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Moringa oleifera Prolongs Lifespan via DAF-16/FOXO Transcriptional Factor in *Caenorhabditis elegans*

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Abstract – Here in this study, we investigated the lifespan-extending effect and underlying mechanism of methanolic extract of *Moringa olelifa* leaves (MML) using *Caenorhabditis elegans* (*C. elegans*) model system. To define the longevity properties of MML we conducted lifespan assay and MML showed significant increase in lifespan under normal culture condition. In addition, MML elevated stress tolerance of *C. elegans* to endure against thermal, oxidative and osmotic stress conditions. Our data also revealed that increased activities of antioxidant enzymes and expressions of stress resistance proteins were attributed to MML-mediated enhanced stress resistance. We further investigated the involvement of MML on the aging-related factors such as growth, food intake, fertility, and motility. Interestingly, MML significantly reduced growth and egg-laying, suggesting these factors were closely linked with MML-mediated longevity. We also observed the movement of aged worms to estimate the effects of MML on the health span. Herein, MML efficiently elevated motility of aged worms, indicating MML may affect health span as well as lifespan. Our genetic analysis using knockout mutants showed that lifespan-extension activity of MML was interconnected with several genes such as *skn-1*, *sir-2.1*, *daf-2*, *age-1* and *daf-16*. Based on these results, we could conclude that MML prolongs the lifespan of worms via activation of SKN-1 and SIR-2.1 and inhibition of insulin/IGF pathway, followed by DAF-16 activation.
Keywords – Lifespan, Stress tolerance, *Moringa oleifera, Caenorhabditis elegans*

Introduction

Aging can be defined as a progressive decline in tissue and organismal function and molecular damage with age. Since aging is one of the most severe risk factor for human disease, many studies have been conducted on the aging pathologies. Although the underlying molecular basis that drives the aging process was not completely understood, exceptional insights into the plasticity of lifespan and health span have gained through the useful model system such as *Caenorhabditis elegans* (*C. elegans*).[1]

Moringa oleifera which belongs to Moringaceae, is also known as 'malunggay' in Philippines. This plant is widely distributed in tropical and subtropical regions and has been used as a traditional herbal medicine with many therapeutic potential.[2] In the Filipino diet, the horseradish can be substituted by roots of this plant and its young leaves, flowers, green pods have been used as vegetables.[3] Previous studies on this plant have noted that leaves of *M. oleifera* have various pharmacological effects including immunomodulation, antioxidant, anti-inflammation, anti-cancer, anti-diabetes and antinociception.[4-7] In addition, previous phytochemical researches have revealed that it has several antioxidant components such as ascorbic acid, carotenoids and phenolic compounds and many of them have been shown to prolong the lifespan of *C. elegans*.[8-11] However, as far as we know, there is no systemic study on the lifespan extending activity of *M. oleifera* until now.

Here in this study, we investigated the effects of methanolic extract of *M. oleifera* leaves (MML) on the lifespan of worms under normal and several stress conditions. Moreover, we tested antioxidant capacities of MML in *C. elegans* by checking intracellular ROS levels and antioxidant enzymes such as superoxide dismutase and catalase. We also analyzed several factors that are associated with aging such as growth, dietary intake, fertility and motility. We further examined the possible genetic mechanism of MML's longevity properties using knockout mutants.

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Experimental

Preparation of sample – Leaves of *Moringa oleifera* were purchased from Kangwonyakcho (Kangwon, South Korea) in March 2015. The plant was identified by Dr. Dae Keun Kim, College of Pharmacy, Woosuk University, Republic of Korea. A voucher specimen (WME090) was deposited at the College of Pharmacy, Woosuk University. The dried leaves (300 g) were extracted with 12,000 mL of MeOH under ultrasonic wave for 2 h. The MeOH extract was concentrated into 58.76 g (Yield: 19.59%) using a rotary evaporator. The resultant was lyophilized and then stored at −20 ºC for further use.

