Limonene attenuates oxidative stress and extends longevity in *Caenorhabditis elegans*

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Abstract

In traditional medicine system, plants from the family Rutaceae have been used against aging and associated debilitating changes. Considering therapeutic potential of Limonene ($C_{10}H_{16}$), the principal component of Rutaceae family plants, present study was designed to decipher lifespan and stress modulating potential of monocyclic terpene Limonene (LM) employing genetically tractable model system *Caenorhabditis elegans*. Furthermore we tested oxidative stress tolerance and *in vivo* ROS build up which was monitored with or without exposure to LM. Additionally, involvement of transcription factor DAF-16 was investigated in terms of nuclear localization. Overall this study has implications for development potential of anti-aging pharmacological strategies which can be taken up for future investigations.

Keywords Aging, *Caenorhabditis elegans*, Limonene, Insulin/Insulin like growth factor signaling, Oxidative stress

Aging is a multifaceted process characterized by various physiological changes along with gradual decline in function. The area of bio-gerontology research has witnessed emergence of aging as genetically regulated phenomenon, which can be delayed and manipulated adopting various approaches[1]. Various phytomolecules like curcumin, resveratrol, stilbenes, cinnamic acid, flavonoids, carotenes and carotenoids have been reported to impart anti-aging effects[2]. These plant based molecules possess therapeutic properties and have least side effects compared to their chemical counterparts, making them suitable alternatives to synthetic drugs.

In traditional medicinal systems, plants of Rutaceae family have been reported to have important effects on aging and associated pathologies[3] Yet none of them have been scientifically evaluated for their anti-aging and stress modulating potential. Therefore, the present study was designed to systematically evaluate anti-aging and stress modulating effects of monocyclic terpene Limonene (LM), an active component of citrus plants employing *Caenorhabditis elegans* model system. *C. elegans* has been exploited extensively for aging studies pertaining to simple culture, rapid regeneration time, ease of genetic manipulations, identification of several gene mutations regulating evolutionary conserved pathways affecting lifespan (LS) and shared homology with human genome[4].

The present study explored the potential longevity promoting and stress modulating properties of LM utilizing advantage of *C. elegans* aging model. The genetic mechanism regulating LM mediated alteration in life span (LS) and stress level was explored using genetic variants of *C. elegans* including mutant and transgenic worms. To a larger extent LS is controlled by evolutionary conserved nutrient sensing pathway, including IIS (Insulin/Insulin like growth factor signaling). Therefore, the present study evaluated the interaction of LM with IGF genes and

expression levels of these genes which play major role in regulating stress and aging in organisms. This study paves a way towards understanding therapeutic potential of LM in terms of age defying properties and has implications for development of potential therapy for modulating age associated decline.

Experimental

C. elegans culture and strains

C. elegans strains namely: N2 Bristol (wild type), TK 22, *mev*-1 (*kn*-1); GR 1307, *daf-16* (*mgdf50*); CB 1370, *daf-2 (e1370)*, TJ 356, *daf*-16::GFP (z*Is* 356) and CF 1553, *sod*-3:: (*muIs* 84) were employed in the present study. The worms were fed over bacterial lawn of *Escherichia coli* variant OP50 at 20 ºC using the protocol described by Brenner[4]. All the *C. elegans* strains and *E. coli* OP50 were obtained from *Caenorhabditis* Genetics Centre (CGC), University of Minnesota, USA.

Test compound

Limonene was dissolved in 10% DMSO to prepare 1mM stock. Toxicity of range dependent concentrations (5µM-250µM) of LM was assessed based on survival of wild type N2 worms. Doses of 5, 25 and 50µM LM were selected for life span assays as they were found to be non-toxic to worms (Figure 1).

Life span assay

Hypochlorite bleaching method was employed in order to obtain synchronous population from gravid adults using standard protocol given in Pant et.al[5]. Worms were treated with different concentrations of LM (5, 25 and 50µM), whereas 0.1% DMSO served as control. Each test concentration of Limonene was spotted directly on 35mm Petri plate containing 2ml nutrient growth medium (NGM) with overnight grown bacterial lawn of OP50. The worms were treated

with test concentrations of LM from L1 stage. The day when L4 larvae molts into adult worm was termed as day 1. Worms were transferred to fresh plates every alternate day and scored daily by prod and watch method[6]. 50µM 5-Fluoro-2-deoxyuridine (FUdR) procured from Sigma Aldrich was used for maintaining the synchronous population by ceasing hatching of laid eggs[7]. All life span assays were performed in triplicate using 70 worms per treatment . The experiment was conducted thrice independently.

Measurement of antimicrobial effects of LM

To evaluate whether LM has any effect on bacteria and LM mediated lifespan extension is independent of change in bacterial metabolism, we employed heat killed (HK) for studying effect of 5µM LM on lifespan of worms. *E. coli* OP50 was killed by incubating at 60˚C for 30 minutes using the protocol described[8].

