**Natural Product Sciences**
21(2) : 128-133 (2015)

# *Lindera obtusiloba* Extends Lifespan of *Caenorhabditis elegans*

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**Abstract** – *Lindera obtusiloba* has been widely used as a traditional medicine for the treatment of lots of diseases, including abdominal pain, bruise, and hepatocirrhosis. Here in this study, we elucidated the lifespan-extending effect of methanolic extract of *Lindera obtusiloba* (MLO) using *Caenorhabditis elegans* model system. We found that MLO has potent lifespan extension activities under normal culture condition. Then, we determined the protective effects of MLO on the stress conditions such as osmotic, thermal and oxidative stress. To reveal possible mechanism of MLO-mediated lifespan, we further investigated the effect of MLO on the antioxidant enzyme activities and intracellular ROS levels. Our results demonstrated that superoxide dismutase and catalase activities were significantly up-regulated by MLO treatment, resulted in reduced intracellular ROS levels. In this work, we also tested whether MLO-mediated longevity activity was associated with aging-related factors such as food intake and growth. Our data revealed that both of pharyngeal pumping rate and body length were significantly shifted by MLO treatment, indicating these factors were involved in MLO's lifespan-extension effects. Although MLO induces reduction in food intake, the body movement of MLO-fed aged worms was not decreased, compared to untreated control worms, indicating MLO might extend lifespan without affecting healthspan.
**Keywords** – Lifespan-extension, Anti-aging, Stress tolerance, *Caenorhabditis elegans, Lindera obtusiloba*

## Introduction

Aging is a complex process and many factors such as environmental, nutritional, and genetic issues can influence the aging process. Due to its complexity, it is not easy to explain the mechanism of aging and to develop anti-aging medications. However, researches on the aging have risen sharply over the past decade. In recent years, anti-aging studies on the traditional herbal medicines and their active compounds have received increasing attention. Because they are promising candidates for the treatment of aging-associated diseases. Previous reports also support that many compounds such as ginsenoside, curcumin, and resveratrol have potent lifespan extension properties in mammals as well as invertebrates.[1]

*Lindera obtusiloba* is widely distributed in northeastern asia. There are many compounds thtat were isolated from *L. obusiloba* and these have been verified their various pharmarcological effects. Lignans and neolignan such as linderin A, (+)-xanthoxyol, pluviatilol, actiforin, (+)-syringaresinol, and phenolic glycosides such as tachioside, isotachioside, koaburaside were isolated from the stems of *Lindera obtusiloba*, and some of them have been found to possess anti-allergic activities.[2,3] In addition, (+)-episesamin from *L. obtusiloba* found to possesses anti-inflammatoy, anti-neoplastic and antifibrotic effects.[4-6] Lee's group also found that secoisolariciresinol derivatives from the stem of *L. obtusiloba* have neuroprotective effects *in vitro*. Moreover, *L. obtusiloba* extract is known to have anti-diabates and hepatoprotective properties.[7,8] However, studies on the lifespan extension activity of *L. obtusiloba* are extremely limited until now. In this regards, this study was undertaken to validate the lifespan extension activity of *L. obtusiloba* using *Caenorhabditis elegans* model system.

*C. elegans* is an excellent experimental model for screening and developing therapeutic agents with lots of advantages compared to other animal models.[1,9] Especially in aging research, *C. elegans* has become a widely accepted model due to their short lifespan, morphological, simplicity, ease of maintenance and genetic manipulation.[10]

In the present study, we determine whether methanolic

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extract of *Lindera obtusiloba* (MLO) has longevity properties in *C. elegans* under normal culture condition as well as stress condition. In addition, we analyzed antioxidant properties of MLO in vivo by checking antioxidant enzyme activities and intracellular ROS levels. Moreover, effects of MLO on the aging-related factors such as food intake and growth rate were investigated.

