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ORIGINAL ARTICLE

5′-Hydroxy-6, 7, 8, 3′, 4′-pentamethoxyflavone extends longevity mediated by DR-induced autophagy and oxidative stress resistance in *C. elegans*

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Abstract 5′-Hydroxy-6, 7, 8, 3′, 4′-pentamethoxyflavone (5-HPF), a polymethoxyflavone compound found in dikamali gum, has been shown to exert a range of beneficial effects on health. We have previously reported that 5-HPF improves the cholinergic dysfunction and also possesses antioxidant properties in *Caenorhabditis elegans*. In this study, we have identified the effect of 5-HPF on the worm lifespan and its underlying molecular mechanisms. Out of the five tested pharmacological doses of 5-HPF, viz. 6.25, 12.5, 25, 50, and 100 μM, the 50 μM dose maximally extended the mean life of *C. elegans* by 28%. The present study revealed that 5-HPF supplementation leads to dietary restriction (DR)-like effects in the worms without altering bacterial metabolism. The analysis of mutant animals fed with 5-HPF suggested that the extended lifespan of *C. elegans* depends upon multiple DR-related signaling pathways, with NRF2 and FOXA being critical factors. Further investigation into the mechanistic aspects indicated that 5-HPF utilizes autophagy pathway induced by DR through the upregulation of autophagy genes *bec-1* and *lgg-1*, evident from the increase in autophagic puncta in the seam cells of *lgg-1*::*gfp* tagged worms. This study identifies the longevity-promoting activity of 5-HPF in *C. elegans* regulated by oxidative stress-resistance genes and DR-induced autophagy pathway.

Keywords 5′-Hydroxy-6, 7, 8, 3′, 4′-pentamethoxyflavone · Dietary restriction · *C. elegans* · Aging · *Gardenia lucida* · Reactive oxygen species

Introduction

Aging is marked with progressive deterioration of physiological functions resulting from the cellular impairment. This stimulates the progression of major ailments such as diabetes, cardiovascular disease, cancer, osteoporosis, arthritis, and neurodegenerative disorders (Franceschi et al. 2018). Major molecular mechanisms regulating the aging process have been distinguished in lower invertebrates, and the same mechanisms are often noted in mammals, providing the evidence of highly conserved aging genes and signaling pathways (Smith et al. 2008). Among all pathways, dietary restriction (DR) is the most extensively studied and established phenomenon of longevity from yeast to mammals (Kennedy et al. 2007). DR prolongs lifespan by curtailing nutrient uptake, altering several nutrient processing pathways. In *Caenorhabditis elegans*, DR-mediated longevity is governed by two transcription factors PHA-4/FOXA and SKN-1/NRF2 (Panowski et al. 2007). Specifically, PHA-4/FOXA enhances the lifespan of *C. elegans* in food-deprived conditions and regulates the expression level of stress modulatory genes (Panowski et al. 2007). PHA-4/FOXA is the

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primary mediator that regulates DR in worms, encodes for the pharynx and foregut development in *C. elegans* (Panowski et al. 2007). SKN-1/NRF2 activates a wide range of genes involved in cellular repair, detoxification, and stress resistance (Oliveira et al. 2009). In *C. elegans*, SKN-1/NRF2 is required for the response to oxidative stress and starvation (An et al. 2005). The expression of SKN-1/NRF2 in ASI neurons has also been reported in lifespan extension (Bishop and Guarente 2007). SKN-1/NRF2 and PHA-4/FOXA both contribute to DR-regulated longevity. The autophagic response induced by LET-363/ MTOR inhibition requires PHA-4 activity. LET-363/MTOR and SKN-1/NRF2 function through a feedback circle for the activation of stress-responsive genes (Hansen et al. 2008; Robida-Stubbs et al. 2012).

