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

Pooja Shukla, Aakanksha Pant and Rakesh Pandey*

Microbial Technology and Nematology Department, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow 226 015, India

In traditional systems of medicine, plants belonging to the family Rutaceae have been used against ageing and associated debilitating changes. Considering the therapeutic potential of limonene ($C_{10}H_{16}$), the principal component of the Rutaceae family plants, the 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* reactive oxygen species build-up, which was monitored with or without exposure to LM. Additionally, involvement of transcription factor DAF-16 was examined in terms of nuclear localization. Overall, this study has implications for developing future anti-ageing pharmacological strategies in the future.

Keywords: Ageing, *Caenorhabditis elegans*, limonene, oxidative stress.

AGEING is a multifaceted process characterized by various physiological changes along with gradual decline in function. The field of biogerontology research has witnessed the emergence of ageing as a 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-ageing 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 systems of medicine, plants of the Rutaceae family have been reported to have important effects on ageing and associated pathologies[3]. Yet they have been scientifically evaluated for their anti-ageing and stress-modulating potential. Therefore, the present study was designed to systematically evaluate anti-ageing 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 ageing studies pertaining to simple culture, rapid regeneration time, ease of genetic manipulations, identification of several gene mutations regulating evolutionarily conserved pathways affecting lifespan (LS) and shared homology with the human genome[4].

The present study explored the potential longevity-promoting and stress-modulating properties of LM utilizing the advantage of *C. elegans* ageing model. The genetic mechanism regulating LM-mediated alteration in lifespan (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 evolutionarily conserved nutrient-sensing pathway, including IIS (insulin/insulin-like growth factor signalling). Therefore, the present study examined the interaction of LM with IGF genes and expression levels of these genes which play a major role in regulating stress and ageing in organisms. This study paves the way towards understanding therapeutic potential of LM in terms of age-defying properties and has implications for development of potential therapy for controlling age-associated decline.

Experimental

C. elegans culture and strains

C. elegans strains, namely N2 Bristol (wild type), TK22, *mev-1* (*kn-1*); GR1307, *daf-16* (*mgdf50*); CB1370, *daf-2 (e1370)*, TJ356, *daf-16*::GFP (*zIs 356*) and CF1553, *sod-3*::GFP (*muIs 84*) were employed in the present study. The worms were fed over bacterial lawn of *Escherichia coli* variant OP50 at 20°C 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

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

Lifespan assay

Hypochlorite bleaching method was employed in order to obtain synchronous population from gravid adults using

*For correspondence. (e-mail: r.pandey@cimap.res.in)

standard protocol given in Pant *et al.*[5]. Worms were treated with different concentrations of LM (5, 25 and 50 µM), whereas 0.1% DMSO served as control. Each test concentration of LM was spotted directly on 35 mm petri plate containing 2 ml 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 molted into adult worms was considered as day-1. Worms were transferred to fresh plates every alternate day and scored daily by prod and watch method[6]. 5-fluoro-2-deoxyuridine (FUdR; 50 µM; Sigma Aldrich, Missourie, USA) was used for maintaining the synchronous population by ceasing the hatching of laid eggs[7]. All lifespan 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 if LM-mediated lifespan extension is independent of change in bacterial metabolism, we used heat-killed (HK) bacteria (*E. coli* OP50) for studying the effect of 5 µM LM on the lifespan of worms. *E. coli* OP50 was killed by incubating at 60°C for 30 min using the protocol described in Garigan *et al.*[8].

Assessment of antioxidant effects of LM

Worms were cultured on nematode growth medium and assays were performed by exposing worms (age-synchronized) to 5 µM LM and 0.1% DMSO as vehicle control. On day-2 of their adulthood, worms ($n = 150$) were washed thrice and collected in phosphate buffered saline with Tween-20 (PBST) buffer. They were then exposed to 50 µM 2,7-dichlorodihydrofluorescein-diacetate (H_2DCFDA) according to the protocol described in Pant *et al.*[9]. Intensity of fluorescence was recorded at time intervals of 20 min for 2 h at 37°C (Agilent, Plate Reader), technologies at 485/530 nm. Further, we employed *C. elegans* null mutant *mev-1 (kn-1)*, wherein the gene encoding complex II of mitochondrial oxidative phosphorylation machinery is mutated[10]. We observed lifespan extension effects of 5 µM LM in *mev-1 (kn-1)* mutants and compared them with control (0.1% DMSO).