## Experimental

**Plant material and sample preparation** – The plant materials were purchased from Hainyakupsa (Chonbuk, South Korea) in June 2010. A voucher specimen (WME068) has been deposited at the Department of Oriental Pharmacy, College of Pharmacy, Woosuk University. An extract was obtained twice from the dried branch (1000 g) with 12,000 mL of MeOH under sonification for 2 h. The resultant methanolic extract was concentrated into 36.5 g (Yield : 3.65%) using a rotary evaporator. The sample was lyophilized and then stored at −20 °C until use.

**Experimental animals** – Bristol N2 (wild-type) and *Escherichia coli* OP50 strain were kindly provided by Dr. Myon-Hee Lee (East Carolina University, NC, USA). All other strains were obtained from the Caenorhabditis Genetic Center (CGC; University of Minnesota, Minneapis, MN). The worms were grown at 20 °C on nematode growth medium (NGM) agar plate with E. coli OP50 as described previously.[11] To prepare plates supplemented with MLO, the stock solution in D.W. was inserted into autoclaved NGM plates (at 50 °C).

**Lifespan assay** – The lifespan assays were performed using mutants as well as wild-type at least 3 times independently at 20 °C. To obtain age-synchronized nematodes, eggs were transferred to NGM plate in the absence or presence of 20 μM and 40 μM of MLO after embryo isolation. Test worms were considered dead when they failed to respond to prodding with the tip of a platinum wire.[12] The worms were transferred to fresh NGM plate every 2 days.

**Assessment of stress resistance** – The age-synchronized N2 worms were bred on NGM agar plates with or without various concentrations of MLO. For the heat tolerance assay worms were transferred to fresh plates and then incubated at 36 °C on the 4th day of adulthood. The viability was scored over 17 h as previously described. Oxidative stress tolerance was assessed as described previously with minor modification.[13] Briefly, On the 4th day of adulthood, worms were subjected to plate containing 60 mM paraquat and then survivals were recorded over 32 h. Resistance to osmotic stress was measured by placing worms to NGM agar plate containing 500 mM NaCl on the 3rd day of adulthood.[14] Survival rate of the worms was calculated after 12 h incubation. The survival of worms was determined touch-provoked movement. Worms which failed to respond to gentle touch with a platinum wire were considered to be dead. Each test was performed at least 3 times.

**Measurement of antioxidant enzyme activities** – To assess enzymatic activity, the worm homogenates were prepared. Briefly, the wild-type worms were harvested from plate with M9 buffer on the 4th day of adulthood and washed 3 times. Then, the collected worms were resuspended in homogenization buffer (10 mM Tris-HCl, 150 mM NaCl, 0.1 mM EDTA, pH 7.5) and homogenized on ice. SOD activity was measured spectrophotometrically analysing the decolorization of formazan using enzymatic reaction between xanthine and xanthine oxidase. The reaction mixture contained 20 μL of worm homogenates and 480 μL of 1.6 mM xanthine, 0.48 mM nitroblue tetrazolium (NBT) in 10 mM phosphate buffer (pH 8.0). After pre-incubation at room temperature for 5 minutes, the reaction was initiated by adding 1 ml of xanthine oxidase (0.05 U/ml) and incubation at 37 °C for 20 min. The reaction was stopped by adding 500 μL of 69 mM SDS, and the absorbance at 570 nm was measured. SOD activity was expressed as a percentage of the scavenged amount per control. Catalase activity was calculated by spectrophotometry as previously described.[15] Briefly, the prepared homogenates were mixed with the 25 mM $H_2O_2$ and after 5 min incubation, absorbance was determined at 240 nm. Catalase activity was expressed in U/mg protein (1 unit will decompose 1.0 μM of $H_2O_2$ per min at pH 7.0 at 25 °C).