***C.* elegans strains and culture conditions** – 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, Minneapolis, MN). The transgenic strain CL2070 (*dvls70*) and CF1553 (*muls84*) were used to visualized HSP-16.2 and SOD-3, respectively. Mechanistic study was performed using several null mutant strains including AM1 (*rm1*), GR1307 (*mgDf50*), VC199 (*ok434*), EU1 (*zu67*), DR1572 (*e1368*), TJ1052 (*hx546*), and KF171 (*ks54*). The worms were grown at 20 ºC on nematode growth medium (NGM) agar plate with *E. coli* as describe previously.[12]

Lifespan assay – The lifespan assays were performed using mutants as well as wild-type at least 3 times independently at 20 ºC To obtain age-synchronized nematodes, eggs were transferred to NGM plate in the absence or presence of 25, 50 and 100 μg/mL of MML after embryo isolation. Test worms were considered dead when they failed to respond to prodding with tip of a platinum wire.[13] The worms were transferred to fresh NGM plate every 2 days.

Stress resistance assay – The age-synchronized N2 worms were bred on NGM plate with or without various concentration of MML. For the heat tolerance assay the adult day 4 worms were transferred to fresh plates and then incubated at 36 ºC. The survivals were scored over 23 h as previously described.[14] Oxidative stress tolerance was assessed as described previously with minor modification.[15] Briefly, the adult day 7 worms were subjected to plate containing 60 mM paraquat and then survival rate was recorded over 22 h. Resistance to osmotic stress was measured by placing the adult day 3 worms to NGM agar plate containing 500 mM NaCl.[16] The viability was scored over 32 h. The survival of worms was determined touch-provoked movement. Worms which failed to respond to gentle touch with a platinum wire consider to be dead. Each test was performed at least 3 times.

Measurement of antioxidant enzyme activity – To assess enzymatic activity, the worm homogenates were prepared. Briefly, the wild-type worms were harvested from plate with M9 buffer on the adult day 5 and washed 3 times. Then, the collected worms were resuspended in homogenization buffer (10 mM Tris-HCl, 150 mM NaCl, 0.1 mM EDTA, and pH 7.5) and homogenized on ice. SOD activity was measured spectrophotometrically analyzing the decolorization of formazan using enzymatic reaction between xanthine and xanthine oxidase. The reaction mixture contained 20 μL of worm homogenates, 480 μL of 1.6 mM xanthine, and 0.48 mM nitroblue tetrazolium (NBT) in 10 mM phosphate buffer (pH 8.0). After pre-incubation at room temperature for 5 min the reaction was initiated by adding 1 mL of xanthine oxidase (0.05 U/mL) and incubation at 37 ºC for 20 min. The reaction was stopped by adding 500 μL of 69 mM SDS, and the absorbance at 570 nm was measured. Catalase activity was calculated by spectrophotometry as previously described.[17] Briefly, the prepared homogenates were mixed with the 25 mM H_2O_2 and after 5 min incubation, absorbance was determined at 240 nm. The enzyme activities were expressed as a percentage of the scavenged amount per control.

Analysis of intracellular ROS – Intracellular ROS in the nematodes was measured using molecular probe 2',7'-dichlorodihydrofluoroscein diacetate (H_2DCF-DA). Equal number of wild-type worms was incubated in the absence or presence of MML. Adult day 4 worms 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_2CDF-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 aging-related factors – The age-synchronized N2 worms were bred on NGM agar plates with or without various concentration of MML. For the growth alteration assay, photographs were taken of wild-type N2 which were 4th days of adulthood, and the body length of each animal was analyzed by the Nikon software (Nikon, Japan), Also on the 4th days of adulthood, single worms were transferred to fresh plate and their pharynx contractions were counted under an inverted microscope for 1 min. To check the body movement, 8th day of adulthood worms were transferred to normal NGM plate and observed their motility using

microscope for 1 min. In order to fertility assay, N2 worms were raised from embryo as in the lifespan assay. L4 larvae were individually transferred to the everyday to distinguish the parent from the progeny. The progeny was counted at the L2 or L3 stage. All the tests were performed at least 3 times.

