**α- and β-Santalols Delay Aging in Caenorhabditis elegans via Preventing Oxidative Stress and Protein Aggregation**

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**ABSTRACT:** α- and β-Santalol (santalol isomers) are the most abundant sesquiterpenoids found in sandalwood, contributing to its pleasant fragrance and wide-spectrum bioactivity. This study aimed at identifying the antiaging and antiaggregation mechanism of α- and β-santalol using the genetic tractability of an in vivo model, *Caenorhabditis elegans*. The results showed that santalol isomers retard aging, improved health span, and inhibited the aggregation of toxic amyloid-β (Aβ42) and polyglutamine repeats (Q35, Q40, and HttQ150) in *C. elegans* models for Alzheimer’s and Huntington’s disease, respectively. The genetic study, reporter gene expression, RNA-based reverse genetic approach (RNA interference/RNAi), and gene expression analysis revealed that santalol isomers selectively regulate SKN-1/Nrf2 and EOR-1/PLZF transcription factors through the RTK/Ras/MAPK-dependent signaling axis that could trigger the expression of several antioxidants and protein aggregation inhibitory genes, viz., gst-4, gcs-1, gst-10, gsr-1, hsp-4, and skr-5, which extend longevity and help minimize age-induced protein oxidation and aggregation. We believe that these findings will further promote α- and β-santalol to become next-generation prolongevity and antiaggregation molecules for longer and healthier life.

**1. INTRODUCTION**

Aging is a natural and inevitable phenomenon characterized by a gradual loss of multiple physiological functions and organ integrity that constitutes a critical risk factor for various life-limiting diseases.5 Several cellular and molecular declines have been identified to be responsible for aging, including genomic instability, telomere attrition, epigenetic alterations, mitochondrial dysfunction, and loss of protein homeostasis (proteostasis).6 Among others, impaired proteostasis has been considered as one of the universal hallmarks of aging.7 As organisms age, the cellular systems responsible for proteostasis become less efficient. Besides, aging is also associated with increased production of reactive species, thus leading to irreversible nitration and oxidation of proteins, which compromise their degradation and facilitates the aberrant aggregation of specific proteins.8 Deposits of such damaged proteins in cellular components and different tissues lie at the heart of several protein misfolding disorders such as Alzheimer’s, Parkinson’s, and Huntington’s diseases. Protein aggregation during the aging process is still unclear, despite overwhelming evidence suggesting a negative correlation with life span. Previous studies have shown that aging in a multicellular organism *Caenorhabditis elegans* is associated with the widespread accumulation of several hundred insoluble proteins.9 Interestingly, recent evidence shows that the aging process induced protein insolubilization, and aggregate formation aggravates the polyglutamine-repeat-related pathologies.9 Indeed, it has been proved that an enhanced SKInHead-1/nuclear factor erythroid 2-related factor 2 (SKN-1/Nrf2) and epidermal growth factor (EGF) signaling, reduced insulin/insulin-like growth factor-1 signaling (insulin/IGF-1), and other protein clearance pathways promote a healthy life span in *C. elegans* via preventing the collapse of the proteostasis network.10–12 Several small molecules and Amyloid-binding compounds exert dual effects that potentially extend longevity and prevent disease protein aggregation during aging.13 These findings imply that pharmacological modulation of protein aggregation pathways and protein homeostasis improve the quality of life and delay or even halt the onset and progression of age-related disorders. Therefore, identifying novel pharmaceutical interventions (naturally occurring or chemically synthesized) that target the protein homeostasis mechanism for the purpose of extending healthy longevity is urgently needed. Santalol isomers (α- and β-santalol), the key constituents of sandal-
wood essential oil, seem promising in this endeavor. \(\alpha\)- and \(\beta\)-Santalol are the most abundant sesquiterpenoids found in sandalwood oil, which together comprise up to 80% of the total content. These isomers contribute to the pleasant fragrance and bioactivity of oil extracted from mature heartwood of East Indian sandalwood trees (Santalum album L.).

\(\beta\)-Santalol is shown to exert antiviral and neuroleptic properties, while \(\alpha\)-santalol has been studied for its cancer chemopreventive and anticancer qualities. In particular, \(\alpha\)-santalol inhibits the growth of human prostate cancer cells via activating caspase-3, enhancing the expression of p53, and reducing angiogenesis. Moreover, it causes G2/M cell-cycle arrest in various cancer cell lines and has chemopreventive activity on UV-B-induced skin tumor development in hairless mice. In addition to anticancer effects, \(\alpha\)-santalol has been shown to exhibit a broad spectrum of pharmacological properties, such as anti-inflammatory, antioxidant, antihyperglycemic, and antibacterial effects. More recently, we reported the life-promoting ability of santalol isomers under neurotoxic and proteotoxic stress in C. elegans models for Parkinson’s disease. However, a clear biological mechanism underlying the longevity-promoting and protein aggregation inhibitory potential of \(\alpha\)- and \(\beta\)-santalol has not yet been described.

In this context, the current study was intended to identify the antiaging and antiaggregation mechanisms of \(\alpha\)- and \(\beta\)-santalol at the organismal level, using a multifaceted animal model C. elegans. This study provides evidence that santalol isomers prolong the mean life span, enhanced the stress resistance, improve the health span, and maintain the protein homeostasis in C. elegans by activating SKN-1/Nrf2 and EOR-1/PLZF transcription factors via the RTK/Ras/MAPK signaling module. We do believe that these findings will further promote \(\alpha\)- and \(\beta\)-santalol to become next-generation prolongevity and antiaggregation drugs for the longer and healthier life of humankind.