Assessment of anti-oxidant effects of LM

Worms were cultured on nematode growth media and assays were performed by exposing worms (age synchronized) to 5µM LM and 0.1% DMSO which was used as vehicle control. On day 2 of their adulthood, worms (n=150) were washed thrice, then collected in Phosphate buffered saline with Tween-20 (PBST) buffer. These were then exposed to 50µM 2,7-dichlorodihydrofluorescein-diacetate (H_2DCFDA) as per described protocol[9] Intensity of florescence was recorded at time intervals of 20 minutes for 2 hours at 37ºC using Agilent technologies' Plate Reader at 485/530 nm. Further, we employed *C. elegans* null mutant *mev-1(kn-1)* where in, gene encoding complex II of mitochondrial oxidative phosphorylation machinery is mutated[10]. We observed life span extension effects of 5µM LM in *mev-1(kn-1)* mutants and compared it with control (0.1% DMSO).

Determination of oxidative stress resistance

As an organism ages, decline in lifespan and health-span occurs[11]. Therefore, response to change in oxidative stress level was assessed by exposing age synchronized treated (5µM LM) and untreated control worms to methyl viologen dichloride hydrate (Sigma Aldrich). A total number of 30 L4 larvae (in triplicates) were transferred to intracellular ROS generator spotted plates. The experiment was repeated thrice.

Green Fluorescent Protein (GFP) visualization and quantification

The transgenic *C. elegans* strains TJ 356; *daf-16*::gfp (*zIs* 356) where *daf-16* is tagged with green fluorescent protein (GFP) was observed for its nuclear localization. Synchronized cohorts were obtained via hypochlorite bleaching assay and further bred as stated for LS with or without 5µM LM treatment. On day 2 of adulthood, one set of worms were subjected to heat shock by keeping at 37ºC for 30 minutes and termed as positive control. All control (n=49), positive control (n=44) and 5µM LM treated (n=50) worms were observed by placing on glass slide containing 2% agarose pad and anesthetized with 1mM sodium azide (Sigma Aldrich). All the worms (control, PC and LM treated) were then assigned to specific groups, depending upon the location of *daf-16* expression as nuclear, cytoplasmic or both[12], Similarly, CF 1553; *sod-3*::gfp+rol-6 (*muIs84*) worms were employed for their relative expression of GFP tagged *sod*-3 in control (n=32) and 5µM LM treated (n=37) transgenic worms. Micrographs were taken using GFP filter (365/420 nm) using DMI 3000 B (Germany) fluorescence microscope at 20X magnification. For CF1553, quantification of GFP expression was performed using image J software (NIH).

mRNA isolation and gene expression analysis

RNAzol (Molecular Research Centre) method was used to isolate total RNA from day 2 wild type worms using manufacturer's protocol. RNA was then reverse transcribed into complementary

DNA using revert aid kit (Invitrogen). cDNA was processed for quantitative PCR (Applied Biosystems 7900 HT) using SYBR green (Puregene) master mix to quantify the expression of *daf-16, sod-3, hsp-70, eat-2* and *pha-4* using endogenous *actin* control. The ΔΔCt method was used for calculating the relative quantification of expression of each target gene[13].

Data analysis

The life span data was analyzed using Kaplan-Meier survival analysis (log-rank test) in GraphPad Prism software. Data other than lifespan are represented as Mean±S.E. and statistically analyzed using one way ANOVA followed by Dunnett's test. The data is considered significant at $P \leq 0.05$.

RESULTS AND DISCUSSION

Limonene extends life span of N2 Bristol wild type *C. elegans*

To elucidate the anti-aging effects of phytomolecule LM, we tested 5, 25 and 50µM concentrations (which were found to be non-toxic to the worms as shown in Figure 1) of LM in *C. elegans* for their life span extending effects. Among these concentrations, maximum life span extension was achieved by 5µM dose which was 17.88% ($P \leq 0.001$) followed by other doses (25 and 50µM LM) represented by 13.23% ($P \leq 0.001$) and 6.62% ($P \leq 0.05$) respectively (Figure 2a, Table 1). Considering it as most effective dose, we performed all other assays with 5µM LM. Additionally, to determine the effect of LM on bacterial metabolism, the lifespan of 5µM LM treated worms was scored in presence of heat killed OP50 bacteria (inactivated bacteria at 60˚C for 30 minutes). The 5µM dose of LM extended lifespan in heat killed OP50 by 19.67% ($P \leq 0.001$) in comparison to vehicle control. This elevation in LS of worms fed over dead OP50, suggested that LM does not interfere with OP50 and extension in life span is a factor of LM treatment (Figure 2b, Table 1).