Fluorescence microscopy and visualization – The age synchronized transgenic nematodes including CL2070 containing a HSP-16.2::GFP reporter and CF1553 containing a SOD-3::GFP reporter were maintenance in the presence or absence of MLL. Prior to microscopy observation, CL2070 mutants were received heat shock at 36 ºC for 2 h and allowed to recover at 20 ºC for 4 h. On the 3rd day of adulthood, both transgenic worms were anesthetized with 2% sodium azide and mounted on 2% agarose pad. The GFP fluorescence of GFP-expressing populations was directly observed under a fluorescence microscope (Nikon Eclipse Ni-u, Japan). To determine the protein expression levels, photographs of the transgenic worms were taken and assayed using Image J software. All experiments were done in triplicate.

Data analysis – The data from the lifespan assay and stress resistance assay 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 group were analyzed by one-way analysis of variance (ANOVA) and post-hoc Tukey's test.

Result and Discussion

M. oleifera has been used as herbal medicine for a long time. Especially, nowadays this plant is in the limelight as "miracle tree" with rich pharmacological profiles. Here in this work, we investigated the possible anti-aging activities of MML using *C. elegans* model system. First, to estimate the effects of MML on the lifespan, lifespan assay was carried out under normal culture condition. We found that the lifespan of wild-type worm was significantly increased by 20.4% in the presence of 100 μg/mL of MML. The mean lifespan of untreated worms was 14.2 ± 0.3 days, while 17.7 ± 0.4 days for the worms 100 μg/mL of MML (Fig. 1, Table 1).

Several previous studies have indicated that the increased lifespan is closely related with improved survival rate under the stress conditions.[18,19] To test whether MML induces lifespan-extension via accelerating stress resistance, stress tolerance assay was performed under extreme stress environment such as heat, oxidative and osmotic conditions. In the thermotolerance assay, MML efficiently increased

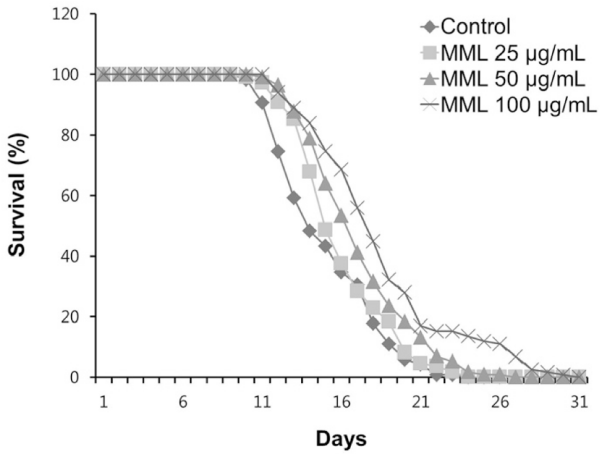

Fig. 1. Effects of MML on the lifespan of wild-type N2. The number of worms used per each lifespan assay experiment was 40 - 50 and three independently experiments were repeated (N=3).

Table 1. Effects of MML on the lifespan of wild-type N2 and various mutants.

Genotype	Mean lifespan[a]		Maximum lifespan		Change in mean lifespan[c] (%)	Log-lank Test[d]
	Untreated	Treated[b]	Untreated	Treated[b]		
Wild-type	14.2 ± 0.3	17.7 ± 0.4	23	30	24.6%	$^{***}p < 0.001$
skn-1 (*zu67*)	11.6 ± 0.4	11.0 ± 0.5	21	19	-5.2%	$p = 0.965$
daf-16 (*mgDf50*)	12.6 ± 0.4	12.1 ± 0.4	21	19	-4.0%	$p = 0.758$
daf-2 (*el368*)	17.1 ± 0.5	16.9 ± 0.7	29	27	-1.2%	$p = 0.169$
age-1 (*hx546*)	17.4 ± 0.5	17.4 ± 0.5	27	28	-0.5%	$p = 0.252$
sir-2.1 (*ok434*)	12.1 ± 0.4	12.4 ± 0.4	19	20	2.5%	$p = 0.240$
mek-1 (*ks54*)	10.9 ± 0.3	12.0 ± 0.4	18	22	10.1%	$p = 0.006$

Differences compared to the control ware consider significant at $^{***}p < 0.001$.
[a]. Mean lifespan presented as mean ± S.E.M.
[b]. MML-treated concentration was 100 μg/mL.
[c]. Change in mean lifespan compared with untreated group of each strain (%).
[d]. Statistical significance of the difference between survival curves was determined by log-lank test using the Kaplan-Meier survival analysis.