The modulation of ribosome biogenesis and autophagy is regulated by target of rapamycin (TOR) (Wullschleger et al. 2006). In *C. elegans*, the components of MTOR, TOR kinase (*let-363* (Long et al. 2002)) and Raptor (*daf-15* (Jia et al. 2004)), regulate growth, protein synthesis, aging, and autophagy (Hansen et al. 2007; Vellai et al. 2003). DR induced by *eat-2* mutation has also been influenced by *let-363*, under the regulation of PHA-4/FOXA (Hansen et al. 2007; Meissner et al. 2004). DR-mediated longevity depends on the nutrient sensing and energy availability, which controls the synthesis and degradation of cellular macromolecules and organelles through a recycling mechanism, "autophagy" (Hansen et al. 2008). In *C. elegans*, longevity is also regulated by autophagy genes *unc-51*/ULK1, *bec-1*/Beclin1, and *lgg-1*/LC3 (Melendez and Levine 2009). Principally, autophagy or self-eating entails scavenging of damaged cellular components, which serve as a major source of reactive oxygen species. The reactive oxygen species (ROS) produced from the aerobic metabolism of an organism causes oxidative stress (Cadet and Wagner 2013). Oxidative stress has been known to possess a crucial role in aging and age-related degenerative diseases (Finkel and Holbrook 2000; Lin and Beal 2006). The characteristic exogenous and endogenous cell defense system tackles free radical formation by several enzymatic and non-enzymatic strategies (Sies 1997). Plant-derived bioactive molecules/extracts have been widely explored for their efficacy in treating different diseases and restorative properties. Previously, phytomolecules have been shown to participate in the regulation of various metabolic processes and have lifespan-extending properties in animal models (Brown et al. 2006; Kampkötter et al. 2008; Koch et al. 2014; Saul et al. 2009). They have also been shown to improve diseases such as cancer, neurodegeneration, and metabolic syndrome. Flavonoids, consisting of mainly polymethoxyflavones (PMFs), have been shown to exhibit a broad spectrum of biological properties including antitumor (Miyata et al. 2008), anti-carcinogenic (Li et al. 2007), anti-inflammatory (Ho et al. 2012), and antioxidant (Li et al. 2007). Hence, with the reported efficacy of PMFs, we undertook the present study to elucidate the effect of 5′-hydroxy-6, 7, 8, 3′, 4′-pentamethoxyflavone (5-HPF) on lifespan. Earlier, we have reported the extraction and identification of 5-HPF and its role in the improvement of cholinergic function and oxidative stress tolerance in *C. elegans* (Trivedi et al. 2017). The systematic evaluation of adequacy, wellbeing, and mechanism of phytomolecule in a mammalian model is expensive and tedious. *C. elegans* is the most favored model inferable from its short life expectancy and life cycle, simple research maintenance, and high homology to mammalian particularly human biochemical and hereditary pathways (Kenyon 2010). The present study highlights the beneficial effect of 5′-hydroxy-6, 7, 8, 3′, 4′-pentamethoxyflavone (5-HPF) on lifespan in the *C. elegans* model system.

Methods

Culture and maintenance of strains

C. elegans strains, Bristol N2; DA1116, *eat-2* (ad116); GR1307, *daf-16* (mgDf50); CB1370, *daf-2* (e1370); EU-31, *skn-1* (zu131); SM190, *smg-1*/*pha-4*; VC424, *bec-1*; BE150, *unc-51* (e369); KR344, *let-363* (h98) *dpy-5* (e61) *unc-13*(e450); DA2123,*lgg-1*::gfp; SM481, *pha-4*::gfp; and *Escherichia coli* OP50 were procured from Caenorhabditis Genetics Center (University of Minnesota, MN, USA) and grown on Nematode growth medium (NGM) and cultured at 20 °C. A synchronized population of worms was obtained by sodium hypochlorite treatment (Porta-de-la-Riva et al. 2012). Worms were maintained on media seeded with *E. coli* OP50 bacteria using established standard protocol (Brenner 1974).