2. RESULTS AND DISCUSSION

2.1. Santalol Isomers-Induced Life Span Extension Requires SKN-1 and EOR-1. We previously confirmed that santalol isomers extended the life span of C. elegans under normal culture conditions. Feeding \(\alpha\)- and \(\beta\)-santalol throughout adulthood extended the mean life span and exhibited a concentration-dependent biphasic effect. Among the tested pharmacological doses, 32 \(\muM\) of \(\alpha\)-santalol and 16 \(\muM\) of \(\beta\)-santalol was the most effective concentration and significantly increased the mean life span of wild-type worms up to 10.31% and 12.56%, respectively (Figure S1 and Table S1). Given that a network of transcription factors can modulate the normal aging process, we examined whether members of this network were required for \(\alpha\)- and \(\beta\)-santalol to extend life span in C. elegans models for Parkinson’s disease.
and longevity-promoting genes in *C. elegans*. It was found that worms fed with α- and β-santalol extended the life span of *hsf-1*(sy441) and *daf-16*(mgDf50) strains (*p < 0.0001) bearing reduction-of-function mutation in genes encoding functional HSF-1 and DAF-16 proteins (Figure 1a,b). Additionally, α- and β-santalol did not alter the localization patterns of DAF-16:GFP and HSF-1:GFP fusion proteins in TJ356 and CF1824 transgenic lines, respectively (Figure S1). These data indicated that santalol isomers do not require functional HSF-1 and DAF-16 for life span extension. We next examined the requirement of SKN-1 in santalol isomers-induced life span extension. SKN-1 is an ortholog of human Nrf2 found to be regulating a wide range of homeostatic functions and oxidative/xenobiotic defense. α-Santalol and β-santalol treatment fail to extend the life span of *skn-1*(zu67) mutant worms, suggesting the participation of SKN-1 in santalol isomers-mediated life span extension (Figure 1c). A genetic study showed that SKN-1 plays an essential role in regulating endoplasmic reticulum unfolded protein response (ER-UPR), an important mechanism that promotes longevity. SKN-1 is directly involved in the activation of many-core regulators of ER-UPR, and SKN-1 expression is, in turn, upregulated by ER stress. Proteotoxic stress is sensed by transmembrane proteins ATF-6, PEK-1, and IRE-1 in the ER. Activation of these sensors increases the protein-folding capacity by producing a human PLZF-like transcription factor shown to regulate longevity and proteostasis in *C. elegans*. As a result, the life span of worms carrying the *cor-1*(cs28) loss-of-function allele showed marginal or no significant changes with α- and β-santalol feeding compared to that of untreated worms, suggesting that santalol isomers might be partially depending on EOR-1. Interestingly, knockdown of *skn-1* using RNAi completely abolishes santalol isomers-mediated life span extension observed in *cor-1*(cs28) mutant worms (Figure 1h). Subsequently, we investigated the effect of santalol isomers on the life span of *eat-2*(ad1116) and *sir-2.1*(ok434) mutants to understand the involvement of the dietary restriction (DR) mechanism. DR is a well-recognized mechanism for the extension of longevity in mammalian and nonmammalian taxa. We found that the mean life span in *eat-2*(ad1116) (a DR-constitutive mutant) and *sir-2.1*(ok434) (a sirtuin mutant) worms treated with santalol isomers was significantly (*p < 0.0001) increased compared to the untreated control (Figure S2 and Table S2). Also, santalol isomers-supplemented worms produced offspring in numbers similar to those seen in the control groups. These observations confirmed that santalol isomers might act independent of a DR-like mechanism. To summarize, these results indicated that SKN-1, IRE-1, XBP-1, and EOR-1 were involved in the longevity phenotype conferred by santalol isomers.

2.2. Santalol Isomers Trigger SKN-1 and EOR-1 Activation through the RTK/Ras/MAPK Signaling Pathway. Multiple upstream cellular signaling components are involved in the activation of SKN-1 and EOR-1 transcription factors. In particular, the receptor tyrosine kinase (RTK)/Ras GTPase/MAP kinase (MAPK) signaling module (RTK/Ras/MAPK signaling) was suggested to control diverse biological processes and acted as an upstream regulator of these transcription factors. In *C. elegans*, activation of LET-23 (RTK), an epidermal growth factor receptor (EGFR), recruits the Ras/ERK signaling cascade, which phosphorylates SKN-1 and EOR-1. Therefore, we wanted to investigate the dependency of each component of the RTK/Ras/MAPK
signaling pathway for the life span response to santalol isomers treatment. We noted that α- and β-santalol-mediated life span extension was dependent on the Ras/MAPK pathway. Our mechanistic study showed that santalol isomers treatment would not further extend the life spans of let-60 (Ras), lin-45 (Raf), mek-2 (MEK), and mpk-1 (ERK) mutants compared to vehicle-treated worms (Figure 2a–d). We next tested the requirement of the RTK function in santalol isomers-mediated life span extension in wild-type C. elegans, and we knocked down let-23 using RNAi and examined the changes in life span. LET-23 is one of two RTKs that stimulates the Ras/MAPK pathway in C. elegans.45 Knockdown of let-23 reduced the survival of wild-type worms by 38.72% (p < 0.0001) under standard conditions and abolishes the longevity-promoting effect of santalol isomers (Figure 2e and Table S3). In addition, LET-23 also acts through phospholipase C γ (PLC-γ) and IP3-inositol (1,4,5) triphosphate receptor (IP3R) signaling to promote longevity via enhancing the release of stored calcium from the ER.44 To determine the role of IP3R in santalol isomers-induced longevity extension, the effect of α- and β-santalol on the life span of itr-1(sa73) mutant C. elegans was measured. The result showed that α-santalol and β-santalol marginally increased the life span of itr-1(sa73) mutant worms (3.44% [p = 0.3104] and 3.16% [p = 0.3029], respectively), suggesting the partial requirement of IP3R in life-span extension (Figure 2f). The above results showed that santalol isomers extended the life span of C. elegans mainly via the RTK/Ras/MAPK signaling pathway.