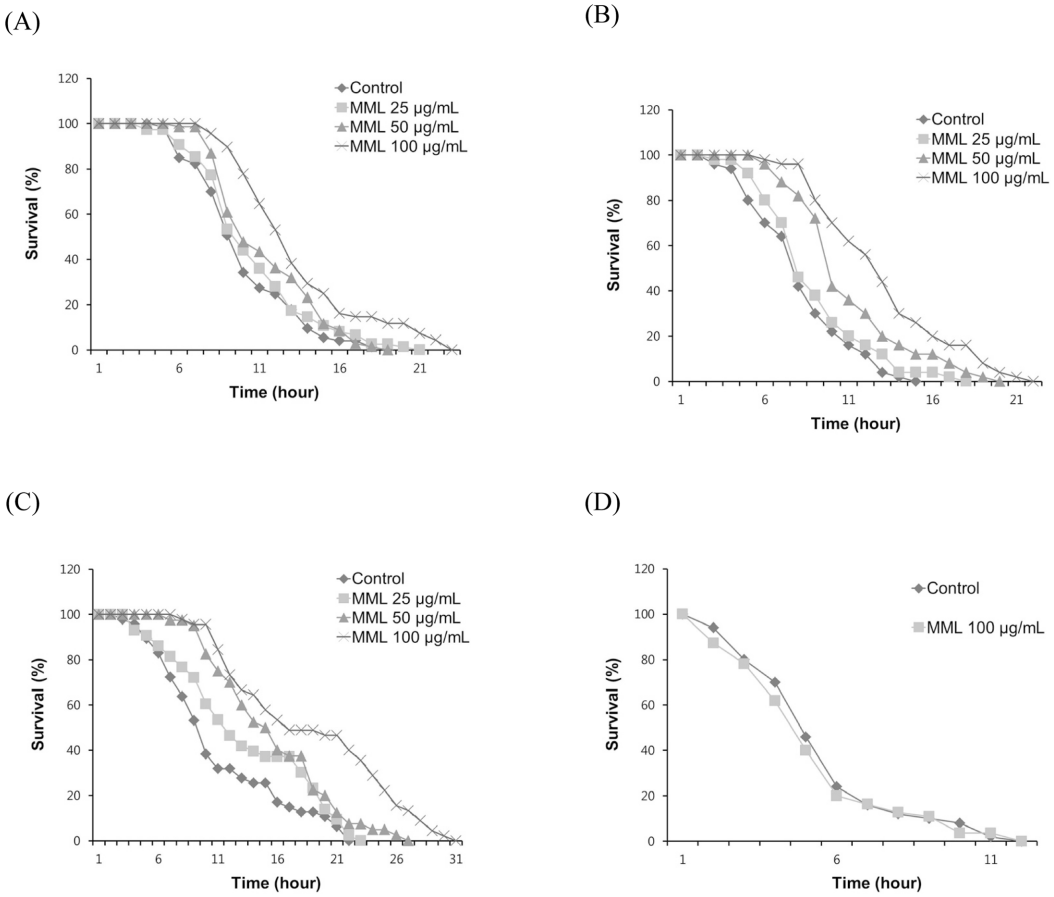

Fig. 2. Effects of MML on the stress resistance of wild-type N2. (A) To assess thermotolerance, worms were incubated at 36 °C and then their viability was scored. (B) For the oxidative stress assay, worms were transferred to NGM agar plate containing 60 mM paraquat, and then their viability was scored. (C) Resistance to osmotic stress was measured by placing 500 mM NaCl and survival rate was calculated. (D) Survival rate of *osr-1* (*rm1*) mutants was recorded under the same osmotic stress condition.

the survival rate of worms, suggesting MML might affect heat tolerance (Fig. 2A), under various stress conditions including heat stress, heat shock proteins (HSPs) are known to play a critical role in the protection of organism against molecular damage.[20] Then, we analyzed the fluorescence intensity of GFP-fused transgenic strain CL2070 to determine the involvement of HSP expression in the MML-mediated increased survival rate under heat condition. Interestingly, HSP-16.2 expression was significantly elevated in the MML-treatment worms, compared with control worms under heat shock condition (Fig. 5A).