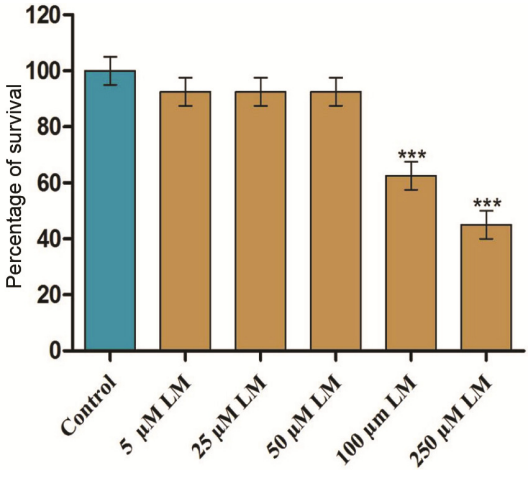

Figure 1. Toxicity profile of monocyclic terpene limonene (LM) at different doses, viz. 5, 25, 50, 100 and 250 µM and 0.1% DMSO as control. No toxicity is observed at 5, 25 and 50 µM concentration of LM, but higher concentrations (100 and 250 µM) are found to be toxic to worms (***$P \leq 0.001$).

Determination of oxidative stress resistance

As an organism ages, there is a decline in health and lifespan[11]. Therefore, response to change in oxidative stress level was assessed by exposing age-synchronized treated (5 µM 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 reactive oxygen species (ROS) generator spotted plates. The experiment was repeated thrice.

Green fluorescent protein visualization and quantification

The transgenic *C. elegans* strains TJ356; *daf-16*::*GFP* (*zIs 356*), where *daf-16* is tagged with green fluorescent protein (GFP) were observed for nuclear localization. Synchronized cohorts were obtained using hypochlorite bleaching assay and further bred as stated for LS with or without 5 µM LM treatment. On day-2 of adulthood, one set of worms was subjected to heat shock at 37°C for 30 min and termed as positive control. All control ($n = 49$), positive control ($n = 44$) and 5 µM LM-treated ($n = 50$) worms were observed by placing them on glass slides containing 2% agarose pad and anesthetized with 1 mM sodium azide (Sigma Aldrich). All the worms were then assigned to specific groups, depending upon the location of *daf-16* expression as nuclear, cytoplasmic or both[12]. Similarly, CF1553; *sod-3*::*GFP+rol-6* (*muIs84*) worms were employed for their relative expression of GFP-tagged *sod-3* in control ($n = 32$) and 5 µM LM-treated ($n = 37$) transgenic worms. Photomicrographs were taken using GFP filter (365/420 nm) using a fluorescence microscope (DMI 3000 B, Germany) at 20× magnification. For CF1553, quantification of GFP expression was performed using image J software (National Institutes of Health, USA).

mRNA isolation and gene expression analysis

RNAzol (Molecular Research Centre, USA) method was used to isolate total RNA from day-2 wild type worms using the manufacturer's protocol. RNA was then

