A Hot Water Extract of *Sideritis scardica* Prolongs Life Span and Enhances Heat Shock Resistance in *Caenorhabditis elegans*

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**Abstract**

*Sideritis scardica* is a Lamiaceae plant that is endemic to the alpine zone of the Balkan Peninsula. The tea of *S. scardica* has been hand-down as a “tea of longevity” in the Rhodope region of Bulgaria for an unknown amount of time. In this study, we prepared a hot water extract of *S. scardica* (SHWE) and examined its effects on both life span and stress response in living tissue using *Caenorhabditis elegans* and its transgenic mutants. The life span of wild-type N2 worms was prolonged by approximately 15% at the SHWE concentration of 5 µg/mL and approximately 22% at the SHWE concentration of 50 µg/mL, as compared with the control group. The effect of SHWE on the expression of heat shock protein 16.2 (HSP-16.2) under heat stress was investigated using TJ375 worms, a transgenic mutant of *C. elegans*. In the TJ375 worms pretreated with SHWE, the fluorescence intensity of green fluorescent protein fluorescence, which indicates the expression of HSP-16.2, was significantly increased. In the assay using TJ356 worms, the worms pretreated with SHWE did not show the translocation of DAF-16, a forkhead transcription factor class O homolog, from the cytoplasm to nucleus under heat stress. Additionally, under heat stress, the pretreatment of SHWE improved the survival rate of GR1307 worms, a knockout mutant of *daf-16*. These results indicate that SHWE enhances HSP-16.2 expression through a stress-response pathway (eg, HSF-1 pathway) other than the DAF-16 pathway, resulting in a prolonged life span of *C. elegans* under heat stress.

**Keywords**

bioactivity, *Caenorhabditis elegans*, *Sideritis scardica*, heat shock response, HSP-16.2, life span extension
used as a raw material of tea and that tea is called a “tea of longevity” in the Rhodope region of Bulgaria. In addition, this plant has also been used as a traditional medicine for respiratory diseases (eg, asthma and bronchitis). Because of such therapeutic effects, S. scardica is currently attracting attention as a medicinal plant. So far, it has been reported that S. scardica has antioxidant, anti-inflammatory, and antibacterial effects, and the potential to improve symptoms of Alzheimer’s disease, as physiological functions. However, to our knowledge, there are only a few reports analyzing the physiological functions of S. scardica in vivo, especially the effects on stress response and life span.

Caenorhabditis elegans, a non-parasitic nematode, has biological characteristics such as a short life cycle and a short life span. Furthermore, the C. elegans gene sequence has high homology with the human gene sequence, so it has been used as a model organism for research on aging and life span. The aim of this study was to elucidate the antiaging effect of S. scardica. As a first step, a hot water extract of S. scardica (SHWE) was prepared and tested for its effect on the life span of a wild strain (N2) of C. elegans. As a second step, we analyzed the expression of HSP and DAF-16/FOXO using transgenic mutants of C. elegans and then investigated the bioactivities of S. scardica.

### Results and Discussion

Figure 1 shows the survival curves of N2 worms cultured on modified nematode growth medium (mNGM) agar plates containing SHWE under normal culture conditions. The results indicated that the SHWE at concentrations of 5 and 50 µg/mL were capable of enhancing the survival of N2 worms at the L4 stage when compared with the control group (Figure 1). From the results of the assay, the average life span of the worms in the sample and control groups was calculated and shown in Table 1. Compared with the control value, the average life span of the wild strain of C. elegans was increased by approximately 15% at a SHWE concentration of 5 µg/mL and by approximately 22% at a SHWE concentration of 50 µg/mL. Among the tested groups, the maximum life span of N2 worms treated with 50 µg/mL SHWE was the longest, at 25 days.