To evaluate the phenotypic effect of α- and β-santalol treatment in C. elegans, we assayed oxidative stress resistance in wild-type C. elegans. As a result, it was found that wild-type N2 worms fed with santalol isomers showed an increased survival after exposure to juglone (240 μM), an intracellular free-radical-generator. The percent survival of worms treated with α- and β-santalol was significantly increased by about 66.61% (p < 0.01) and 69.31% (p < 0.01), respectively, in comparison with that of unexposed worms (37.08%). This increased resistance to oxidative stress is abolished in the RNAi knockout of let-23 and mutation in let-60, lin-45, mek-2, mpk-1, and skn-1. It is interesting to note that α- and β-santalol feeding marginally increased the percent survival of cor-1(cs28) mutant worms under juglone-intoxicated conditions (p < 0.05). Conversely, skn-1 RNAi completely inhibited the stress-resistance phenotype observed in cor-1(cs28) worms (Figure 3a). Similarly, α- and β-santalol feeding protects C. elegans from DTT-induced reductive ER stress (p < 0.05) in an skn-1-dependent manner. DTT is a reducing agent that affects protein folding or initiates the UPR through disrupting the disulfide-bond formation in the ER. DTT exposure results in an increase in protein misfolding and completely inactivates the protein-folding process in C. elegans.8 We found that santalol isomers-mediated stress protection was found to rely on skn-1, ire-1, and xbp-1 but did not rely on gene pck-1 and atf-6 (Figure S2). These results were in line with the life span experiments (Figure 1d–g). The enhanced survival under oxidative stress conditions is further associated with altered intracellular redox status.45 To test this, we measured the intracellular ROS levels using H$_2$DCF-DA in wild-type worms grown on NGM plates carrying either santalol isomers or vehicle control. Results showed that wild-type worms treated with juglone exhibited significantly higher ROS levels, and vehicle treatment does not alter the intracellular ROS levels. In contrast, α- and β-santalol significantly reduced the ROS levels by 58.85 and 68.76%, respectively. On the contrary, let-23 RNAi and mutation in let-60, lin-45, mck-2, mpk-1, and skn-1 prevent the santalol isomers-induced reduction in ROS levels.

Figure 3. Role of RTK/Ras/MAPK pathway components in santalol isomers-mediated stress resistance and endogenous ROS levels. (a) Survival percentage of worms treated with α- and β-santalol under juglone-intoxicated conditions. (b) Relative changes in endogenous ROS levels were measured using H$_2$DCF-DA after being treated with α- and β-santalol under juglone-induced oxidative stress. Combined data of three independent biological trials were presented (mean ± SEM). *p < 0.05 and **p < 0.01 vs juglone-treated group; p < 0.01 vs untreated control.
results showed that SKN-1 but not EOR-1 is required for exogenous oxidative stress resistance and ROS inhibition following santalol isomers treatment. Collectively, these data specifically implicate the RTK/Ras/MAPK signaling pathway in santalol isomers-induced life span extension and stress resistance.

2.3. Santalol Isomers Induce SKN-1 and EOR-1 Gene Targets. Given the dependency of SKN-1, IRE-1/XBP-1, and EOR-1 in santalol isomers-induced life span extension, we further examined the expression pattern of target genes of these transcription factors using transcriptional reporter C. elegans strains. First, we tested the effect of santalol isomers feeding on the expression and nuclear accumulation of the skn-1b/c::GFP transcription factor. It was found that α- and β-santalol triggered the constitutive localization of skn-1 b/c::GFP into nuclei of intestinal cells (Figure 4a) as well as in ASI neurons (Figure 4b). Under the basal condition, the activity of SKN-1 was suppressed by the binding of a WD40 repeat-containing protein, WDR-23. Once activated, SKN-1 is uncoupled from WDR-23 and translocates into the cells’ nucleus, where it transactivates multiple cellular antioxidant and detoxifying genes. We then examined the effect of santalol isomers on the expression of direct targets of SKN-1 using transgenic transcriptional reporter strains gst-4::GFP, gcs-1::GFP, and gsr-1::GFP transgene following α- and β-santalol feeding at 20 °C (p < 0.01 vs the untreated control; p < 0.01 vs santalol isomers-treated). (c) Effect of santalol isomers on the expression of gst-4::GFP in worms bearing the skn-1 null mutation (CL691) (p < 0.05). Data were acquired from three independent biological experiments and are presented as mean ± SEM. The scale bar represents 100 μm.
santalol on the expression of ER chaperon hsp-4 using the transcriptional reporter strain SJ4005. HSP-4 is upregulated during ER stress, and it is a direct target of the IRE-1/XBP-1 axis. α- and β-santalol treatment significantly upregulates hsp-4::GFP expression compared to unexposed worms in an SKN-1-dependent manner (Figure 4c,d).