Lifespan assay

Lifespan assays were performed at 20 °C as described previously (Srivastava et al. 2017). Briefly, age-

synchronized eggs were added to NGM plates seeded with *E. coli* OP50 along with different concentrations of test compound 5-HPF and 0.05% DMSO serves as vehicle control. At L4 stage, worms ($n = 60–70$ per 6 cm Petri plate) were transferred to new treatment plates supplemented with 50 μM 5-fluoro-2′-deoxyuridine (FUdR) (Sigma-Aldrich) to inhibit the progeny. Worms were observed daily for survival, scoring until the last worm survived. Worms were transferred to fresh treatment plates, every 48 h to avoid bacterial contamination and maintain a specific concentration of 5-HPF. Any worms desiccated or damaged were excluded from the study.

Measurement of brood size and reproductive span

For reproduction assay, age-synchronized N2 worms at the L1 stage were transferred to pre-treated NGM plates as described in the lifespan assay without FUdR. Individual L4-stage worm was transferred to fresh NGM plates ($n = 5$) treated with and without 5-HPF each day until the worms ceased to lay eggs. Numbers of hatched worms per plate were counted daily (Pant et al. 2014).

Lipid staining assay by Nile Red

The effect of 5-HPF treatment on lipid levels in worms was measured using Nile Red (a fluorescent dye used to stain intracellular lipid droplets) staining. A 0.5-mg/ml stock solution of Nile Red was prepared in acetone, further diluted with OP50 in a ratio of 1:250 and spotted onto NGM plates along with or without 5-HPF treatment. Thereafter, age-synchronized L1 worms were transferred to treatment plates and incubated at 20 °C. After 72 h, worms were transferred to 3 different treatment plates according to the day of observation under a microscope. At the day of imaging (day 3/6/12), worms were washed off from the plates using M9 buffer. Worms were anesthetized using 100 mM sodium azide, mounted onto slides, and were observed using rhodamine filter. The fluorescence intensity was calculated semi-quantitatively using ImageJ (Ashrafi et al. 2003).

Lipofuscin assay

Lipofuscin comprises highly oxidized and cross-linked proteins, which are considered as prominent "biomarkers of aging". The intestinal accumulation of auto-fluorescent lipofuscin was quantified in treated/control worms. For lipofuscin assay, treated and control day 3/6/12 worms ($n = 30$) were washed off and monitored on DAPI filter using a fluorescent microscope as previously described (Berdichevsky et al. 2010; Lee et al. 2015a). Briefly, age-synchronized L1 worms were transferred to treatment plates and incubated at 20 °C. After 72 h, worms were transferred to three different treatment plates according to the day of observation under microscope. At the day of imaging (day 3/6/12), the worms were washed off from the plates using M9 buffer. The worms were anesthetized using 100 mM sodium azide, mounted onto slides, and were observed under the fluorescent microscope (excitation/emission 358 nm/461 nm). The fluorescence intensity was calculated semi-quantitatively using ImageJ and represented in terms of normalized values of corrected total cell fluorescence (CTCF)(CTCF = Integrated Density − (Area of selected cell × Mean fluorescence of background readings)).

Stress resistance assay through juglone exposure in *C. elegans*

The accumulation of oxidized toxic products and free radicals may cause oxidative stress which leads to impaired mitochondrial function (López-Otín et al. 2013). Thus, sensitivity to oxidative stress can be measured by quantifying the survival of worms following exposure to free radical generating compounds like juglone and paraquat. To analyze the oxidative stress tolerance, control and 50 μM 5-HPF-treated worms were subjected to 250 μM concentration of juglone (5-Hydroxy-1, 4-naphthoquinone). The survivals of the worms were scored after 6 h (Cong et al. 2015).

Measurement of intracellular ROS in *C. elegans*

Intracellular ROS levels were quantified using the H_2DCF-DA method (Labuschagne and Brenkman 2013). Adult worms were washed thrice using M9 buffer and finally collected in 300 μl of 0.1% PBST buffer and transferred to a 96well black plate (Thermo Scientific). Fifty micromolar working concentration of H2DCF-DA was added to each well. Fluorescence was measured using a microplate reader (BMG polarstar Omega) at 485 nm excitation and 520 nm emission. Observations were recorded for 120 min with intervals of 20 min each at 37 °C.