HSP-16.2 is a small HSP in C. elegans, and its expression is induced by heat and oxidative stress. It has been pointed out that increasing the expression level of HSP-16.2 in cells has a prolonged life span effect on C. elegans. The TJ375 strain of C. elegans is a genetically modified worm with a hsp-16.2 promoter linked to a green fluorescent protein (GFP) reporter, enabling visualization of HSP-16.2 expression in response to the stress. The effect of SHWE on HSP-16.2 expression under heat stress was tested using TJ375 worms. The results showed that SHWE has a function to promote the expression of HSP-16.2 under heat stress. In order to confirm the improvement of heat stress tolerance by SHWE treatment, the survival rate under heat stress was measured using N2 worms. As a result of culturing at 35°C, N2 worms precultured on NGM agar plates containing SHWE (500 and 1000 µg/mL) maintained a significantly higher survival rate than the control group (Figure 3). Six hours after the start of the experiment, there was a remarkable difference in the survival rate of the worms, and the difference between the sample and control groups was approximately 34 points. These results strongly suggest that SHWE enhances heat stress tolerance in C. elegans through activation of HSP-16.2 expression.

### Table 1. Effect of SHWE on Life Span of C. elegans N2 Strain

|       | N   | Average life spana (day) | P value (log-rank test) | Maximum life spanb (day) |
|-------|-----|--------------------------|-------------------------|--------------------------|
| Control | 50  | 15.67 ± 0.26             | -                       | 23                       |
| SHWE (5 µg/mL) | 50  | 17.99 ± 0.18             | <0.001                  | 23                       |
| SHWE (50 µg/mL) | 50  | 19.09 ± 0.16             | <0.0001                 | 25                       |

SHWE, hot water extract of Sideritis scardica.

aThe data are presented as mean ± standard error.

bMaximum life span is the survival day of the last worm alive.
DAF-16 is the only homolog of the FOXO family of transcription factors in *C. elegans* and has been shown to be involved in the expression of HSP-16.2.17,18 Normally, DAF-16 is phosphorylated by DAF-2 and AKT-1/AKT-2 in the insulin/insulin-like growth factor-1 signaling pathway and is present in the cytoplasm.19 Under stress conditions such as heat shock, peroxidation, and starvation, phosphorylation of DAF-16 is suppressed, and as a result, dephosphorylated DAF-16 translocates to the nucleus and induces the expression of stress-responsive genes such as *hsp-16.2* and *sod-3*18,20. To determine whether DAF-16 is involved in the activation of HSP-16 expression induced by SHWE treatment, changes in the subcellular localization of DAF-16 were analyzed using TJ356 worms, genetically modified strain of *C. elegans*. In this strain, a DAF-16::GFP fusion protein is constructed as a reporter to reveal the subcellular localization of DAF-16. Fluorescence microscopy revealed that in the control group, 56% of DAF-16 was localized in the nucleus under heat stress (Figure 4). On the other hand, in the worms precultured on NGM containing SHWE at 500 µg/mL, most of DAF-16 was present in the cytoplasm (Figure 4). These results suggest that the increase of HSP-16.2 expression by SHWE treatment under heat stress does not involve the activation of DAF-16 by nuclear translocation. Furthermore, a survival test under heat stress was performed using GR1307 worms, genetically modified strain of *C. elegans*. In this strain, a DAF-16:GFP fusion protein is constructed as a reporter to reveal the subcellular localization of DAF-16. Fluorescence microscopy revealed that in the control group, 56% of DAF-16 was localized in the nucleus under heat stress (Figure 4). On the other hand, in the worms precultured on NGM containing SHWE at 500 µg/mL, most of DAF-16 was present in the cytoplasm (Figure 4). These results suggest that the increase of HSP-16.2 expression by SHWE treatment under heat stress does not involve the activation of DAF-16 by nuclear translocation. Furthermore, a survival test under heat stress was performed using GR1307 worms, genetically modified strain in which the *daf-16* gene region was knocked out. As a result of the test, the worms pretreated with SHWE significantly maintained a higher survival rate than the control group (Figure 5). The largest difference compared with the control was about approximately 40 points and was found after 6 hours. Increased HSP expression has also been shown to be mediated by activation of the transcription factor HSF-1, which is in an inactive monomeric state in unstressed cells.21,22 These results and findings suggest that under heat stress, SHWE may promote HSP-16.2 expression through activation of HSF-1 but not the DAF-16 pathway.