A recent study uncovered the new function of EOR-1 in the regulation of the cytoprotective gene gst-4 independent of SKN-1. Hence, to further understand the possibility that santalol isomers could modulate the gst-4::GFP expression through EOR-1, novel C. elegans strains were used to critically investigate this idea. At first, we tested the effect of santalol isomers in worms bearing the skn-1 null mutant background (gst-4::GFP;skn-1[zu67]; CL691 strain). α- and β-Santalol feeding marginally upregulated the expression of the gst-4::GFP transcriptional reporter (p < 0.05, Figure 4e), suggesting the implication of EOR-1 in the gst-4 expression in CL691 C. elegans. To clarify this possibility, we next assayed the effect of santalol isomers on the gst-4 transcription level in worm populations carrying a loss-of-function mutation in cor-1 (LSC969) and gain-of-function mutation in let-23 (LSC1255) (***p < 0.01 vs the untreated control; *p < 0.01 vs santalol isomers-treated). (c, d) Expression of gst-4::GFP under the oxidative stress condition in CL691 and LSC969 C. elegans (***p < 0.01 vs the untreated control; *p < 0.01 vs juglone-treated). Data were acquired from three independent biological experiments and are presented as mean ± SEM. The scale bar represents 100 μm.

Figure 5. Interplay between SKN-1 and EOR-1 in santalol isomers-induced activation of antioxidant and geroprotective gene gst-4. (a, b) Effect of santalol isomers on the expression gst-4::GFP in worms carrying loss-of-function mutation in cor-1 (LSC969) and gain-of-function mutation in let-23 (LSC1255) (***p < 0.01 vs the untreated control; *p < 0.01 vs santalol isomers-treated). (c, d) Expression of gst-4::GFP under the oxidative stress condition in CL691 and LSC969 C. elegans (***p < 0.01 vs the untreated control; *p < 0.01 vs juglone-treated). Data were acquired from three independent biological experiments and are presented as mean ± SEM. The scale bar represents 100 μm.
Santalol do not require functional EOR-1 to upregulate gst-4::GFP expression following endogenous oxidative stress, and the EOR-1 acts largely independent of the classical oxidative stress response pathway. These findings are highly consistent with the previous report.49 Taken together, these results suggested that santalol isomers confer longevity and stress resistance in *C. elegans* via inducing the expression of SKN-1 and EOR-1 target genes.

### 2.4. Santalol Isomers Help Maintain Protein Homeostasis in *C. elegans*

Given the relationship between life span and proteostasis and the well-established role for SKN-1, IRE-1/XBP-1, and EOR-1 in both longevity and protein homeostasis,7,8,40 we hypothesize that α- and β-santalol might increase the life span of *C. elegans* via inhibiting the age-dependent accumulation of insoluble proteins. To validate this hypothesis, we measured santalol isomers’ effect on the age-related accumulation of the insoluble proteins α- and β-santalol delayed the Aβ-induced paralysis phenotype in the transgenic *C. elegans* strain CL4176. (b) Effect of santalol isomers on the paralysis caused by the polyglutamine-repeat protein (Q35) in the transgenic *C. elegans* strain AM140. (c–e) Influence of α- and β-santalol on the aggregation of Q35::YFP and Q40::YFP in AM140 and AM141 *C. elegans*, respectively. (f) Neuronal viability in HA759 worms expressing Q150 tracts (HtnQ150::GFP) in ASH neurons treated with santalol isomers. (g) Relative expression rate of geroprotective and protein aggregation inhibitory genes in worms expressing Q40 repeats in body wall muscle cells. Data were acquired from three independent biological experiments and are presented as mean ± SEM. *p < 0.05 and **p < 0.01 vs the untreated control; #p < 0.01 vs santalol isomers-treated. The scale bar represents 100 μm.