**Analysis of intracellular ROS** – Intracellular ROS in the nematodes was measured using molecular probe 2',7'-dichlorodihydrofluoroscein diacetate ($H_2$DCF-DA). Equal number of wild-type worms was incubated in the absence or presence of MLO. On the 4th day of adulthood, animals were exposed to NGM agar plate containing 30 mM paraquat for 3 h. Subsequently, 5 worms were transferred into the wells of a 96-well plate containing 50 μL of M9 buffer. Immediately after addition of 50 μL of 25 μM $H_2$DCF-DA solution resulting in a final concentration 12.5 μM, basal fluorescence was quantified in a microplate fluorescence reader at excitation 485 nm and emission 535 nm. Plates were read every 30 min for 2 h.

**Measurement of pharyngeal pumping, body length and movement** – The age-synchronized N2 worms were bred on NGM agar plates with or without various concentrations of MLO. On the 4th and 8th days of

adulthood, single worms were transferred to fresh plate followed by pharynx contractions and body movements of animals were counted under an inverted microscope for 1 min. For the growth alteration assay, photographs were taken of worms, and the body length of each animal was analyzed by the Nikon software (Nikon, Japan). All the tests were repeated at least 3 times.

**Data analysis** – In The data from the lifespan assay and stress resistance assays were plotted using Kaplan-Meier analysis and statistical significance was analyzed by log-rank test. Other data were presented as mean ± standard deviation or standard error of the mean, as indicated. Statistical significance of differences between the control and treated groups were analyzed by one-way analysis of variance (ANOVA).

## Results and Discussion

*Lindera obtusiloba*, is belong to *Lauraceae,* has been used as traditional medicine in Korea, Japan and China. Here in this study, we evaluated the longevity properties of MLO using *C. elegans* model system. First, to test the effects of MLO on the lifespan of worms, we carried out lifespan assay using wild-type N2 worms under normal culture condition. We found that MLO treatment significantly prolonged the lifespan of worms in a dose dependent manner and the mean lifespan was significantly increased about 17.6% at 250 μg/ml of MLO (***$p < 0.001$, Fig. 1, Table 1).

Since there are many evidences about correlation between longevity and the enhanced stress tolerance,[16] we determined the protective effect of MLO on the stress resistance of worms against osmotic, thermal, and oxidative conditions. In the current study, MLO-fed worms exhibited significant increase in survival rate under osmotic stress condition, compared to control worms (Fig. 2A). In addition, MLO permitted worms live longer under heat stress condition, indicating MLO could enhance thermotolerance of worms (Fig. 2B). Moreover, the results from paraquat-induced oxidative stress assay showed that MLO exposure strongly elevated the survival time of paraquat-treaeted worms compared with controls (Fig. 2C). These results suggest that the MLO's lifespan-extension activity is possibly associated with increased stress tolerance.

Previous reports have shown that reactive oxygen species (ROS) accumulation resulted in oxidative stress is closely involved in the aging process.[17] Indeed ROS-mediated oxidative stress is a major factor limiting lifespan in *C. elegans* as well as humans. Thus, here in this study, we validate the possible antioxidant capacity of MLO. Herein, we determine the effect of MLO on the antioxidant enzyme activities using worm homogenate. Our results demonstrated that MLO effectively up-regulated the activities both of superoxide dismutase (SOD) and catalase

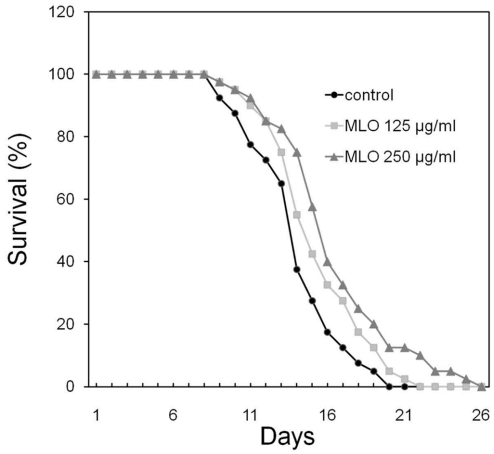


**Fig. 1.** Effects of MLO on the lifespan of wild-type N2 nematodes. Worms were grown in the NGM agar plate at 20 °C in the absence or presence of MLO. The number of worms used per each lifespan assay experiment was 40 and three independent experiments were repeated (N = 3). The mortality of each group was determined by daily counting of surviving and dead animals. Statistical difference between the curves was analyzed by log-rank test.