This is the first report to evaluate and analyze the antiaging effect of *S. scardica*. Experiments with *C. elegans* revealed that
SHWE has a prolonged life span effect and a function to enhance stress tolerance. It has been reported that plant extracts such as blueberry, apple, and green tea show life-extending effects and stress-relieving effects in experiments using *C. elegans*\(^\text{23-25}\) In these extracts, polyphenols (proanthocyanidins) are contained as a main component, and it has been pointed out that they contribute to the antiaging effects.\(^\text{23-25}\) In another study,\(^\text{10}\) it has been reported that polyphenols are contained in the water-soluble extract of *S. scardica*. As a result of thin-layer chromatography analysis using the Folin-Ciocalteu reagent as a spray reagent, several spots suggesting phenolic compounds were detected in SHWE (data not shown). Furthermore, some phenylethanoid glycosides and flavonoid glycosides were detected in SHWE by liquid chromatography/mass spectrometry analysis with a C18 column. The antiaging effects of SHWE revealed in this study may be due partially to the phenolic compounds contained.

### Experimental

#### Materials

The dried plant of Bulgarian *S. scardica* was kindly donated by Koshin bussan. The plant used for the preparation of SHWE was pulverized via pulverizing mill (ABS-W, Osaka Chemical). The obtained powder was stored at approximately 25°C until use. All reagents used in this study were of analytical grade.

#### Preparation of SHWE

Four hundred milliliter of ultrapure water was added to the dried powder of *S. scardica* (10 g), and the suspension was autoclaved for 20 minutes at 121°C. After paper filtration, the filtrate was centrifuged at 14,000 rpm for 30 minutes at 5°C. The supernatant was freeze-dried using lyophilizer FDU-2200 (Eyela), and the obtained lyophilized powder (1.66 g) was used as SHWE. The SHWE was completely dissolved in ultrapure water and sterilized using a filter (polar diameter: 0.2 µm, Minisart, Sartorius) for sterilization. The sterilized SHWE solution was used for all experiments.

#### C. elegans Strains and Maintenance

The *C. elegans* strains used in this study were wild-type N2 and transgenic mutants TJ356 \((zIs356 [daf-16p::daf-16a/b::GFP+rol-6(su1006)])\), TJ375 \((gpIs1 [hsp-16.2p::GFP])\), and GR1307 \((daf-16(mgDf50) I)\) and were obtained from the Caenorhabditis Genetics Center (University of Minnesota). All the strains were cultured on nematode growth medium (NGM) agar plate with *Escherichia coli* OP50 as a feed and kept in an incubator at 20°C.\(^\text{26}\) To prevent bacterial contamination, streptomycin at a final concentration of 275 µg/mL was added to the NGM agar plate. Age-synchronization of the worms was performed according to the method of Guerrero-Rubio et al.\(^\text{26}\)

#### Life Span Assay Using N2 Worms

L4 larvae of age-synchronized N2 worms were transferred via a platinum wire to mNGM (peptone free) agar plates containing 1 mL of SHWE solution to the final concentrations of 5.0 and 50 µg/mL. Ultrapure water sterilized by filtration was used as a control solution. The mNGM plates contained 200 µM 2′-deoxy-5-fluorouridine to prevent the growth of progeny and streptomycin to prevent bacterial contamination. The worms were cultured at 25°C, and their viability was determined every 2 days. The worms were scored as dead if they failed to respond to a gentle stimulus delivered via a platinum wire.

#### Assay of HSP-16.2 Expression

Each TJ375 worm L1 larvae was placed on the mNGM agar plate supplemented with SHWE to give final concentrations of 100, 500, and 1000 µg/mL, and preincubated for 72 hours at 20°C. For the control group, a medium supplemented with sterilized water was used. After that, randomly selected worms were transferred to sample-free NGM plates and maintained at 20°C. The expression of HSP-16.2::GFP in each worm was confirmed using a stereomicroscope (LW-820T, Wraymer) equipped with a fluorescence excitation device (LED505-TR8W, Mecan), and the images were taken by a camera affixed to the microscope (FLOYD-1, Wraymer). The relative fluorescence intensity was determined using Image J software.