LM mediated longevity dependent on ROS scavenging

The impaired balance of pro-oxidants, oxidants and anti-oxidants is an attribute of defective cellular machinery and contributes to accelerate aging[14]. Various phytomolecules have been shown to possess anti-oxidant activity leading to ROS scavenging and thus, ultimately influencing lifespan[15]. In our study, significant reduction ($P \leq 0.01$) in intracellular ROS was observed for day 2 worms, pre-treated with 5µM LM as compared to control worms (Figure 3a) in DCFDA method of ROS estimation. These ROS scavenging effects indicate antioxidant effects of LM against aging induced oxidative damage, conferring LM induced anti-aging in worms. Additionally, ROS ameliorating effects of LM were explored using stress hypersensitive *C. elegans* null mutant *mev-1(kn-1).* In life span assay, control worms exhibited reduction in normal mean life span of 13.06±0.56 days in comparison to N2 wild type, whereas 5µM LM treated worms showed an elevated (15.29% increase) life span of 15.06±0.55 ($p \leq 0.01$) as shown in Table 1, Figure 3b. This shows that LM imparts its positive effects via reducing of oxidative stress thus imparting an increase of life span to the worms.

LM protects against oxidative stress:

Previous studies suggest existence of a positive correlation between longevity and tendency to tolerate stress[16]. In order to elucidate whether LM has any effect on stress responsiveness in *C. elegans,* the oxidative stress assay was performed. The untreated control and 5µM LM treated worms were exposed to methyl viologen (ROS inducer) and survival was recorded. Worms treated with LM demonstrated higher mean survival ($P \leq 0.001$) in comparison to untreated control worms (Figure 4). Altogether, these results suggest LM treatment attenuated intracellular ROS accumulation and enhances oxidative stress tolerance in worms.

LM activates transcription factor DAF-16

The transcription factor DAF-16 present in *C. elegans* is orthlogous to FOXO transcription factors in mammals[17]. This transcription factor gets activated in scenarios of stress viz. food deprivation, heat shock and overcrowding. In these conditions, *daf-16* gets dephosphorylated and ultimately activated after its translocation from cytoplasm to nucleus. We quantified translocation of *daf-16* in transgenic strain TJ 356*(zIs356)* wherein, *daf-16* is tagged with GFP, with or without 5µM LM treatment. In control TJ356 worms, *daf-16* resided in cytoplasm as seen in representational image (Figure 5a). In positive control (worms received heat shock) and 5µM LM treated worms, nuclear translocation of *daf-16* is evident as pointed out by red arrows in representational images (Figure 5 b,c). We then quantified the GFP tagged expression profile of *daf-16* by assigning worms to specific groups as nuclear, cytoplasmic and both. The percent of worms expressing these reveals that LM treatment induces *daf-16* nuclear localization and thus, imparts longevity mediating effects (Figure 5d). The same was ascertained by gene expression analysis, as *daf-16* was found to be elevated by 1.98 fold ($P \leq 0.01$) compared to endogenous control *actin* (Figure 10). In our study, we used single gene null mutant *daf-16(mgdf50)* and explored effect of LM on its life span. The log rank test between LS analysis of control (14.08±0.63) and 5µM LM pre-treated (14.62±0.59) worms revealed no significant difference indicating the involvement of *daf-16* in LM mediated longevity (Figure 5e, Table 1).Taken together, these results indicate that LS extending and stress culminating effects of LM are driven by change in expression of master transcription factor DAF-16.

LM extends life span via modulating stress responsive genes

Aging is modulated by several metabolic pathways and cellular processes, which are evolutionarilyy conserved in mammals, flies and worms, insulin/insulin like signaling (IIS) being of integral importance[18]. IIS exerts its effects on age and related parameters by regulating various

intercalated cellular responsive mechanisms[19]. The terpene moiety recruited in our study LM possesses longevity promoting potential as it showed anti-oxidant, ROS scavenging and stress modulating effects which prompted us to know the effect of LM on anti-oxidant enzyme SOD-3 (superoxide dismutase-3). We tested expression levels of *sod-3* gene which was found to be up-regulated 1.56 folds ($P \leq 0.05$) as compared to internal control *actin* (Figure 7). This was also confirmed by transgenic strain CF1553*(muIs84),* having *sod-3* tagged withGFP, which showed enhanced levels of *sod-3* expression between control and 5µM LM treated worms as shown in representational images (Figure 6a,b). Images were quantified for difference in florescence via Image J (NIH); they revealed significant increase ($P \leq 0.01$) in *sod-3* expression mediated by LM supplementation (Figure 6c). In *C. elegans, daf-2* encodes a tyrosine kinase receptor orthologous to IGF-1 receptor which is required for life span maintenance in worms and also regulates activity of transcription factor DAF-16[20]. Worms with loss of function of *daf-2* have an increased life span and are resistant to stress more than wild worms. We employed single gene mutant *daf-2(e1370)* and performed life span analysis with control and 5µM LM treated worms. Life span analysis for *daf-2* null mutants revealed no significant difference between control (29.27±1.30) and 5µM LM treated (30.7±0.89) worms indicating involvement of *daf-2* in LS extending effects of LM (Figure 6d, Table 1).