GFP reporter assay

Protein reporter assay was performed using transgenic strains expressing LGG-1 and PHA-4 tagged with GFP. Age-synchronized L1 worms were transferred to NGM plates treated with or without 5-HPF and incubated for 3 days at 20 °C allowed to egg lay and hatch. Second-generation worms were washed off at the L3 stage from the plates using M9 buffer. Worms were anesthetized using 100 mM sodium azide, mounted onto slides, and were observed using GFP filter. GFP-autophagy puncta and localization of PHA-4 were analyzed using × 20 and × 100 magnification by confocal laser scanning microscopy (CLSM) using a Zeiss Confocal LSM700 microscope equipped with Plan-Apochromat × 20/0.8 M27 and Plan-Apochromat × 100/1.40 Oil DIC M27 objectives. Acquisitions were realized in a plane scan mode, with excitation at 488 nm and emission at 530 nm. Images were processed with the Zeiss ZEN 2 software, in which background was reduced using brightness and contrast adjustments applied to the full set of images and finally exported as TIFF files.

RNA isolation, cDNA synthesis, and quantitative qPCR

Total RNA was extracted from adult worms using RNAzol reagent (Molecular Research Centre Inc., Cat. No. RN190) according to the manufacturer's protocol. cDNA synthesis was done from 1 μg of total *C. elegans* RNA in a 96-well thermal cycler using a cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's protocol. qRT-PCR studies were done using the QuantStudio 3 and 5 Real-Time PCR Systems (Applied biosystem, USA). Differential expression was calculated by the 2-ΔΔCT method. *gpd-1* was used as housekeeping control. Primers were procured from Eurofins.

Statistical analysis

Statistical analysis and Graphical representation of data were done using GraphPad Prism version 5. Analysis of variance and independent *t* test was used to calculate statistical significance where ever applicable. Significant differences between the lifespan of treated and control worms were determined using the Kaplan–Meier survival assay. All the experiments were performed thrice independently, and the results were plotted as mean ± SEM (standard error of the mean).

Results

5-HPF enhances longevity and reduces the aging biomarkers

We evaluated the lifespan extension potential of 5-HPF in wild-type N2. The population of worms was treated with different concentrations (6.25, 12.5, 25, 50, and 100) μM of 5-HPF and control (0.05% DMSO) at the early embryonic stage on NGM plates. Subsequently, we observed a significant increase in mean lifespan of 5-HPF-treated worms at 6.25 μM (18.05 ± 0.42, $p \leq 0.01$), 12.5 μM (18.50 ± 0.28, $p \leq 0.001$), 25 μM (18.98 ± 0.38, $p \leq 0.001$), 50 μM (20.76 ± 0.49, $p \leq 0.001$), and 100 μM (17.67 ± 0.41, $p \leq 0.05$), as compared with control (16.18 ± 0.34) (Fig. 1 a–c, Table S1). The maximum longevity was observed at 50 μM 5-HPF treatment (28.30%). We also evaluated the effect of control and treated 50 μM 5-HPF treatment starting from adult day 1 stage; 5-HPF significantly increased the mean lifespan by 31% as compared to control (Fig. S3 b). The result suggested that the effect is fully penetrant whether the treatment is initiated at any stage of life. Next, we examined aging biomarker, lipofuscin (an intestinal auto florescent age pigment), and found that 5-HPF treatment delays the accumulation of lipofuscin at 50 μM as compared with control (Fig. 1d, e and Fig. S5 a-b,). Fifty micromolars 5-HPF treatment also alleviated the total neutral lipid levels in Fig. 1f, g (Table S3) and specifically triglycerides (Fig.S5 c-e,) in comparison to their respective controls.

5-HPF enhances oxidative stress tolerance and reduces intracellular ROS levels in wild-type worms

Oxidative stress in terms of reactive oxygen species (ROS) is a by-product of normal metabolism and is known to cause deleterious cellular damage leading to instability of lipids and proteins, resulting in the normal aging phenomenon. To ascertain the antioxidant potential of 5-HPF, we performed oxidative stress tolerance assay. 5-HPF treatment was found to enhance the percentage survival of worms under juglone-induced oxidative stress (Fig. 2a). The outcome suggested increased percentage survival in 5-HPF-treated worms at 50 μM dose of 5-HPF (88.33 ± 3.19, $p \leq 0.001$) as compared to control (51.66 ± 1.67).