#### Assay of Subcellular DAF-16 Localization

L1 stage of age-synchronized TJ356 worms was cultured on the mNGM under the same conditions described above for 72 hours at 20°C. After incubation, randomly selected worms were transferred to sample-free NGM plates and maintained at 18°C.
for 25 minutes at 35°C. Fifty worms per group were imaged on a fluorescence microscope (AXI-5300/TPHFL, Wrayer) with a camera affixed to the microscope (FLOYD-1, Wrayer). Distribution of DAF-16::GFP in each worm can be in the nucleus, cytoplasm, or intermediate region between the nucleus and cytoplasm. Worms were sorted and counted according to observed amounts of localized DAF-16::GFP.

Survival Assay Under Heat Stress
Age-synchronized N2 and GR1307 (DAF-16 loss-of-function mutant) worms at the L1 stage were treated with mNGM supplemented with SHWE at a final concentration of 500 µg/mL for 72 hours at 20°C. For the control group, the mNGM plate with sterilized water was used. After incubation, each strain of worm was transferred to a sample-free NGM plate and exposed for 8 hours to 35°C. The viability of worms was determined every 2 hours. The experiment was performed with 50 worms of type N2 and 70 worms of type GR1307.

Statistical Analysis
For the life span assay, the statistical significance was determined by a log-rank test. In the experiments other than the DAF-16 localization assay, the differences among groups were mined by a log-rank test. In the experiments other than the life span assay, the statistical significance was determined by a t-test.