Figure 6. Santalol isomers feeding prevents the aggregation of insoluble toxic proteins and its associated pathologies in *C. elegans*. (a) α- and β-santalol delayed the Aβ-induced paralysis phenotype in the transgenic *C. elegans* strain CL4176. (b) Effect of santalol isomers on the paralysis caused by the polyglutamine-repeat protein (Q35) in the transgenic *C. elegans* strain AM140. (c–e) Influence of α- and β-santalol on the aggregation of Q35::YFP and Q40::YFP in AM140 and AM141 *C. elegans*, respectively. (f) Neuronal viability in HA759 worms expressing Q150 tracts (HtnQ150::GFP) in ASH neurons treated with santalol isomers. (g) Relative expression rate of geroprotective and protein aggregation inhibitory genes in worms expressing Q40 repeats in body wall muscle cells. Data were acquired from three independent biological experiments and are presented as mean ± SEM. *p < 0.05 and **p < 0.01 vs the untreated control; #p < 0.01 vs santalol isomers-treated. The scale bar represents 100 μm.
dependent aggregation of neurotoxic peptides, amyloid β (Aβ), and polyglutamine repeats (polyQ35/Q35 and polyQ40/Q40). Initially, the antiaggregation potential of α- and β-santalol was investigated by employing the *C. elegans* strain CL4176. This strain expresses an aggregation-prone Aβ_{1-42} peptide in body wall muscles cells and exhibits Aβ aggregation-induced paralysis phenotype following temperature upshift from permissive (15 °C) to restrictive (25 °C). The results showed that santalol isomers feeding significantly delayed the proportion of paralyzed worms in comparison with that of control levels, and it was canceled by either let-23 or skn-1 RNAi (Figure 6a). Furthermore, α- and β-santalol feeding increased the mean life span of CL4176 worms by 33.35% (p < 0.0001) and 39.41% (p < 0.0001), compared with that of the untreated control group at 16 °C in a let-23- or skn-1-dependent manner (Figure S4 and Table S4). We also checked santalol isomers’ potential to inhibit protein aggregation using AM140 and AM141 *C. elegans*. These strains express muscle-specific polyglutamine repeats (Q35 and Q40, respectively), driven by the unc-54 promoter. After treatment with α- and β-santalol, Q35-dependent paralysis phenotype and visible aggregate formation in AM140 worms significantly decreased (Figure 6b–d) and the mean life span was extended by 27.25 and 28.01%, respectively (Figure S4 and Table S4). In addition, AM141 worms treated with santalol isomers exhibited a very significant reduction in Q40 aggregates (63.23 and 68.87%, respectively, p < 0.01) (Figure 6e,c) and extended the mean life span (p < 0.0001) (Figure S4 and Table S4) compared to the unexposed control group. We next investigated the protective effect of santalol isomers against polyglutamine-mediated neuronal death in HA759 worms expressing Q150 tracts (HtnQ150::GFP), especially in ASH neurons weekly in other neurons, leading to neuronal death. As shown in Figure 6f, only 33.30 ± 0.03% of the neurons survived in control group worms, indicating the neurotoxic property of Q150, while α- and β-santalol were proficient in increasing the neuronal survival to 74.71 ± 2.50% (p < 0.01) and 77.71 ± 1.94% (p < 0.01), respectively (Figure S4). Moreover, santalol isomers feeding improved the polyglutamine-repeat-related pathologies, including reduced chemosensory behavior and mean life span in HA759 worms (Figure S5 and Table S4). Interestingly, either let-23 or skn-1 RNAi completely blocks the santalol isomers-induced beneficial effect in AM140, AM141, and HA759 *C. elegans*, indicating the importance of SKN-1 and EOR-1. We noticed that the effect of santalol isomers on Alzheimer’s and Huntington’s disease models of *C. elegans* was very similar to the positive control EGCG (50 μM) across the experiments. Adding up, we also found an increased mRNA transcript level of antioxidant and geroprotective genes gst-4, gst-10, gsr-4, and hsp-4 in worms expressing Q40 tracts treated with santalol isomers. Also, α- and β-santalol upregulates the mRNA transcript levels of skr-S, a gene that controls global protein turnover in *C. elegans* (Figure 6g). The santalol isomers-induced expression of such genes was abolished by skn-1 RNAi in AM141 worms. Comparatively, in wild-type worms, a significant upregulation of skn-1, gst-4, gsr-1, gst-10, and gsr-1 was observed. Santalol isomers did not obviously alter the expression rate of hsp-4; however, they marginally upregulated hsp-4, but it was found to be statistically insignificant (p > 0.05) (Figure S4). These
results demonstrated that santalol isomers could prevent detrimental effects of age-related protein aggregation in C. elegans mainly via activating SKN-1 and EOR-1 transcription factors and its direct transcriptional readouts.

2.5. Santalol Isomers Also Interact with hpa-1 and hpa-2 to Promote Life Span and Health Span. To further understand the possibility that α- and β-santalol could enhance life span and health span, we examined their effect on hpa-1(tm3256) and hpa-2(tm3827) (high performance in advanced age) mutant worms. It has been proven that HPA-1 and HPA-2 negatively regulate the EGF ligand possibly by binding and sequestering its function, and they control the overall aging process and locomotory aging in C. elegans, respectively.44 In the absence of HPA-1/2, LIN-3/EGF freely binds with RTK (LET-23/EGFR) and recruits the downstream RTK/Ras/MAPK and PLC-γ/IP3R signaling axis to confer health span and life span.44 As a result, we found that α- and β-santalol feeding failed to enhance the mean life span of worms deficient in hpa-1 and hpa-2 (p > 0.05) (Figure 7a,b; Table S2). These results indicate that santalol isomers required both hpa-1 and hpa-2 to extend longevity and proteostasis in C. elegans. To further strengthen these results, we, therefore, performed a molecular modeling analysis to predict the interaction of α- and β-santalol with HPA-1, HPA-2, and LIN-3. As shown in Figure 7c−h, the results showed that α- and β-santalol bound to the active site pocket of HPA-1 through strong hydrogen bonds and hydrophobic interaction with binding affinities of −6.3 and −6.6 kcal mol$^{-1}$, respectively; it clearly indicates that santalol isomers showed high binding affinity toward HPA-1 possibly by interacting with active site residues Thr148, Ile82, Tyr125, Leu175, and Ile82. The santalol isomers have a similar binding affinity of −5.5 kcal mol$^{-1}$ with HPA-2. α-Santalol forms two hydrogen bonds with HPA-2 active site residues (Phe8,10), whereas β-santalol forms a single hydrogen bond and hydrophobic interaction with Leu3 and Phe7. α- and β-Santalol have a similar profile with the hydrophobic pocket of LIN-3, form two hydrogen bonds with binding site residues Tyr336 and Asn159, and have different binding affinities of −6.6 and −5.7 kcal mol$^{-1}$. The docking scores and postdocking analysis of ligand-bound protein complexes clearly indicate that α- and β-santalol can bind HPA-1, HPA-2, and LIN-3 and have favorable interactions with active site residues. The hydroxyl group of santalol isomers is actively involved in hydrogen bonding with active site residues, and the presence of hydrogen bonding between the receptor and santalol isomers plays a crucial role in the formation and stabilization of the protein-inhibitor/-inducer complex. From these observations, it was concluded that the direct interaction of santalol isomers with HPA-1 and HPA-2 inhibits their function and promotes the release of LIN-3. Another plausible explanation is that the interaction of α- and β-santalol may trigger the binding of LIN-3 to the extracellular domain of the RTK and thus enhance the signaling through downstream components. Currently, we have been working to validate the biophysical interaction of santalol isomers with HPA-1/2 and LIN-3 in C. elegans.