We also studied the effect of 5-HPF on intracellular ROS levels using a cell-permeant dye, H_2DCFDA. 5-

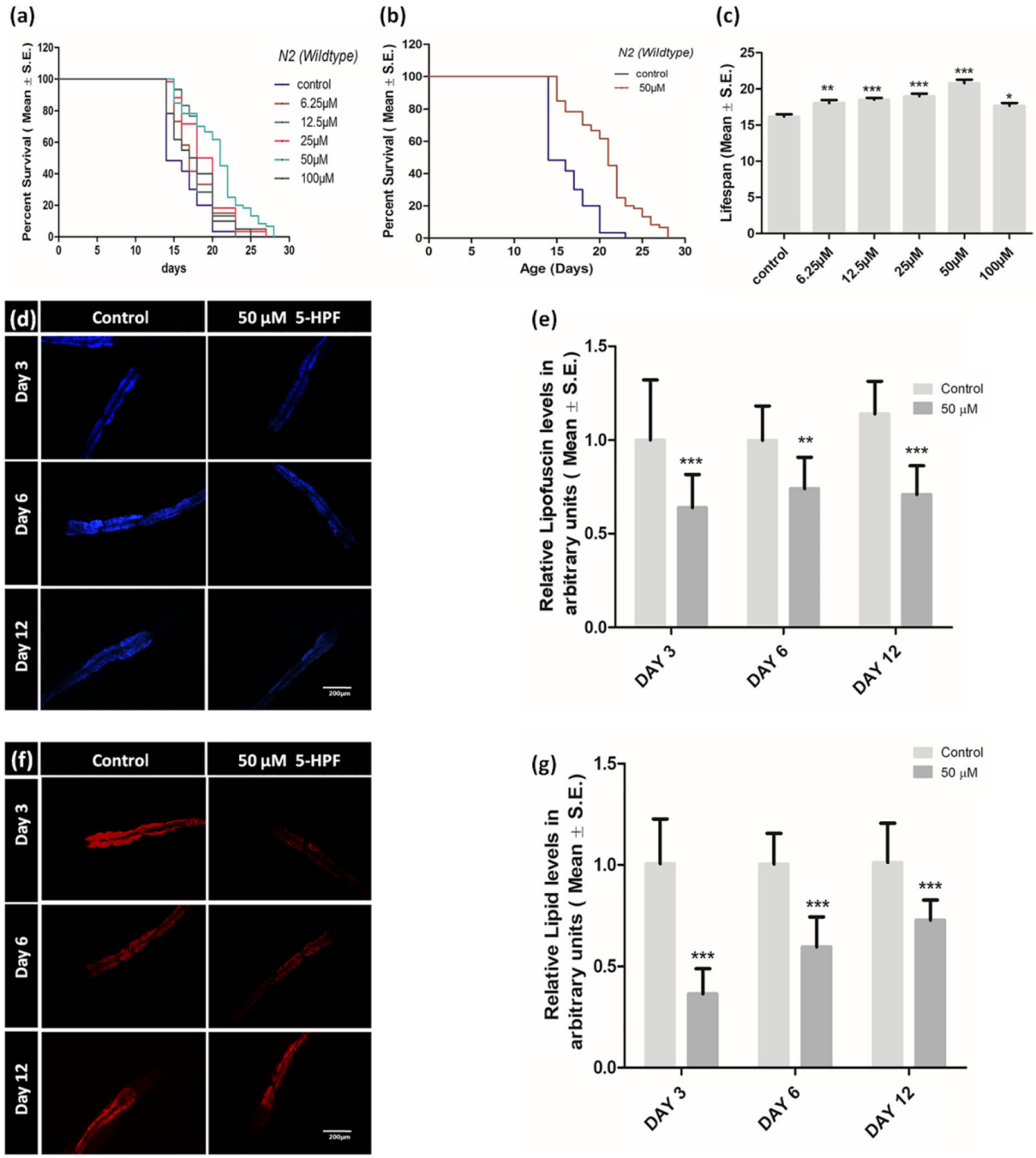

Fig. 1 5-HPF extends the mean lifespan and modulates aging parameters in *C. elegans*. **a** Survival curves of animal control and treated with different doses (5-HPF treated worms at 6.25 μM, 12.5 μM, 25 μM, 50 μM, 100 μM) of 5-HPF. 5-HPF significantly increased the mean lifespan of treated worms. **b** 50 μM 5-HPF significantly increased the mean lifespan by 28% as compared to control. **c** Bar graph representing mean lifespan treated with different concentration of 5-HPF. **d** Representative images of lipofuscin levels in control and treatment group, respectively. **e** 50 μM 5-HPF treatment alleviates relative lipofuscin levels. **f** Representative images of lipid levels in control and treatment group, respectively. **g** 5-HPF significantly reduces lipid levels in 50 μM 5-HPF-treated worms. The images were quantified by determining the average pixel intensity in each worm using ImageJ software (NIH). The data were analyzed using the Kaplan–Meier survival analysis and independent *t* test wherever applicable in GraphPad Prism version 5. Differences between the data were considered significant at $p \leq .05$ (*p value < 0.05 and *** p value < 0.001, Scale bar = 200 μm, $N = 30$/group)

HPF alleviates the ROS levels at 50 μM by 59.20% as compared to control (Fig. 2b). Moreover, to further validate the result, we have determined the ROS level under juglone exposure. Interestingly, 5-HPF curtailed the ROS levels against juglone-exposed condition significantly (Fig. 2c). The augmentation of oxidative stress tolerance and decline in ROS levels in 5-HPF-treated worms implies considerable ROS scavenging properties of 5-HPF.

5-HPF works independently of IS/ILS signaling