**Table 1.** Effects of MLO on the Lifespan of of wild-type N2 nematodes

| Treatment (μg/ml) | Mean lifespan (days) | Maximum lifespan | Change in mean lifespan (%) | Log-rank test |
|---|---|---|---|---|
| Control | 14.0 ± 10.5 | 19 | – | – |
| MLO 125 | 15.4 ± 0.6 | 21 | 9.6 | $P = 0.052$ |
| MLO 250 | 16.5 ± 0.4 | 25 | 17.6 | $P < 0.001$*** |
| MLO 500 | 13.4 ± 0.5 | 17 | –4.6 | $P = 0.135$ |

Mean lifespan presented as mean ± S.E.M. data.
Change in mean lifespan compared with control group (%).
Statistical significance of the difference between survival curves was determined by log-rank test using the Kaplan-Meier survival analysis.
Differences compared to the control were considered significant at ***$p < 0.001$

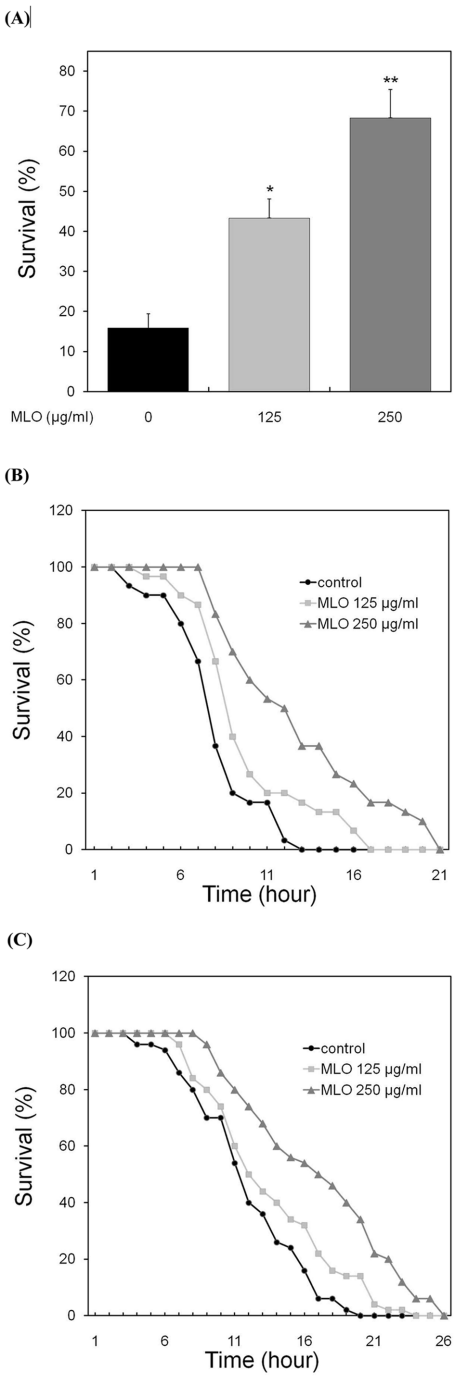


**Fig. 2.** Effects of MLO on the stress tolerance of wild-type N2 nematodes. (A) Resistance to osmotic stress was measured by placing worms to NGM agar plate containing 500 mM NaCl and survival rate was calculated after 12 h incubation. (B) To assess thermal tolerance, worms were incubated at 36 °C and then their viability was scored. (C) For the oxidative stress assays, worms were transferred to NGM agar plate containing 80 mM of paraquat, and then their viability was scored. Statistical difference between the curves was analyzed by log-rank test. Error bars represent the standard error of mean (S.E.M.). Differences compared to the control were considered signicant at $*p < 0.05$, $**p < 0.01$ by one-way ANOVA.