Here in this work, MML also demonstrated strong protective effects against 60 mM paraquat-induced oxidative stress at all designated concentrations (Fig. 2B). Since antioxidant capacity of MML might be responsible for increased survival rate under oxidative stress, we measured antioxidant enzyme activities such as superoxide dismutase (SOD) and catalase (CAT) using worm homogenates. Our results showed that SOD and CAT activities were significantly enhanced by MML treatment compared with control group (Fig. 3A, B). To validate whether this MML-mediated up-regulation of SOD activity was due to change in enzyme expression, we quantified the SOD expression level using GFP-expressing transgenic strain CF1553. Interestingly, MML exposure significantly elevated the SOD-3GFP intensity, suggesting that the SOD expression was increased by MML (Fig. 5B). Using molecular probe H_2DCF-DA, we further tested whether MML treatment decreases intracellular ROS levels. As expected, MML-fed worms were appeared to show lower intracellular ROS levels compared to vehicle-treated worms (Fig. 3C).

In addition, MML-treatment significantly prolonged the survival of worms under osmotic stress condition (Fig. 2C). OSR-1 is known to regulate osmosensation, adaptation and survival in hypertonic environments via CaMKII and a conserved p38 MAP kinase signaling cascade.[21] To address the possible involvement of OSR-1 in the MML-mediated elevated osmotic stress tolerance, the hypertonic

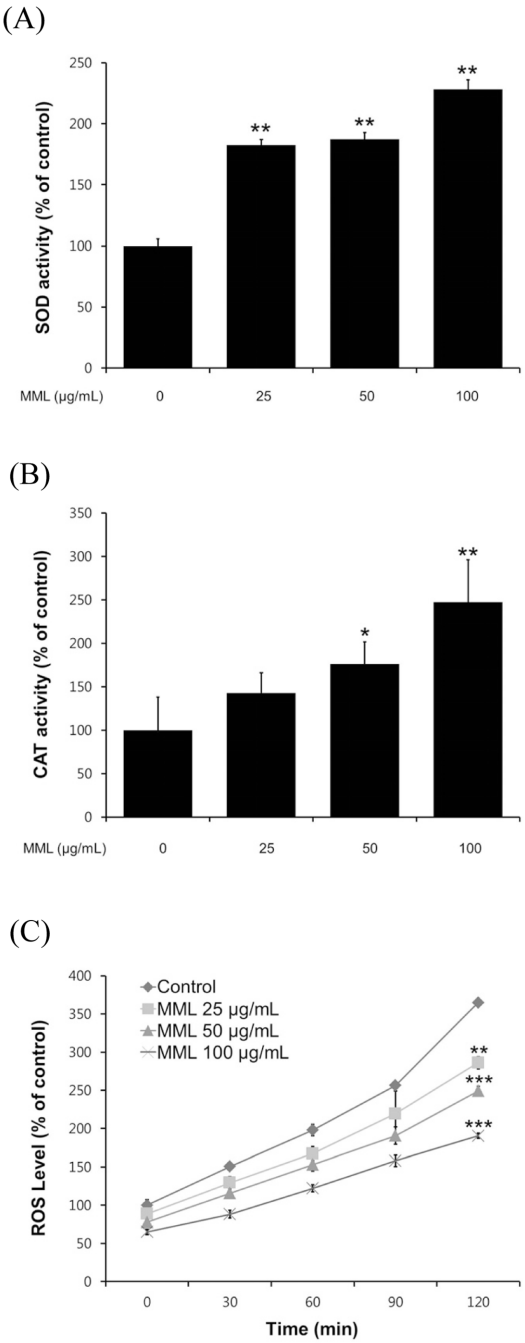

Fig. 3. Effects of MML on the antioxidant enzyme activity and intracellular ROS levels of wild-type N2. (A-B) Enzyme activity was expressed as a percentage of of the scavenged amount per control. (C) Intracellular ROS accumulation was quantified spectrophotometrically at excitation 485 nm and emission 535 nm. Plates were read every 30 min for 2 h. All data was expressed as the mean ± S.E.M. of three independent experiments (N=3). Differences compared to the control were considered significant at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ by one-way ANOVA.