2.6. α- and β-Santalols Confer Favorable Health Span Benefits. Interventions that extend the life span of C. elegans do not necessarily improve the health span with age;51 to test
this idea, we, therefore, studied the health-promoting activities of santalol isomers. Initially, we measured the level of lipofuscin accumulation in control and santalol isomers-treated wild-type worms. Lipofuscin is a complex molecular waste that accumulates within the lysosome-like gut granules in *C. elegans* and is often used as an aging marker. The amount of autofluorescent lipofuscin granules steadily increases throughout *C. elegans* adulthood, and it emits an intense blue fluorescence under ultraviolet illumination.\(^\text{52}\) We noticed that \(\alpha\)- and \(\beta\)-santalol treatment significantly reduced the lipofuscin levels in late-age (day 10) wild-type worms by 55.27\% (\(p < 0.01\)) and 62.97\% (\(p < 0.01\)), respectively, showing that worms fed with santalol isomers age more slowly than the control worms (Figure 8a,b). The learning ability and motor activity were reported to decline with age and have been considered to be physiological parameters of a worm’s healthiness.\(^\text{53}\) Therefore, we checked the effect of santalol isomers on a few physiological parameters such as pharyngeal pumping, motor activity (i.e., body bends), and chemotaxis behavior. It was found that the optimal dose of \(\alpha\)-santalol (32 \(\mu\)M) and \(\beta\)-santalol (16 \(\mu\)M) significantly improved the age-dependent reduction in pharyngeal pumping (Figure 8c) and its morphology in aged worms (Figure S6). Also, we observed that wild-type worms pre-exposed to santalol isomers showed an enhanced chemotaxis behavior on day 10 compared to age-matched control worms; it indicates the healthy status of chemosensory neurons (Figure 8d). The motor activity of *C. elegans* is also reported to decay with age. Therefore, we compared body bends between santalol isomers-treated and untreated worms in old age. Worms maintaining the loss-of-function alleles, *hpa-1*(tm3256) and *hpa-2*(tm3827), were used as positive controls, which exhibit an enhanced swimming vigor during the later adulthood stage.\(^\text{44}\) As shown in Figure 8e, both \(\alpha\)-santalol- and \(\beta\)-santalol-treated worms showed an enhanced swimming activity/body bends at old age (day 10; \(p < 0.01\)) compared to the unexposed control. Besides, santalol isomers treatment significantly increased the average body bends compared to *hpa-1* and *hpa-2* mutant worms (\(p < 0.01\) and \(p < 0.056\); \(p < 0.01\) and \(p < 0.03\), respectively). In *C. elegans*, the ability to sense and respond to touch (touch response) is primarily controlled by mechanoreceptor neurons, and it declines with age.\(^\text{44}\) We observed that late adulthood worms (day 10) exhibited an age-dependent reduction in posterior and anterior touch response, while \(\alpha\)- and \(\beta\)-santalol feeding improved the anterior (16.98 and 18.87\%; \(p < 0.05\)) and posterior (39.68 and 41.2\%; \(p < 0.05\)) touch response late in life (Figure 8f). Overall, these results imply that \(\alpha\)- and \(\beta\)-santalol treatment improved several health-span measures in *C. elegans* during their late adulthood stages.

In conclusion, as shown in Figure 9, santalol isomers act on regulators of EGF (such as HPA-1/2) and promote the release of LIN-3 or bind to LIN-3, which in turn promotes EGF signaling. Activation of RTK by santalol isomers recruits the Ras/MAPK signaling cascade, which phosphorylates SKN-1 and EOR-1 transcription factors. Once activated, SKN-1 and EOR-1 translocate to the cells’ nucleus and trigger the transcription of several antioxidant and detoxification genes, which help minimize protein oxidation and aggregation and extend the life span. Furthermore, santalol isomers-mediated RTK activation also recruits PLC-\(\gamma\)/IP3R signaling to promote longevity via enhancing the release of stored calcium from the ER. Also, santalol isomers help maintain proteostasis in *C. elegans* via activating the IRE-1/XBP-1 pathway, which increases the protein-folding capacity by activating the ER chaperon HSP-4. Overall, \(\beta\)-santalol exhibits reactive higher activity at lower doses than \(\alpha\)-santalol; it may be due to the presence of an unsaturated active alkene group in the second position over the eight-member ring. Together, the proposed antiaging and antiaggregation mechanism of santalol isomers may open the possibility of developing a novel therapeutic intervention to counteract aging and its associated declines.