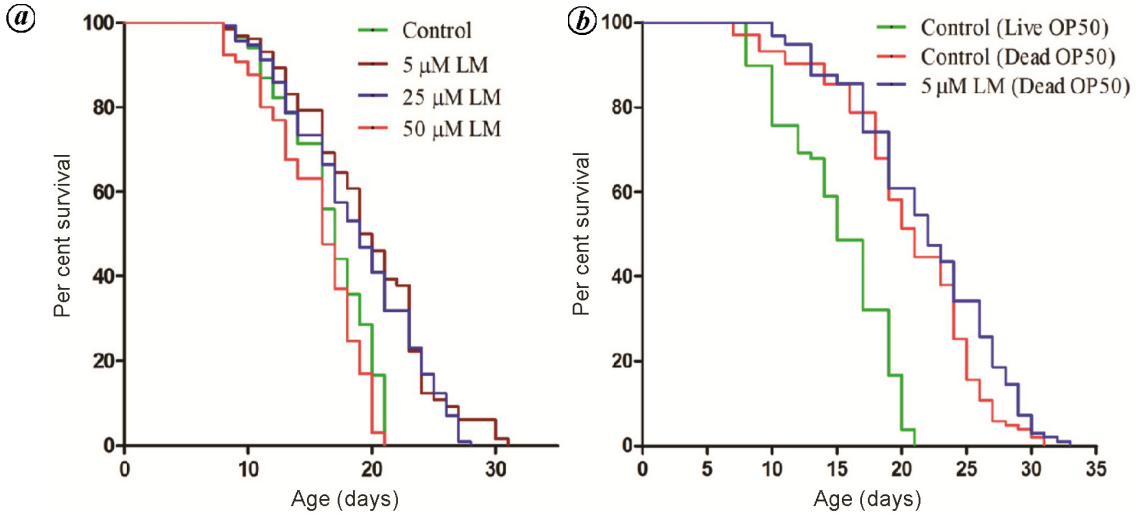

Figure 2. Effect of LM on the lifespan wild type *Caenorhabditis elegans* at 20°C: ***a***, Worms treated with different concentrations of LM, viz. 5, 25 and 50 µM, and 0.1% DMSO as control. Survival curve shows dose-assisted increase in LS, 5 µM LM being foremost with mean lifespan extension of 17.88% ($P \leq 0.001$) which is higher compared to mean lifespan extension achieved by 25 and 50 µM LM showing 13.23% ($P \leq 0.001$) and 6.62% ($P \leq 0.05$) respectively. ***b***, Lifespan assay employing heat-killed OP50 *Escherichia coli* (maintained at 65°C for 30 min) in response to 5 µM LM treatment. Survival curve shows 19.67% increase in LS when treated with 5 µM LM ($P \leq 0.001$), suggesting that LM does not interfere with bacterial metabolism and lifespan extension.

Table 1. Lifespan analysis of wild type (WT) and mutant strains at 20°C

Strain	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 ± 0.498	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		

Effect of limonene (LM) treatment on the lifespan of *Caenorhabditis 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) with $*P \leq 0.05$; $**P \leq 0.01$ and $***P \leq 0.001$ as the criteria for significance. NS, No significant change.

reverse-transcribed into complementary DNA using RevertAid kit (Invitrogen, California, USA). 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 lifespan data were analysed using Kaplan–Meier survival analysis (log-rank test) in GraphPad Prism software. Data other than lifespan were represented as mean ± SE and statistically analysed using one-way ANOVA followed by Dunnett's test. The data were considered significant at $P \leq 0.05$.

Results and discussion

LM extends lifespan of N2 Bristol (wild type)

To elucidate the anti-ageing effects of LM, we tested 5, 25 and 50 µM concentrations of the phytomolecule (which were found to be non-toxic to the worms) in *C. elegans* for their lifespan-extending effects. Among

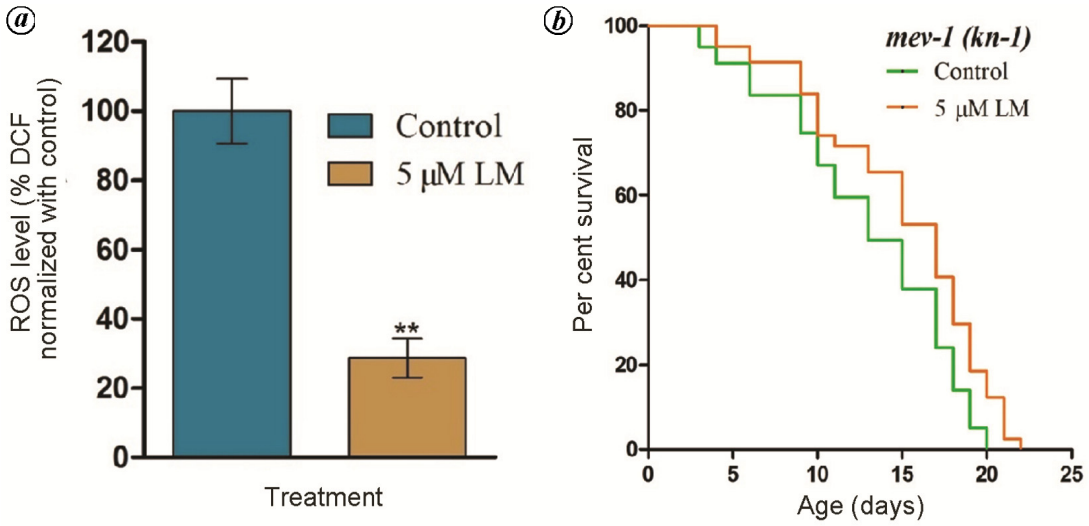

Figure 3. LM scavenges intracellular reactive oxygen species (ROS): ***a***, ROS scavenging capacity recorded using fluorescent dye 2,7-dichlorodihydrofluorescein-diacetate (H_2DCFDA). The 5 µM LM treatment significantly reduces ROS levels in comparison to control (**$P \leq 0.01$). ***b***, Null mutant *mev-1 (kn-1)* worms were exposed to LM and control (0.1% DMSO) treatment and recorded for survival. Treatment with LM enhances the lifespan up to 15.09% ($P \leq 0.01$), suggesting ROS terminating properties of LM.