assay was performed again using *osr-1* null mutants (AM1). Interestingly, the MML failed to increase the survival time of *osr-1* mutant under same osmotic condition (Fig. 2D). Based on these results, we could conclude that OSR-1 is essentially required for the MML's protective activities against osmotic stress.

Next, we studied whether MML might influence aging-related factors such as growth, food intake, and fertility. Previous evidences have suggested that these factors and lifespan-extension are closely interconnected even in *C. elegans*.[22-26] In this study, MML-fed worms decreased the body length by 11.2% at 100 μg/mL of MML compared to vehicle-fed worms, indicating lifespan extending effects of MML is related with diminished growth (Fig. 4A). To investigate the involvement of dietary restriction in MML's pharmacological action, we counted the number of pharyngeal pumping. Our data revealed that MML failed to change the pharyngeal pumping rate of worms, suggesting MML exerts longevity properties independent of affecting food intake (Fig. 4B). We further conducted fertility assay to determine whether reproductive change is involved in the MML-mediated lifespan extension. Here in this study, MML-treatment led to a decrease in total progeny (14.7% at 100 μg/mL), indicating MML possibly causes altered germline developmental events such as spermatogenesis and oogenesis which is known to affect longevity (Fig. 4D).

Since lifespan is not a sufficient parameter to describe complex aging process, we also focused on the age-associated functional declines in *C. elegans* such as body movements which represents health span. Herein, we showed that the movement of aged worm was diminished significantly and MML supplementation efficiently overcomes the reduced movement of aged worms (Fig. 4C). These results demonstrate that MML could affect not only worm's lifespan but health span.

Lastly, we dissected the underlying mechanism of MML-mediated longevity properties using selected mutant strains which are relevant to aging process. We first investigated the possible involvement of DAF-16 in MML-mediated longevity. The forkhead transcription factor, DAF-16 has several target genes which provide endurance for stress conditions and extended lifespan.[27] Interestingly, MML-treatment failed to prolong the lifespan of daf-16 mutants, indicating DAF-16 is required for MML-induced lifespan extension (Table 1). This result is consistent with our findings that expression of downstream targets of DAF-16 such as HSP-16.2 and SOD-3 were up-regulated by MML exposure (Fig. 5B). Furthermore, the DAF-16 activity has been known to be negatively modulated by gonad-dependent signals.[28] As mentioned above, MML-fed worms were defective in