### 3. MATERIALS AND METHODS

**3.1. Materials.** Santalol isomers were obtained from Santalis Pharmaceuticals Inc. (San Antonio, TX). The purity of \(\alpha\)- and \(\beta\)-santalol used in this study was determined through gas chromatography/flame-ionization detector (GC/FID) analysis and had 98.2 and 93.2\% purity, respectively.\(^\text{27}\) The detailed isolation procedure is provided in the Supporting Information. S-Hydroxy-1,4-naphthoquinone (juglone), 2',7'-...
dichlorodihydrofluorescein diacetate (H$_2$DCF-DA), 5-fluoro-2′-deoxyuridine (FUDR), epigallocatechin gallate (EGCG), isopropyl β-D-thiogalactopyranoside (IPTG), and dithiothreitol (DTT) were obtained from Sigma Aldrich (St. Louis, MO). Culture media components, dimethyl sulfoxide (DMSO), sodium hydroxide (NaOH), household bleach (NaClO), sodium azide (Na$_3$NO$_2$), ampicillin, streptomycin, and tetracycline were purchased from HiMedia Laboratories Pvt. Ltd. (Mumbai, India).

3.2. Worm Strains, Molecular Genetics, and Maintenance. C. elegans strains were maintained and cultured under standard conditions at 20 °C on nematode growth media (NGM) agar plates containing lawn of live Escherichia coli strain OP50 as described.28 Worms carrying temperature-sensitive mutation (itr-1) were maintained at the permissive temperature of 15 °C. Bristol N2 was used as the wild-type strain. The mutant strains used in this study are PS1839 (let-23[sa62]), ZB2844 (hpa-1[Tn3256]), ZB2845 (hpa-2[tm3827]), MT4866 (let-60[n2021]), WUS1 (lin-45[n2520]), MT8666 (mek-2[n1989]), MH37(mpk-1[ku1]), UP233 (cor-1[cs28]), EU1 (skn-1[zu67]), JT73 (itr-1[sa73]) CB1370 (daf-2[e1370]), GR1307 (daf-16[mgD50]), and PS3551 (hsf-1[y441]). The following transgenic lines were used: CF1824 (muEx265 [hsl-1::GFP]), TJ356 (zls356 [daf-16::GFP]), CL691 (dvi19[pAF15(gst-4::GFP::NLS)]) ; skn-1[zu67] IV/ nT1[unc-22(7[n574]; let-4)], LSC969 (cor-1[cs28];gst-4::GFP), LSC1255 (let-23[sa62];gst-4::GFP), LD1 (ldIs7[skn-1b::GFP]), CL2166 (dvi19[gst-4::GFP]) ; LD1171 (ldIs3[gcs-1::GFP]), grl-1::GFP, SJ4005 (zIs4 [hsp-4::GFP]), AM140 (rnIs132[unc-54::Q35::YFP]), AM141 (rnIs133[unc-54::Q40::YFP]), HA759 (rlIs11[osm-10::GFP + osm-10::HhnQ150 + dpy-20(+)]) and CL4176 (dvi27[myo-3p::Abf1-42::let-851 3′UTR + rol-6(su1006)]) . The isogenic worm populations were obtained by treating the gravid adults with 5% NaClO + 5 N NaOH as described.29

3.3. Life-Span Assay. The life span of C. elegans was measured at 20 °C, as previously described.25 Briefly, age-sorted L4-stage worms (15–20 worms per plate for a total of 100–130 individuals per experiment) were transferred to NGM plates with and without santalol isoforms. Animals were transferred to a new plate at every 3–4 day interval, until death. Worms were scored as dead if they failed to respond to mild physical contact with metal wire pick and loss of pharyngeal pumping. Censoring occurred if the worms suffered from internal hatching, desiccation due to crawling off the plates, ruptured, or mechanical death. At least three independent biological repeats were performed with appropriate replicates as indicated at similar conditions.

3.4. Assay for Stress Resistance. The age-synchronized L4 larvae (15–20 individuals per replicate) were grown on NGM plates with and without santalol isoforms or 3 days at 20 °C. Subsequently, treated and untreated adult worms were transferred to fresh NGM plates containing 240 μM DTT, an intracellular redox cycle. The viability of worms was scored after 3 h of continuous exposure. To measure the protective effect of santalol isoforms against DTT-induced reductive ER stress, worms were raised on the NGM plates spotted with and without santalol isoforms from the embryo. On day 2 of adulthood, the worms were transferred to new plates containing 5 mM DTT, and the viability was scored after 12 h.8