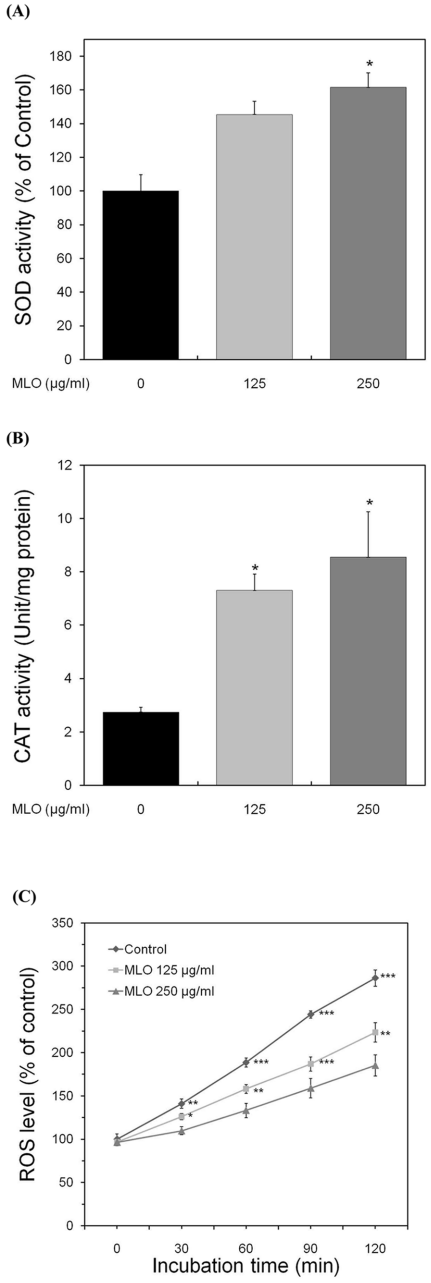


**Fig. 3.** Effects of MLO on the antioxidant enzyme activity and intracellular ROS accumulation of wild-type N2 nematodes. (A) The enzymatic reaction of xanthine with xanthine oxidase was estimated spectrophotometrically through formazan formation by NBT reduction. SOD activity was expressed as a percentage of the scavenged amount per control. (B) Catalase activity was calculated from the concentration of residual $H_2O_2$, as determined by a spectrophotometric method. Catalase activity was expressed in U/mg protein. (C) The worms were incubated with 60 mM paraquat for 3 h, and subsequently treated with the uorescent probe $H_2$DCF-DA. Intracellular ROS accumulation was quantified spectrometrically at excitation 485 nm and emission 535 nm. Plates were read every 30 min for 2 h. Data are expressed as the mean ± S.E.M. of three independent experiments (N = 3). Differences compared to the control were considered signicant at $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ by one-way ANOVA.

(Fig. 3A, B). 250 μg/ml of MLO increased SOD and catalase activities about 61.5% and 211.8%, (*$p < 0.05$) respectively. Both 1enzymes are known to play a crucial role in reduced generation of hydroxyl radicals, and may be effective to prolong the lifespan. Then, we studied the effects of MLO on the accumulation of intracellular ROS using $H_2$DCF-DA probe. As can be seen in Fig. 3C, diminished intracellular ROS level in the MLO-fed nematode was observed. MLO decreased the ROS level of worms about 35.3% at 250 μg/ml compared to control. Thus, it is conceivable that antioxidant properties of MLO might be attributed to extended lifespan and increased survival rate under oxidative stress condition.