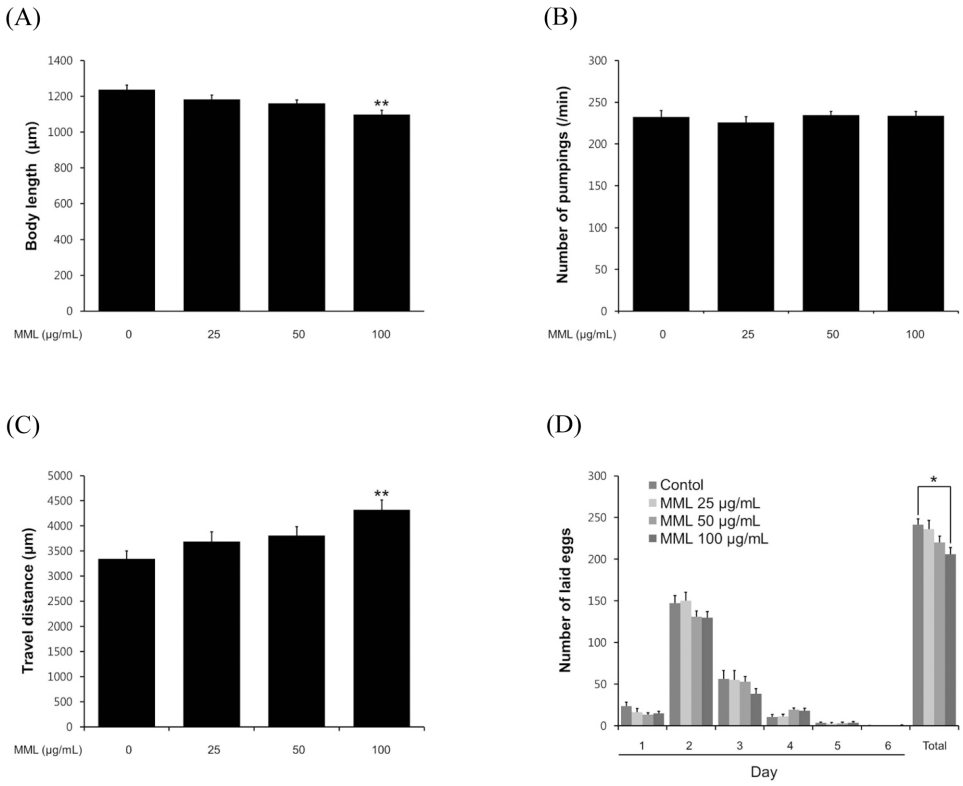

Fig. 4. Effects of MML on the various aging-related factors of wild-type N2. (A) For the grown alteration assay, photographs were taken on 4th days of adulthood worms. (B) On the 4th days of adulthood, the pharyngeal pumping rates were counted under a dissecting microscope for 1 min. (C) To check the body movement, the analysis was taken on 8th days of adulthood worms. Both assessments were analyzed by the Nikon software (Nikon, Japan). (D) In order to fertility assay, daily and total laid eggs were counted. The progeny was counted at the L2 or L3 stage. Data are expressed as the mean ± S.E.M. of three independent experiments (N=3). Differences compared to the control were considered significant at $^{*}p < 0.05$ and $^{**}p < 0.01$ by one-way ANOVA.

fertility and thus it is also possible that MML-mediated altered germline signaling might accelerate the nuclear localization of DAF-16.

Since DAF-16 can be negatively regulated by insulin/IGF signaling (IIS) pathway, we further evaluated whether MML contributes its longevity activity via IIS pathway dependent mechanism. To test this notion, we measured the lifespan of loss-of-function mutants lacking genes such as *daf-2* and *age-1*. These genes are known to play an important role in IIS pathway by encoding insulin/IGF receptor and phosphoinositide 3-kinase (PI3K), respectively. Our results showed that MML did not extend the lifespan of both mutants, indicating down-regulation of IIS pathway might cause DAF-16 activation (Table 1).

We also confirmed the possible involvement of SIR-2.1 which encodes NAD^{+}-dependent protein deacetylases with similarity to mammalian SIRT1. Previous studies have noted that overexpression of SIR-2.1 can increase the lifespan of *C. elegans* via either indirect activation of DAF-16 through inhibition of IIS signaling or direct activation of DAF-16 in a parallel to IIS signaling.[12,29,30] Herein, we observed no significant lifespan extension of sir-2.1 mutant by MML-treatment (Table 1). This result indicated that SIR-2.1 is also participated in MML-mediated DAF-16 activation. Recent study revealed that SIR-2.1 prevents age-associated metabolic decline and improves lifespan via activation of mitochondrial unfolded protein response (UPR^{mt}) in parallel to DAF-16-dependent mechanism. Since overexpression of SIR-2.1 up-regulates the UPR^{mt} gene HSP-6, which encodes a mitochondria-specific chaperone.[31,32] MML might also improve mitochondrial function which is beneficial for oxidative stress.