Another stress modulating factor in *C. elegans* is heat shock protein (HSP). Over expression of HSP family proteins (including *hsp-70*) is directly associated with enhanced survival under stress conditions, especially thermal stress[21]. Interestingly, *hsp-70* was found to be over-expressed with fold change value of 1.63 ($P \leq 0.05$) as shown in Figure 7 which is in concordance with the stress culminating effects of LM. We tested transcript levels of genes *eat-2* and *pha-4* , which are

involved in dietary restriction mediated longevity. The change in transcript levels for both *eat-2* and *pha-4* were found to be 1.50 ($P \leq 0.05$) and 1.96 ($P \leq 0.05$) fold change respectively (Figure 7).

CONCLUSION

The present study reveals a novel pharmacological intervention Limonene (LM) imparting longevity and stress reducing properties. These are attained by modulation of cellular, physiological changes of genetic machinery in *Caenorhabditis elegans*. This study for the first time has revealed that LM shows its anti-aging effects by activating transcription factor DAF-16 in terms of its nuclear inclusion, as well as, simultaneously modulating stress responsive genetic machinery. Implying the evolutionary conserved nature of these pathways, it is interesting to speculate that LM could be subjected to further investigations in organisms of higher complexity.

Acknowledgments Authors are thankful to *Caenorhabditis* Genetic Centre (Minneapolis, MN) which is funded by NIH office of Research Infrastructure Programs (P40OD01440) for providing strains used in this study. The authors are also thankful to the Director of CSIR–CIMAP, Lucknow, India for his valuable support. Author PS thanks SERB, New Delhi for providing N-PDF as scholarly grant (PDF/2015/000489).

Conflict of Interests Authors disclose no conflict of interests.

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Figure legends

Figure 1: Toxicity profile of monocyclic terpene LM at different doses 5, 25, 50, 100 and 250µM. 0.1% DMSO served as control. No toxicity was observed at 5, 25, 50µM concentrations of LM, but higher concentrations of LM (100 and 250µM) were found to be toxic to worms (***=P≤0.001).

Figure 2: Effect of LM on life span (LS) of wild type *C. elegans* at 20ºC: **(a)** Worms were treated with varied concentrations of 5, 25 and 50µM Limonene while 0.1% DMSO served as control. Survival curve shows dose assisted increase in LS, 5µM LM being foremost with mean life span extension of 17.88% (P≤0.001) which was higher compared to mean life span extension achieved by 25 and 50µM LM that showed 13.23% (P≤0.001) and 6.62% (p≤0.05) respectively. **(b)** Life span assay employing heat killed OP50 *E. coli* (kept at 65ºC for 30 minutes) in response to 5µM LM treatment. Survival curve shows 19.67% increase in LS when treated with 5µM LM (P≤0.001) suggesting LM does not interfere with bacterial metabolism and LS extension observed due to LM treatment.

Figure 3: LM scavenges intracellular ROS: **(a)** Reactive oxygen species (ROS) scavenging capacity was recorded using florescent dye 2,7-dichlorodihydrofluorescein-diacetate (H_2DCFDA). 5µM LM treatment significantly reduced ROS levels in comparison to control (**P≤0.01) **(b)** Null mutant *mev-1(kn-1)* worms were bred 5µM LM or control (0.1% DMSO) treatment and recorded for survival. Treatment with LM enhanced the LS up to 15.09% (P≤0.01) suggesting ROS terminating properties of LM.

Figure 4: LM attenuates oxidative stress: Worms were treated with known ROS inducer methyl viologen from L1 stage and scored for survival. LM exhibited oxidative stress culminating properties in this assay as observed in terms of enhanced survival of 5µM LM treated worms compared to control (**P≤0.01; ***P≤0.001).