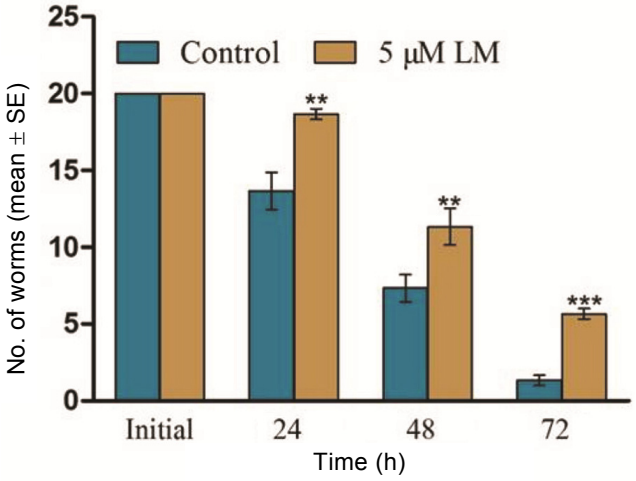

Figure 4. LM attenuates oxidative stress: worms were treated with known ROS inducer, methyl viologen from L1 stage and scored for survival. LM exhibits oxidative stress culminating properties in this assay as observed in terms of enhanced survival of 5 µM LM-treated worms compared to control (**$P \leq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

these, maximum lifespan extension was achieved by 5 µM dose which was 17.88% ($P \leq 0.001$), followed by other doses, viz. 25 and 50 µM LM represented by 13.23% ($P \leq 0.001$) and 6.62% ($P \leq 0.05$) respectively (Figure 2 *a* and Table 1). Considering it as most effective dose, we performed all other assays with 5 µM LM. Additionally, to determine the effect of LM on bacterial metabolism, the lifespan of 5 µM LM-treated worms was scored in the presence of heat-killed OP50 bacteria (inactivated bacteria at 60°C for 30 min). The 5 µM dose of LM extended the lifespan in heat-killed OP50 by 19.67% ($P \leq 0.001$) in comparison to vehicle control. This suggests that LM does not interfere with OP50 and extension in lifespan is a factor of LM treatment (Figure 2 *b* and Table 1).

LM-mediated longevity depends on ROS scavenging

The impaired balance of pro-oxidants, oxidants and antioxidants is an attribute of defective cellular machinery and contributes to accelerate ageing[14]. Various phytomolecules have been shown to possess antioxidant activity leading to ROS scavenging and thus, ultimately influencing lifespan[15]. In the present study, significant reduction ($P \leq 0.01$) in intracellular ROS was observed for day-2 worms, pre-treated with 5 µM LM compared to control worms (Figure 3 *a*) using H2-DCFDA method of ROS estimation. These ROS scavenging activities indicate antioxidant activity of LM against ageing-induced oxidative damage, conferring LM-induced anti-ageing in worms. Additionally, ROS ameliorating effects of LM were explored using stress hypersensitive *C. elegans* null mutant *mev-1 (kn-1)*. In lifespan assay, control worms exhibited reduction in normal mean lifespan of 13.06 ± 0.56 days in comparison to N2 wild type, whereas 5 µM LM-treated worms showed an increased (15.29% increase) lifespan of 15.06 ± 0.55 days ($P \leq 0.01$; Table 1 and Figure 3 *b*). This shows that LM imparts its positive effects by reducing the oxidative stress, thus resulting in an increase in lifespan of the worms.