3.5. Scoring of GFP Reporter Accumulation or Expression. Nuclear localization/accumulation of SKN-1 was measured using the transgenic strain LD1 carrying the skn-1b/c::GFP transgene, which encodes two of the three SKN-1 isoforms. Synchronized L1 larvae were treated with santalol isomers for 72 h at 20 °C. After treatment, the worms were washed thrice with M9 buffer to remove adhering bacteria, anesthetized with 25 mM Na$_3$NO$_2$ and mounted on glass slides for fluorescence microscopy (BX51, Olympus, Japan). Scoring of skn-1b/c::GFP was done as follows: none, no visible nuclear accumulation; low, few nuclei showed GFP; medium, more than half of the intestinal nuclei exhibits GFP positive; high, all nuclei showed GFP.27 To analyze the gst-4::GFP, gcs-1::GFP, and grl-1::GFP expressions, synchronized L1 larvae were transferred to fresh NGM plates with or without α- and β-santalol. On the first day of adulthood, the green fluorescence intensity of 40–50 worms/condition was observed and imaged after mounting on glass slides. The GFP signal was quantified by determining the mean pixel intensity using ImageJ software (NIH, Bethesda, MD) and expressed as a relative fluorescent unit (RFU).31

3.6. Quantification of ROS Generation. Intracellular ROS generation was quantified in C. elegans using the molecule probe H$_2$DCF-DA. The worms were maintained and treated as described above. After exposure to α-santalol or β-santalol, the worms were washed off from the plates with M9 buffer and pelleted by centrifugation. The pellet was reconstituted in 1 mL of 50 μM H$_2$DCF-DA solution and incubated for 30 min in the dark at 20 °C. Fluorescence images were obtained, and the relative ROS level was analyzed densitometrically using ImageJ software.32

3.7. Assay for Protein Aggregation. Protein aggregation assay was performed using transgenic C. elegans strains expressing polyQ35::YFP (AM140) and polyQ40::YFP (AM141) fusion proteins in the body wall muscle cells as described.33 In brief, age-sorted L1 larvae (30–40 individuals/treatment) were continuously exposed to santalol isoforms at 20 °C for indicated time points. After that, the photomicrographs of control and treated worms were taken under a fluorescence microscope and scored for polyQ35::YFP and polyQ40::YFP aggregates across various adulthood stages.

3.8. Neuronal Survival Assay. C. elegans strain HA759 expressing polyQ tract (HtnQ150) in ASH neurons was used for neuronal survival assay.33 Synchronized L1 larvae were treated with α- and β-santalol until their day 6 adulthood stage at 20 °C. After treatment, the worms were collected and mounted on 3% agarose padded microscopic slides, as said above. About 40–50 randomly selected individuals per treatment were scored for GFP positive or negative in ASH neurons using a fluorescence microscope. Further, a chemotaxis behavior assay was performed to evaluate the polyQ inhibitory effect of santalol isoforms according to the method described previously.33

3.9. RNA Interferences (RNAi). C. elegans skn-1 and let-23 genes were silenced via the regular feeding RNAi method.34 NGM plates containing 2 mM IPTG and 100 μg/mL ampicillin were seeded with E. coli HT115(DE3) bacteria cloned with either the pH4440 vector or the test RNAi construct. Age-synchronized L3-stage larvae were transferred to RNAi plates and propagated at 20 °C unless noted otherwise. To prevent adulthood lethality due to internal hatching in the worms fed with HT115 bacteria containing the RNAi construct for let-23, FUdR with a final concentration of 100 μM was overlaid onto each assay plate before transferring.
the worms. After propagation in RNAi plates (after two/three generations), L1-/L4-stage worms were used for various assays.

3.10. Quantitative Real-Time PCR Analysis. The worms were frozen in liquid nitrogen, thawed on ice, and homogenized at 4 °C, and the total RNA was extracted using TRIzol reagents (Invitrogen) according to the manufacturer’s instructions. cDNA was synthesized by reverse transcription using a RevertAid First-stand cDNA synthesis kit (Invitrogen). Quantitative real-time PCR was performed with SYBR Green Super-mix (Applied Biosystems), and the relative gene expression was analyzed using a comparative C\(^{\Delta \Delta C_{t}}\) method. The housekeeping gene act-1 was chosen as the reference gene.

3.11. Molecular Modeling. The ab initio modeling approach was implemented in Rosetta to build a high-quality three-dimensional structure of proteins HPA-1, HPA-2, and LIN-3. For structural optimization, 200 ns GPU accelerated molecular dynamics (MD) simulation was implemented in the GROMACS 2016.4 package. The molecular docking was performed using AutoDock Vina (v.1.1.2), as previously described. The detailed procedure is provided in the Supporting Information.

3.12. Statistical Analysis. Statistical differences between each treatment were analyzed using a one-way analysis of variance (ANOVA; 95% confidence interval), followed by Bonferroni’s post hoc test (SPSS 16, IBM Corporation, NY). Survival curves of C. elegans were constructed using the Kaplan–Meier survival method and analyzed by the log-rank (Mantel–Cox) test in MedCalc statistical software (MedCalc 14, Ostend, Belgium). Data represent the results of three independent biological repeats performed with appropriate replicates at similar conditions, as indicated. The probability level of \(p < 0.05\) was considered to be statistically significant between means.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c05006.

Detailed experimental procedures (materials and methods a–k); MD simulation (results and discussion); life span of N2 worms and localization of transcription factors (Figure S1); involvement of the DR pathway and ER stress response in life-span extension mediated by sEH59, atal, and AM141 worms and gene expression (Figure S4); effect of sEH59 on HA758 worms (Figure S5); pharynx morphology, MD simulation, and modeled protein (Figures S6–S8); life span of wild-type, mutant, wild-type under RNAi, and neurodegenerative disease models (Tables S1–S4); references (PDF)

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Notes

The authors declare no competing financial interest.

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