical difference between the curves was analyzed by log-rank test. All experiments were repeated three independent times (n = 3).

exclusion (16). Also, under oxidative stress conditions, sir-2.1 can bind to daf-16 and induce its deacetylation resulting in direct activation of daf-16 and consequent lifespan-extension, independent of IIS pathway in *C. elegans* (17). Since our findings showed the involvement of both IIS components and sir-2.1 in MLJ-mediated longevity, MLJ treatment likely induces daf-16 activation through both IIS pathway and sir-2.1, independently.

Previously, we reported that leonurine, an active compound of *L. japonicus,* could extend the lifespan of worms (12.5% at 100 μM) (3). In the current study, MLJ treatment increased the lifespan to 27.2%, which was more than 2 folds higher, compared to leonurine alone. Thus, we could assume that other constituents of *L. japonicus*, in addition to leonurine, might also play a role in promoting longevity. Consistent with this assumption, research on leonurine has revealed that it has therapeutic potential in the mouse models of D-galactose-induced aging, ischemic stroke, and kidney injury through the activation of Nrf2 pathway, while our finding suggested that skn-1 was not essentially required for the MLJ-mediated lifespan-extension (4,18,19). Indeed, many bioactive flavones contained in *L. japonicus* such as quercetin, luteolin, and kaempferol glycosides are known to have longevity potential (20). In addition to their well-known radical scavenging activities, these compounds could activate sirtuin-1 in vertebrate cells (21). Thus, we could speculate that the polyphenols in MLJ might play roles in the resistance of worms against oxidative stress and lifespan extension through direct scavenging of ROS and sir-2.1-dependent activation of antioxidant defense mechanism (at least in part). Collectively, our results provide evidence that *L. japonicus* may contribute to boosting longevity in worms. The identification of bioactive compounds from extracts and determining their molecular mechanisms are subjects for future studies.

Conclusion

MLJ could extend both the lifespan and healthspan of worms without affecting food intake or growth. This longevity potential of MLJ was attributed to the activation of sir-2.1 and daf-16. Conclusively, motherwort seems to be a valuable agent for boosting longevity, and thus additional studies are warranted in mammalian models.

Authors' contribution

DSC designed the experiment and wrote the manuscript. WY and YJY performed experiments. YDJ, SYL, and HJ gave technical support and advice. All authors confirmed the final version for submission and approved the publication.

Conflict of interests

The authors declare no conflict of interest.

Ethical considerations

All authors carefully checked the ethical issues including plagiarism, misconduct, data fabrication, falsification, double publication, or submission redundancy.

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