Figure 5: LM activates transcription factor DAF-16 **(a)** Transgenic TJ 356 (*daf-16*::gfp) transgenic worms receiving no treatment (control) exhibited no nuclear localization of *daf-16*. **(b)** Positive control worms on day 2 of adulthood received heat shock (37ºC for 20 minutes) and show heat shock induced nuclear localization of *daf-16* as indicated by red colored arrows. **(c)** 5µM LM treated worms exhibited LM mediated nuclear localization of *daf-16* (red arrows). **(d)** Nuclear and cytoplasmic location of transcription factor *daf-16* was quantified by counting number of worms exhibiting each such condition or both. Control worms were mostly having cytoplasmic retention of *daf-16*. Worms which received heat shock (HS) show highest nuclear localization of *daf-16* (***P≤0.001;*P≤0.05) and LM treated worms also have large number of worms with nuclear localization (***P≤0.001;*P≤0.05). **(e)** Survival assay of null mutant *daf-16(mgdf50)* revealed no difference between life span (LS) of control and 5µM LM treated worms (p=0.76) suggesting requirement of *daf-16* in LS extending effect of LM.

Figure 6: Effect of LM on *sod-3* and *daf-2*: Transgenic strain CF1553 (*sod-3*::gfp) worms were treated with or without 5µM LM. **(a)** Representational image of transgenic strain CF1553 (*sod-3*::gfp) control worms with expressing *sod-3* **(b)** Representational image of transgenic strain CF1553 (*sod-3*::gfp) worms treated with 5µM LM expressing higher *sod-3* levels **(c)** Quantified relative expression of *sod-3* between control and LM treated worms expressed in terms of Relative florescence units (RFU). Treatment with LM significantly elevates expression anti-oxidant enzyme *sod-3* (**P≤0.01). **(d)** Single gene null mutant *daf-2(e1370)* worms were treated with 5µM LM while 0.1% DMSO served as control. Survival analysis revealed no significant change between control and 5µM LM treated worms (p=0.16) suggesting LM requires *daf-2* to impart its longevity properties.

Figure 7: Effect of 5µM LM on expression of age associated genes employing RT-PCR with *p≤0.05, **P≤0.01 as criterion for significance.

Figure 1

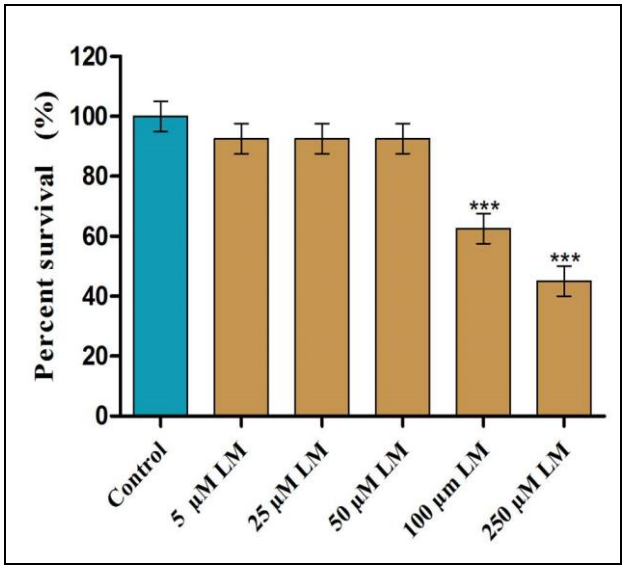

Figure 2 a, b

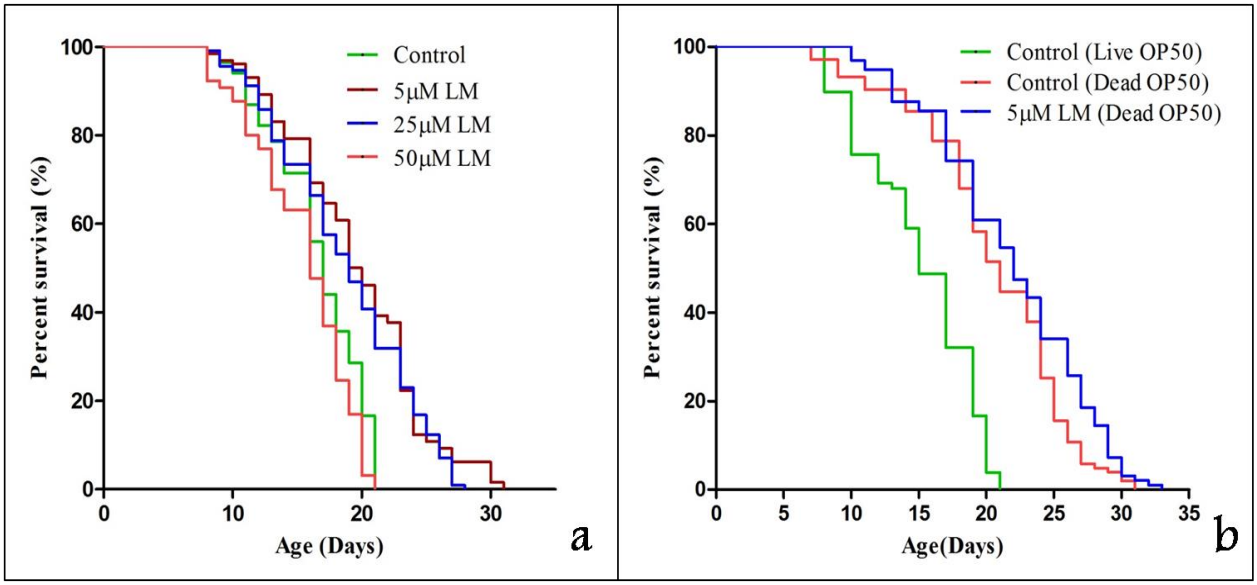
Control
5µM LM
25µM LM
50µM LM
Percent survival (%)
Age (Days)
a
Control (Live OP50)
Control (Dead OP50)
5µM LM (Dead OP50)
Percent survival (%)
Age(Days)
b