In addition to its association with elevated stress tolerance, longevity and several aging-related factors are often interconnected in many species including *C. elegans*. Previous studies suggest that reductions in body size, and food intake prolong the lifespan of many species including *C. elegans*.[18,19] In this study, we could detect significant decrease in body length of MLO fed worms compared to control worms, indicating MLO's lifespan-extension activity is associated with change in growth rate (Fig. 4A). To address the possibility that MLO treatment might affect food intake, we also measured pharyngeal pumping rate of worms. Our results showed that there was significant difference in feeding behavior between MLO fed worms and untreated worms, indicating that MLO's longevity action is connected with dietary restriction, at least in parts (Fig. 4B). We also investigated whether MLO affects age-related changes in behavior of worms. Herein, the body movement of aged worms was not changed by MLO treatment, despite of decrease in food intake compared with untreated worms. This result suggests that MLO-induced caloric restriction did not affect the healthspan of worms.

Previously, Freise's group reported that *L. obtusiloba* suppresses insulin like growth factor-1 (IGF-1) in human cancer cell.[8] Since IGF-1 is structurally similar to insulin and plays an important role on nutritious metabolism and growth of organism, we speculate that regulation of insulin signaling might be involved in MLO-mediated longevity properties.

In summary, MLO exhibited significant lifespan extension properties in C. elegans under normal and stress condition. We found that MLO can accelerate antioxidant enzyme activities and reduce intracellular ROS concentration. We also revealed that aging-related factors such as growth and food intake are associated with MLO-mediated longevity. However, further genetic studies are required to evaluate detail anti-aging mechanism of MLO.

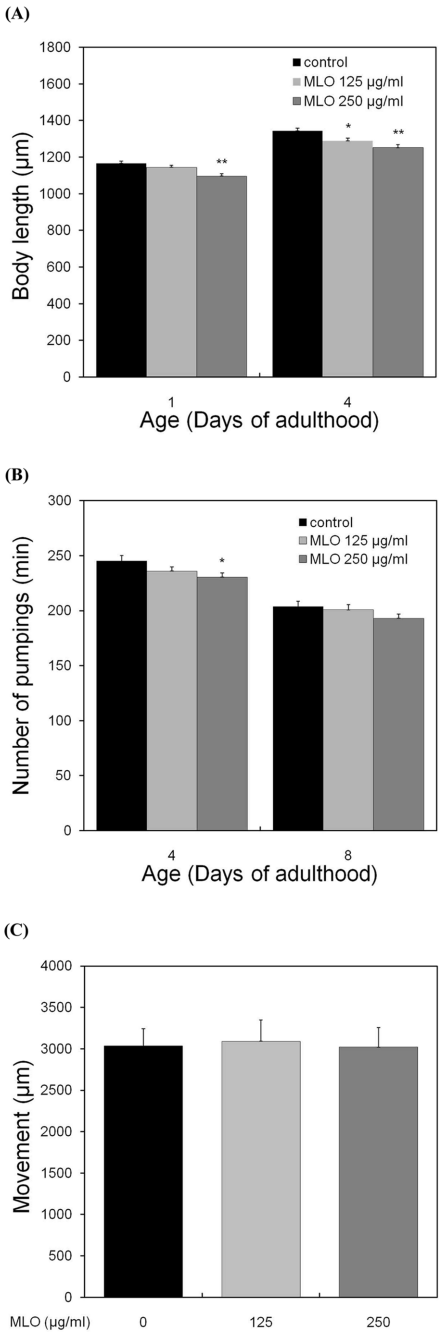


**Fig. 4.** Effects of MLO on the various aging-related factors of wild-type N2 nematodes. (A) For the growth alteration assay, photographs were taken of worms and the body length of each animal was analyzed on the 4th and 8th days of adulthood. (B) On the 4th and 8th days of adulthood, the pharyngeal pumping rates were counted under a dissecting microscope for 1 min. For the growth alteration assay, photographs were taken of worms and the body length of each animal was analyzed on the 4th and 8th days of adulthood. (C) On the 8th day, body movement was counted under a dissecting microscope for 1 min. Data are expressed as the mean ± S.E.M. The number of worms used per each experiment was 28 - 34 and three independent experiments were repeated (N = 3). Differences compared to the control were considered signicant at *$p < 0.05$ by one-way ANOVA.

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Received January 26, 2015
Revised March 20, 2015
Accepted March 20, 2015