LM protects against oxidative stress

Previous studies suggest the existence of a positive correlation between longevity and tendency to tolerate stress[16]. In order to elucidate whether LM has any effect on stress

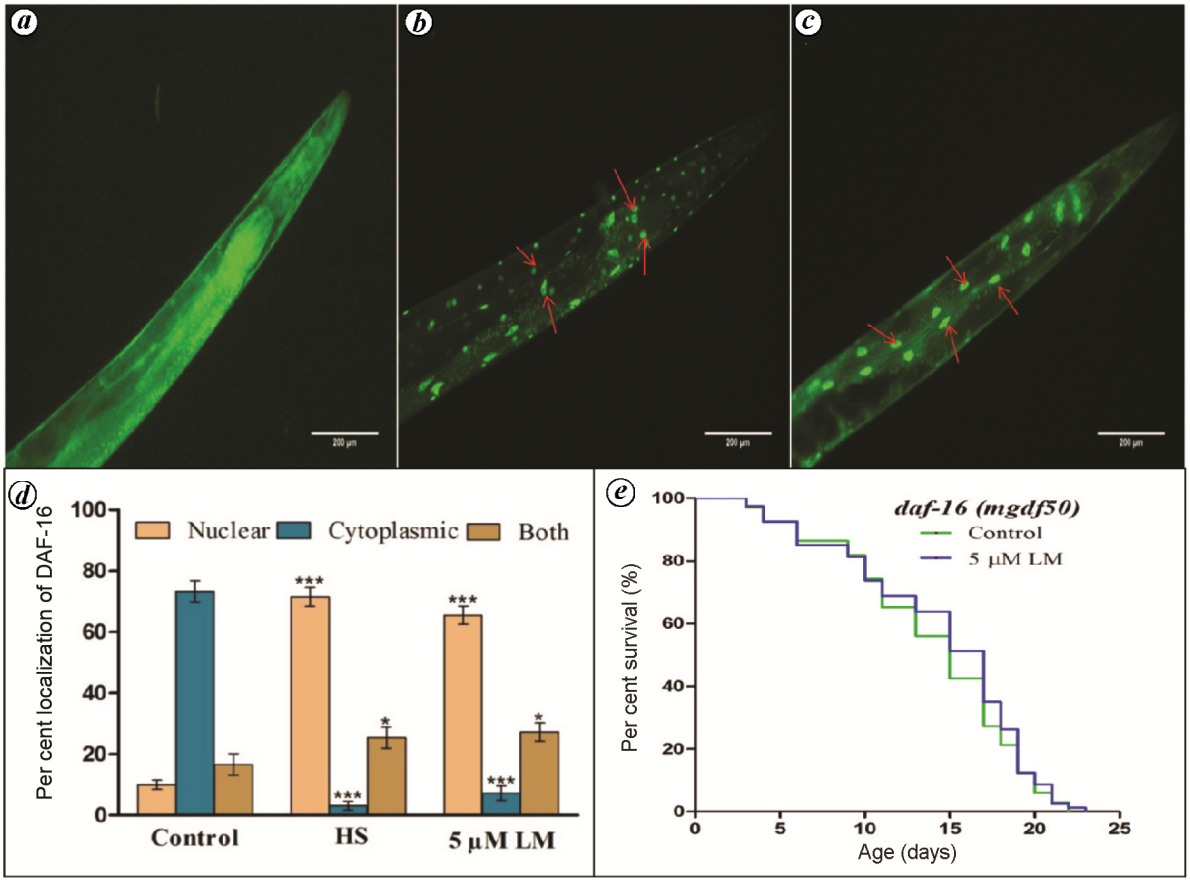

Figure 5. LM activates transcription factor DAF-16. ***a***, Transgenic TJ 356 (*daf-16*::*gfp*) worms (untreated control) exhibit no nuclear localization of *daf-16*. ***b***, Positive control worms on day-2 of adulthood subjected to heat shock (37°C for 20 min) and showing heat-shock induced nuclear localization of *daf-16* as indicated by red-coloured arrows. ***c***, 5 µM LM-treated worms demonstrated LM induced *daf-16* nuclear localization (red arrows). ***d***, Nuclear and cytoplasmic location of transcription factor *daf-16* quantified by counting the number of worms exhibiting each such condition, or both. Control worms mostly show cytoplasmic retention of *daf-16*. Worms subjected to heat shock show highest nuclear localization of *daf-16* (***$P \leq 0.001$; *$P \leq 0.05$). LM-treated worms also have large number of worms with nuclear localization (***$P \leq 0.001$; *$P \leq 0.05$). ***e***, Survival assay of null mutant *daf-16 (mgdf50)* reveals no difference between lifespan of control and 5 µM LM-treated worms ($P = 0.76$), suggesting the role of *daf-16* in the lifespan-extending effect of LM.