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References
1. Niccoli T, Partridge L. Ageing as a risk factor for disease. *Curr Biol*. 2012;22(17):R741-R752. doi:10.1016/j.cub.2012.07.024
2. Beckman KB, Ames BN. The free radical theory of aging matures. *Physiol Rev*. 1998;78(2):547-581. doi:10.1152/physrev.1998.78.2.547
3. Swanson JM, Kregel KC, Oberley TD. Autophagy following heat stress: the role of aging and protein nitration. *Autophagy*. 2008;4(7):936-939. doi:10.4161/auto.6768
4. Lang IA, Scarlett A, Guralnik JM, Depledge MH, Melzer D, Galloway TS. Age-related impairments of mobility associated with cobalt and other heavy metals: data from NHANES 1999-2004. *J Toxicol Environ Health A*. 2009;72(6):402-409. doi:10.1080/15287390802647336
5. Back P, Bryce FN, Mathijssens F. ROS in aging *Caenorhabditis elegans*: damage or signaling? *Oxid Med Cell Longev*. 2012;2012:608478. doi:10.1155/2012/608478
6. Kregel KC. Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. *J Appl Physiol*. 2002;92(5):2177-2186. doi:10.1152/japplphysiol.01267.2001
7. Kim S, Koh H. Role of FOXO transcription factors in cross-talk between mitochondria and the nucleus. *J Bioenerg Biomembr*. 2017;49(4):335-341. doi:10.1007/s10863-017-9705-0
8. Sahli DAM, Brunet A. FOXO transcription factors in the maintenance of cellular homeostasis during aging. *Curr Opin Cell Biol*. 2009;20(2):126-136. doi:10.1016/j.cceb.2008.02.005
9. Todorova M, Trendafilova A. *Sideritis scardica* Griseb., an endemic species of Balkan peninsula: traditional uses, cultivation, chemical composition, biological activity. *J Ethnopharmacol*. 2014;152(2):256-265. doi:10.1016/j.jep.2014.01.022
10. Kratchanova M, Denev P, Matthijssens F. ROS in aging *Caenorhabditis elegans* by comparative proteomics. *Oxid Med Cell Longev*. 2012;2012:608478. doi:10.1155/2012/608478
11. Tadić VM, Jeremic I, Dobrice S, et al. Anti-inflammatory, gastro-protective, and cytotoxic effects of *Sideritis scardica* extracts. *Planta Med*. 2012;78(5):415-427. doi:10.1055/s-0031-1298172
12. Tadić V, Bojović D, Arsić I, et al. Chemical and antimicrobial evaluation of *Sideritis scardica* extracts. *Molecules*. 2012;17(3):2683-2703. doi:10.3390/molecules17032683
13. Heiner F, Feistel B, Wink M. *Sideritis scardica* extracts inhibit aggregation and toxicity of amyloid-β in *Caenorhabditis elegans* used as a model for Alzheimer’s disease. *PeerJ*. 2018;6:e4683. doi:10.7717/peerj.4683
14. Lai CH, Chou CY, Ch’ang LY, Liu CS, Lin W. Identification of novel human genes evolutionarily conserved in *Caenorhabditis elegans* by comparative proteomics. *Genome Res*. 2000;10(5):703-713. doi:10.1101/gr.10.5.703
15. Jaturjan P, Chalorak P, Siamgcham T, et al. *Holothuria scabra* extracts possess anti-oxidant activity and promote stress resistance and lifespan extension in *Caenorhabditis elegans*. *Exp Gerontol*. 2018;110:158-171. doi:10.1016/j.exger.2018.06.006
16. Mendenhall AR, Tedesco PM, Taylor LD, Lowie A, Cyper JR, Johnson TE. Expression of a single-copy hsp-16.2 reporter predicts life span. *J Gerontol A Biol Sci Med Sci*. 2012;67(7):726-733. doi:10.1093/gerona/glr225
17. Lin K, Dorman JB, Roslan A, Kenyon C. daf-16: An HNF-3/forkhead family member that can function to double the lifespan of *Caenorhabditis elegans*. *Science*. 1997;278(5341):1319-1322. doi:10.1126/science.278.5341.1319
18. Walker GA, Lithgow GJ. Lifespan extension in *C. elegans* by a molecular chaperone dependent upon insulin-like signals. *Aging Cell*. 2003;2(2):131-139. doi:10.1046/j.1474-9728.2003.00045.x
19. Henderson ST, Johnson TE. Daf-16 integrates developmental and environmental inputs to mediate aging in the nematode Caenorhabditis elegans. *Curr Biol.* 2001;11(24):1975-1980. doi:10.1016/S0960-9822(01)00594-2
20. McElwee J, Bubb K, Thomas JH. Transcriptional outputs of the Caenorhabditis elegans forkhead protein DAF-16. *Aging Cell.* 2003;2(2):111-121. doi:10.1046/j.1474-9728.2003.00043.x
21. Crombie TA, Tang L, Choe KP, Julian D. Inhibition of the oxidative stress response by heat stress in Caenorhabditis elegans. *J Exp Biol.* 2016;219(Pt 14):2201-2211. doi:10.1242/jeb.135327
22. Dayalan Naidu S, Dinkova-Kostova AT. Regulation of the mammalian heat shock factor 1. *Febs J.* 2017;284(11):1606-1627. doi:10.1111/febs.13999
23. Wilson MA, Shukitt-Hale B, Kalt W, Ingram DK, Joseph JA, Wolkow CA. Blueberry polyphenols increase lifespan and thermotolerance in Caenorhabditis elegans. *Aging Cell.* 2006;5(1):59-68. doi:10.1111/j.1474-9726.2006.00192.x
24. Sunagawa T, Shimizu T, Kanda T, Tagashira M, Sami M, Shirasawa T. Procyanidins from apples (Malus pumila Mill.) extend the lifespan of Caenorhabditis elegans. *Planta Med.* 2011;77(2):122-127. doi:10.1055/s-0030-1250204
25. Prasanth M, Sivamaruthi B, Chaiyasut C, Tencomnao T. A review of the role of green tea (Camellia sinensis) in antiphotoaging, stress resistance, neuroprotection, and autophagy. *Nutrients.* 2019;11(2):474-498. doi:10.3390/nu11020474
26. Guerrero-Rubio MA, Hernández-García S, García-Carmona F, Gandía-Herrero F. Extension of life-span using a RNAi model and in vivo antioxidant effect of Opuntia fruit extracts and pure betalains in Caenorhabditis elegans. *Food Chem.* 2019;274(15):840-847. doi:10.1016/j.foodchem.2018.09.067