Previous genetic analysis suggested that the JNK pathway also participated in stress resistance and longevity as a positive regulator of DAF-16 transcriptional factor in *C.elegans*.[33,34] To identify whether JNK pathway is connected with MML's pharmacological action, we performed the lifespan assay using *mek-1* mutant which lacks the MEK-1 (MAPKK) in the JNK pathway. We demonstrated that MML extended the lifespan of *mek-1*

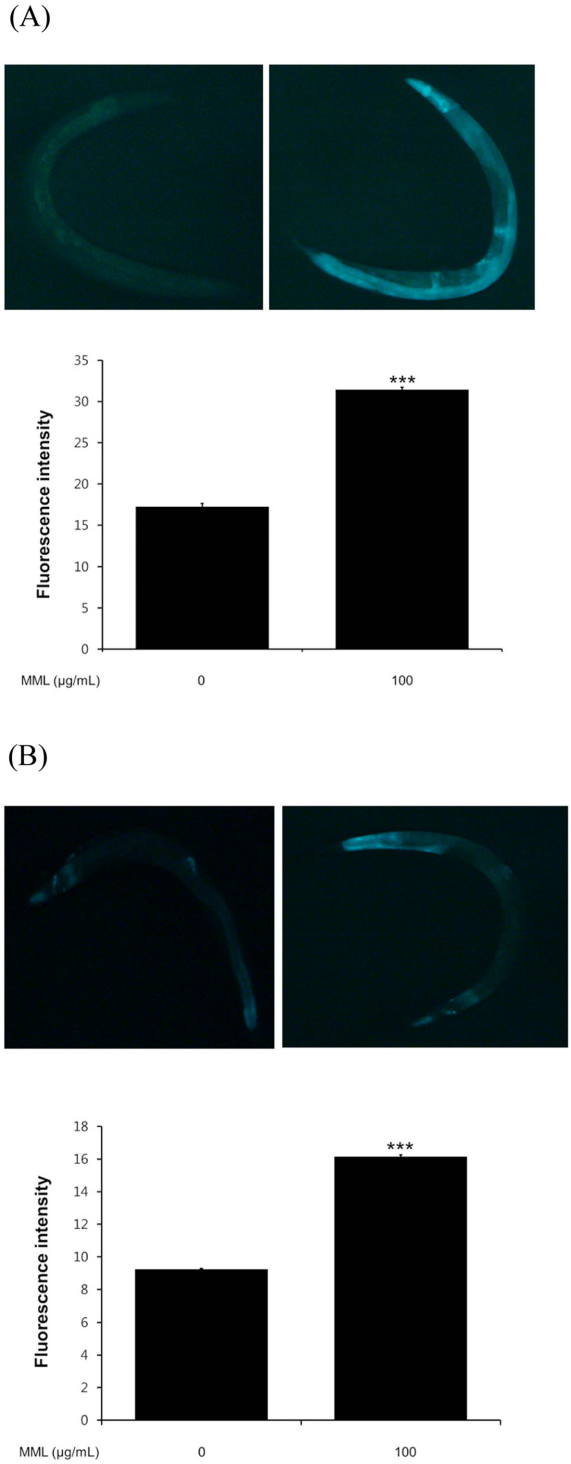

Fig. 5. Effects of MML on expression of HSP-16.1 and SOD-3 in *C. elegans* (CL2070 and CF1553 Respectively). (A) Image of HSP-16.1::GFP expression in control and MML-treated worms. (B) Image of SOD-3::GFP expression in control and MML-treated worms. The GFP intensity was quantified using Image J software by determining average pixel intensity. Data are expressed as the mean ± S.E.M. of three independent experiments (N=3). Differences compared to the control were considered significant at $^{***}p < 0.001$ by one-way ANOVA.

mutants, indicating MML might act independently of JNK pathway (Table 1). Our genetic studies revealed that SKN-1 is also involved in MML's longevity properties (Table 1). The *skn-1* gene encodes a worm ortholog of the Nrf2 that is critical for oxidative stress resistance and promotes longevity.[35]

In summary, MML prolonged the lifespan of worms under normal conditions and stress conditions. In addition, MML exhibited enhanced antioxidant enzyme capacity and suppressed ROS formation. Furthermore, we found that MML-mediated lifespan-extension is associated with aging-related factors such as growth and fertility. MML also increased body movement of aged worms, indicating that MML might also attenuate the time-dependent functional declines. Based on our genetic study, we could conclude that MML-medicated longevity property is possibly associated with increased transcriptional activity of DAF-16 via both of SKN-1 and SIR-2.1 activation and insulin/IGF signaling inhibition.

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