responsiveness in *C. elegans*, the oxidative stress assay was performed. The untreated control and 5 µM 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). Thus, these results suggest LM treatment attenuates 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. Under 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 TJ356 (*zIs356*), wherein *daf-16* was tagged with GFP, with or without 5 µM LM treatment. In control TJ356 worms, *daf-16* resided in cytoplasm (Figure 5 *a*). In positive control (worms subjected to heat shock) and 5 µM LM-treated worms, nuclear translocation of *daf-16* was evident (red arrows, Figure 5 *b* and *c*). We then quantified the GFP-tagged expression profile of *daf-16* by assigning worms to specific groups as nuclear, cytoplasmic and both. The percentage of worms expressing these reveals that LM treatment induces *daf-16* nuclear localization and thus imparts longevity-mediating effects (Figure 5 *d*). 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 7). In the present study, we used single gene null mutant *daf-16 (mgdf50)* and explored the effects of LM on its lifespan. The log rank test between LS analysis of control (14.08 ± 0.63) and 5 µM LM pre-treated

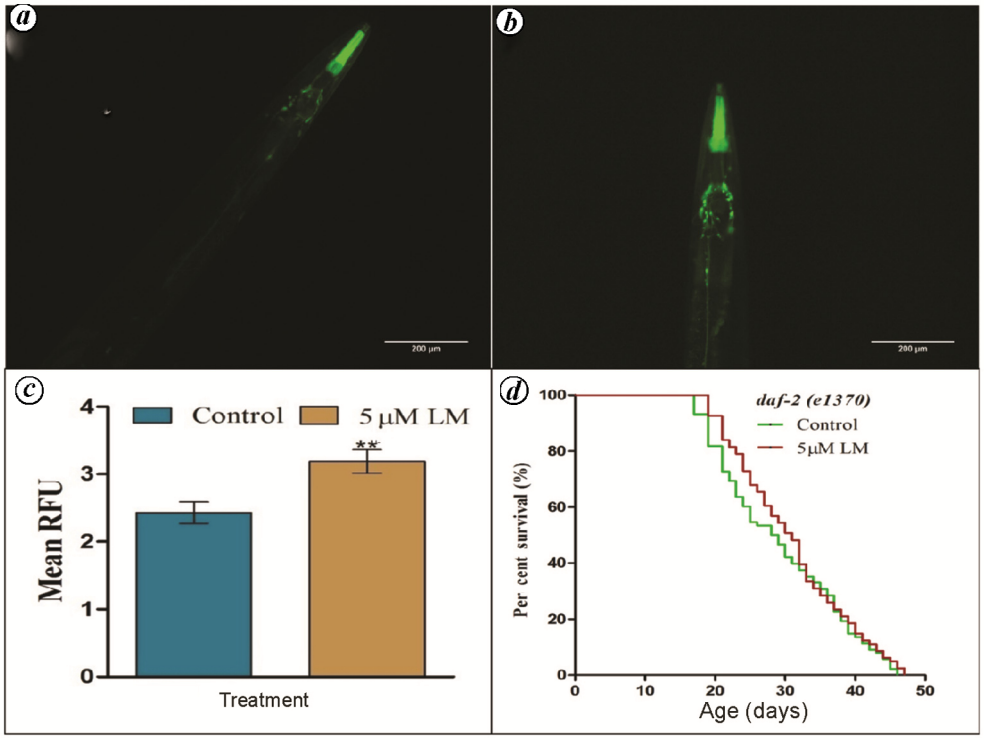

Figure 6. Effect of LM on *sod-3* and *daf-2*: transgenic strain CF1553 (*sod-3*::*gfp*) worms treated with or without 5 µM LM. ***a***, Representational image of transgenic strain CF1553 (*sod-3*::*gfp*) control worms expressing *sod-3*. ***b***, Microscopic image of transgenic strain CF1553 (*sod-3*::*gfp*) worms treated with 5 µM LM expressing higher *sod-3* levels. ***c***, Quantified relative expression of *sod-3* between control and LM-treated worms in terms of relative florescence units (RFU). Treatment with LM significantly elevates expression of anti-oxidant enzyme *sod-3* (**$P \leq 0.01$). ***d***, Single-gene null mutant *daf-2 (e1370)* worms treated with 5 µM LM and 0.1% DMSO as control. Survival analysis reveals no significant change between control and 5 µM LM-treated worms ($P = 0.16$), suggesting LM mediated longevity is mediated by altered *daf-2* signalling.