Figure 3 a, b

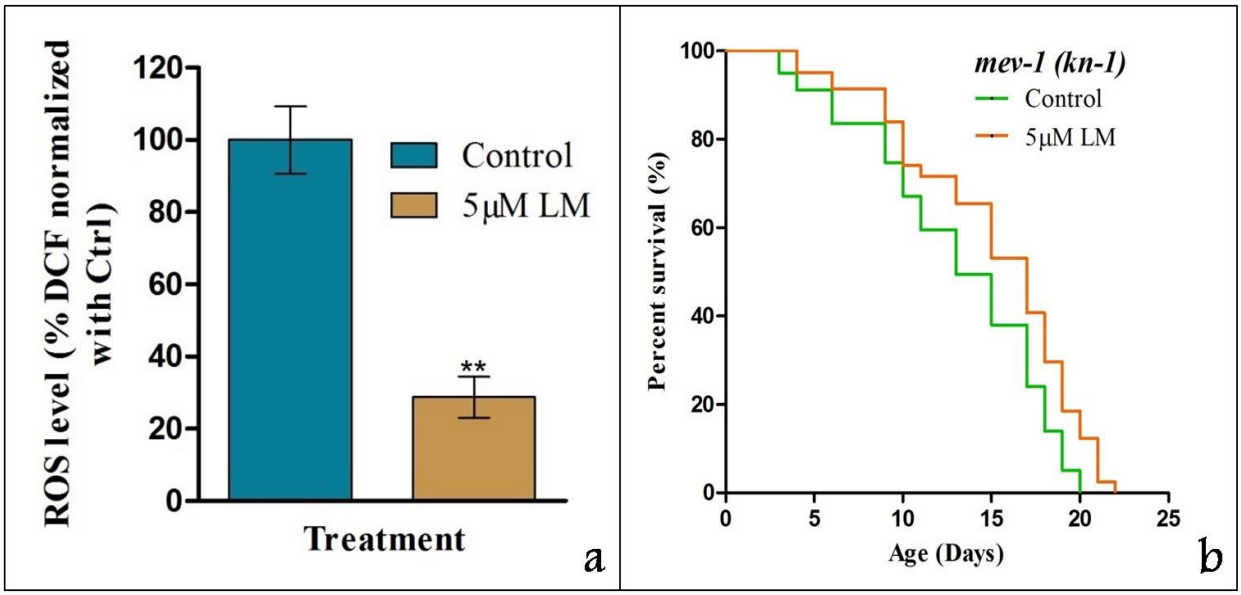

Figure 4

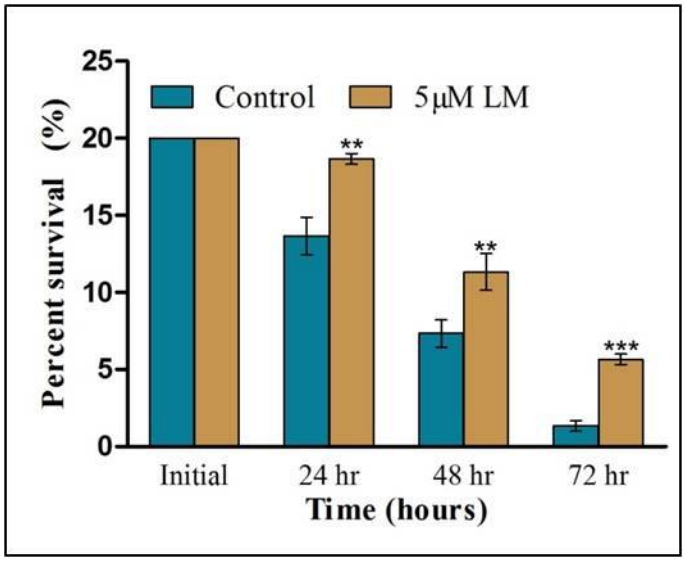

Figure 5 a, b, c, d, e

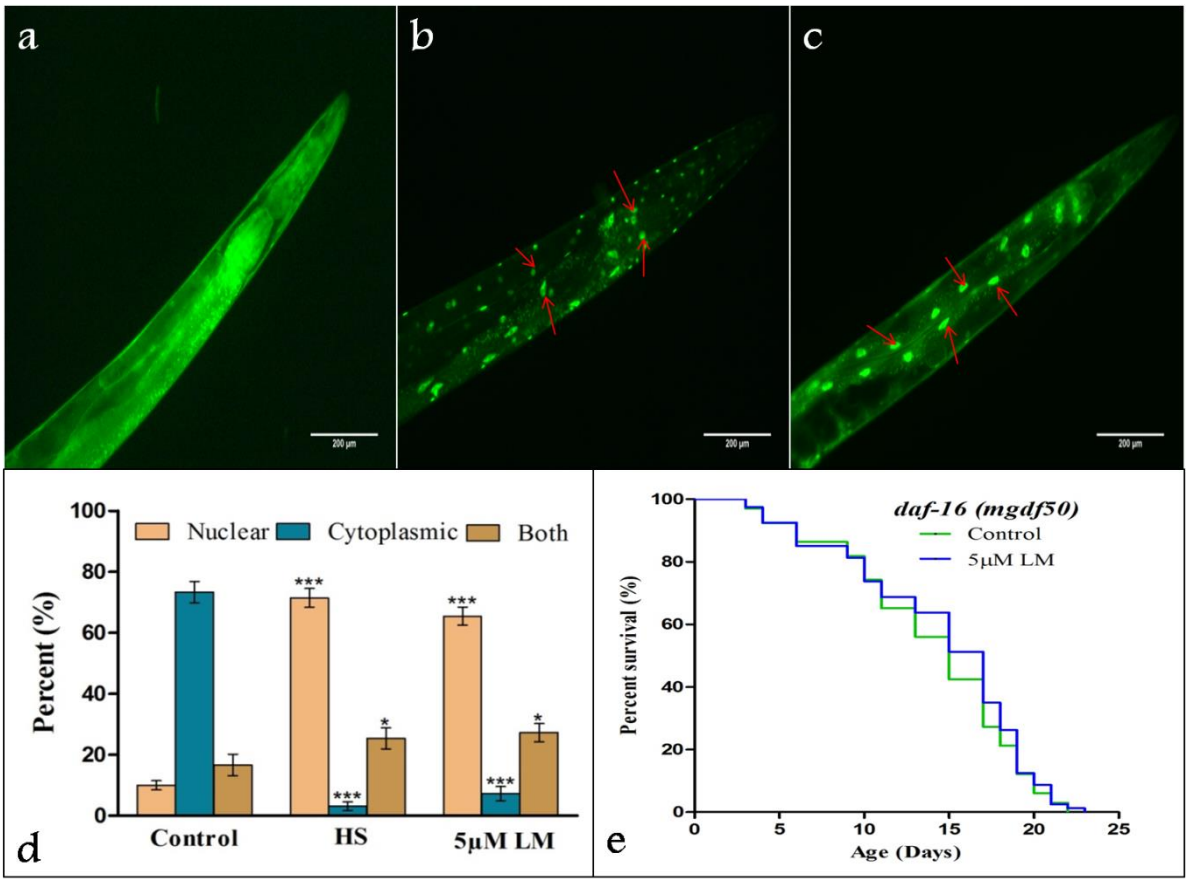

Figure 6 a, b, c, d

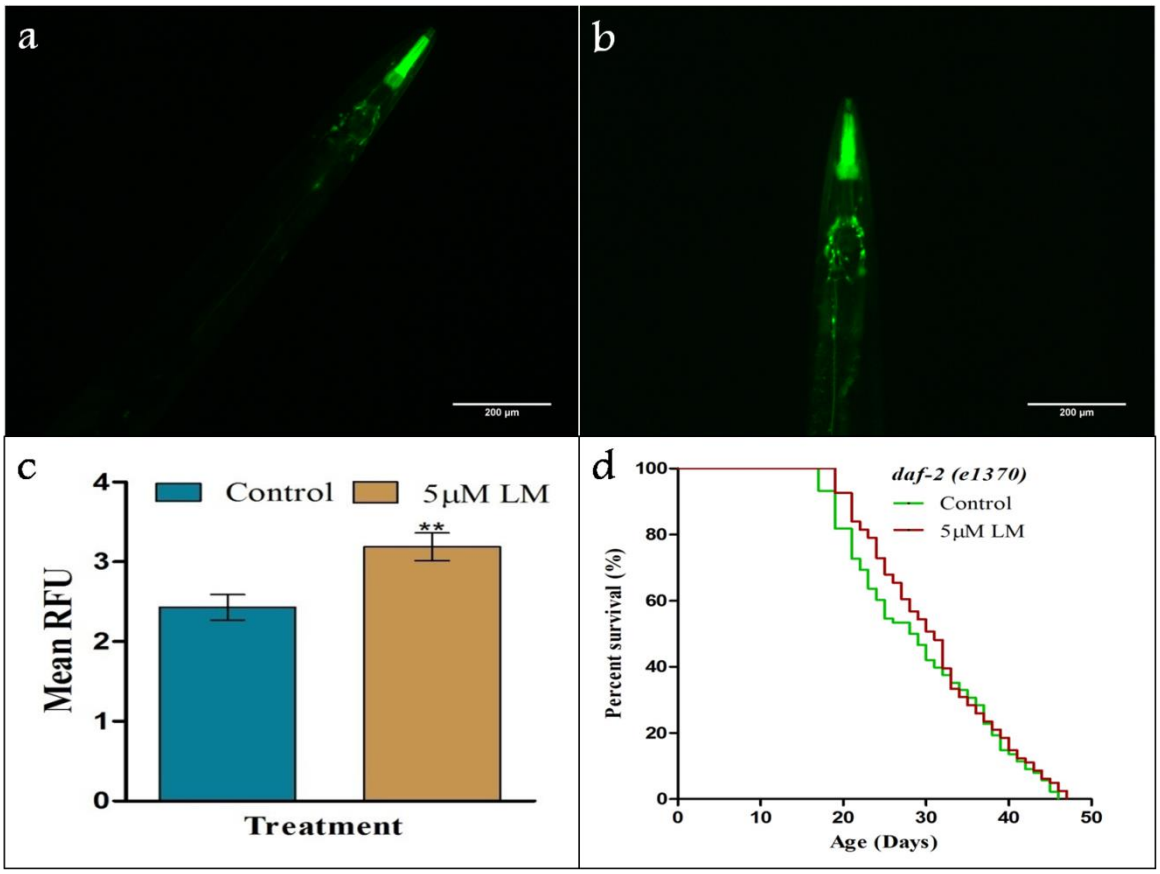

Figure 7

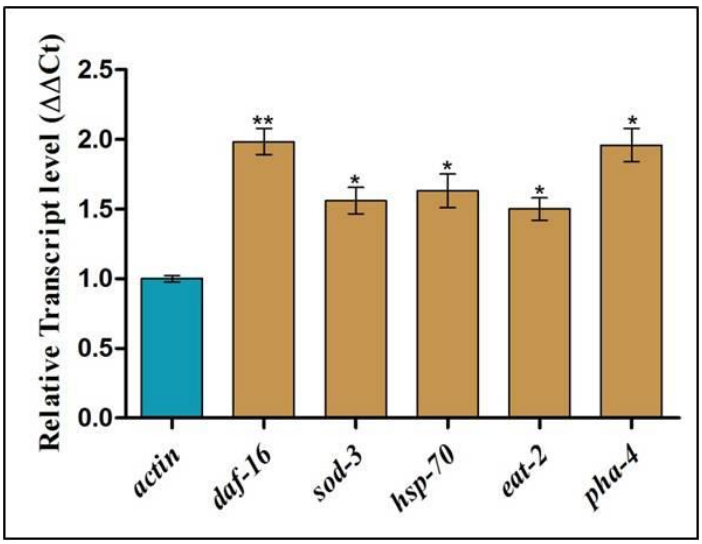

Table 1: Lifespan analysis of wild type and mutant strains at 20 ˚C

Strains	**Treatment**	**Mean LS ±SE**	**% change**	**P value**
N2 (WT)	Control	16.60±0.39		
	5 µM LM	19.57±0.46	17.88	≤0.001
	25 µM LM	18.8±0.49	13.23	≤0.001
	50 µM LM	15.50±0.47	6.62	≤0.05
N2 (WT)	Control (Live OP50)	15.69±0.69		
	Control (Dead OP50)	18.41±0.40		
	5 µM LM (Dead OP50)	22.04±0.35	19.67	≤0.001
mev-1(kn-1)	Control	13.06±0.56	15.29	≤0.01
	5 µM LM	15.06±0.55		
daf-16 (mgdf50)	Control	14.08±0.63	3.77	=0.76 NS
	5 µM LM	14.62±0.59		

daf-2(e1307)	Control	29.27±1.30	4.9	=0.16 NS
	5 µM LM	30.7±0.89		

Table 1 Effect of Limonene (LM) treatment on the life span (LS) of *C. elegans* wild type (WT) and mutant worms. Between control and treatment conditions, log rank test was employed as the test of significance and survival curves plotted using Kaplan-Meier survival analysis in GraphPad Software (San Diego, CA, USA) using P*≤0.05; **≤0.01 and ***≤0.001 as a criterion for significance whereas, NS represents no significant change.