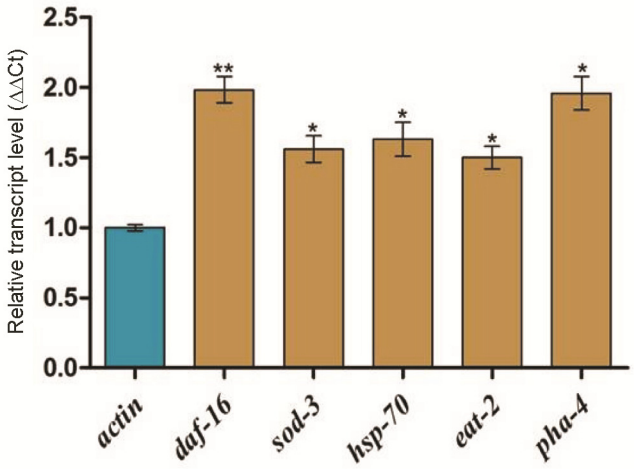

Figure 7. Effect of 5 µM LM on the expression of age-associated genes employing RT-PCR with *$P \leq 0.05$, **$P \leq 0.01$ as criteria for significance.

(14.62 ± 0.59) worms revealed no significant difference, indicating the involvement of *daf-16* in LM-mediated longevity (Figure 5 *e* and Table 1). Thus, these results indicate that LS-extending and stress-culminating effects of LM are driven by change in the expression of DAF-16.

LM extends lifespan via modulating stress responsive genes

Ageing is modulated by several metabolic pathways and cellular processes, which are evolutionarily conserved in mammals, flies and worms, with 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 used in our study possesses longevity-promoting potential, as it shows antioxidant, ROS scavenging and stress-modulating effects, which prompted us to examine the effect of LM on antioxidant enzyme SOD-3 (superoxide dismutase-3). We tested expression levels of *sod-3* gene which was found to be upregulated 1.56-fold ($P \leq 0.05$) compared to internal control *actin*. This was also confirmed by transgenic strain CF1553 (*muIs84*), having *sod-3* tagged with GFP, which showed enhanced levels of *sod-3* expression between control and 5 µM LM-treated worms as (Figure 6 *a* and *b*). Images were quantified for difference in fluorescence using Image J (NIH); they revealed significant increase ($P \leq 0.01$) in *sod-3* expression mediated by LM supplementation (Figure 6 *c*). In *C. elegans*, *daf-2* encodes a tyrosine kinase receptor orthologous to IGF-1

receptor, which is required for lifespan maintenance in worms and also regulates activity of transcription factor DAF-16 (ref. 20). Worms with loss of function of *daf-2* have an increased lifespan and are resistant to stress more than wild type. We employed single gene mutant *daf-2* (*e1370*) and performed lifespan analysis with control and 5 μM LM-treated worms. Lifespan analysis for *daf-2* null mutants revealed no significant difference between control (29.27 ± 1.30) and 5 μM LM-treated (30.7 ± 0.89) worms, indicating involvement of *daf-2* in LS extending effects of LM (Figure 6 *d* and Table 1).

Another stress-modulating factor in *C. elegans* are heat shock proteins (HSPs). Overexpression 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 overexpressed with fold change value of 1.63 ($P \leq 0.05$; Figure 7) which is in agreement 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 fold change in transcript levels for both *eat-2* and *pha-4* was found to be 1.50 ($P \leq 0.05$) and 1.96 ($P \leq 0.05$) respectively (Figure 7).

Conclusion

The present study reveals a new pharmacological intervention LM imparting longevity and stress-reducing properties. These are attained by modulation of cellular and physiological changes in the genetic machinery of *C. elegans*. This study revealed that LM displays its anti-ageing effects by activating transcription factor DAF-16 in terms of its nuclear inclusion as well as simultaneously modulating stress responsive genetic machinery. Implying the evolutionarily conserved nature of these pathways, it is interesting to speculate that LM could be subjected to further examination in more complex organisms.

Conflict of interest: Authors declare no conflict of interest.

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