IS/ILS signaling is an established mechanism of lifespan extension in *C. elegans* (Altintas et al. 2016). To elucidate the molecular mechanism underlying the 5-HPF-mediated lifespan extension, we started screening for IS/ILS signaling pathway. DAF-16/FOXO is the major transcription factor that regulates lifespan extension by upregulating the genes responsible for cell survival and maintenance (Sun et al. 2017). 5-HPF extended the mean lifespan of DAF-2/IGF1R mutant significantly by 26.80% (31.50 ± 0.72, $p \leq 0.001$) at 50 μM concentration as compared with control (24.83 ± 0.68) (Fig. 3a). In addition to *daf-2* mutant, 50 μM 5-HPF treatment also enhanced the mean lifespan of *daf-16* mutant by 26.72% (18.19 ± 0.46, $p \leq 0.001$) compared to control (14.36 ± 0.41) (Fig. 3c). These data show that 5-HPF asserts longevity independent of the IS/ILS pathway.

5-HPF mimics DR and regulates autophagy

5-HPF was found to act independently of the IS/ILS pathway, so we next examined other longevity pathways. To examine the connection of the DR pathway, we studied the effect of 5-HPF in *eat-2* mutants (Fig. 4b) and found that 50 μM 5-HPF failed to enhance the mean lifespan of *eat-2* (23.63 ± 0.46, $p = 0.660$) compared to the control (23.66 ± 0.67). We also observed that 50 μM 5-HP- treated wild-type worms possess reduced brood size (270.8 ± 3.6) in comparison to control (289.8 ± 7.3) (Fig. 4 c and d).

PHA-4/FOXA transcription factor is vital for DR-mediated lifespan extension, and its activity is independent of the IS/ILS pathway. So next, we evaluated the effect of 5-HPF treatment on *pha-4* mutant worms. Fifty micromolars 5-HPF was unable to enhance the mean lifespan of *pha-4* (13.88 ± 0.326, $p = 0.761$) compared to the control (13.98 ± 0.3) (Fig. 5a). Therefore, like *eat-2* mutant, 5-HPF also requires PHA-4 for longevity. Besides, we also found increased nuclear localization

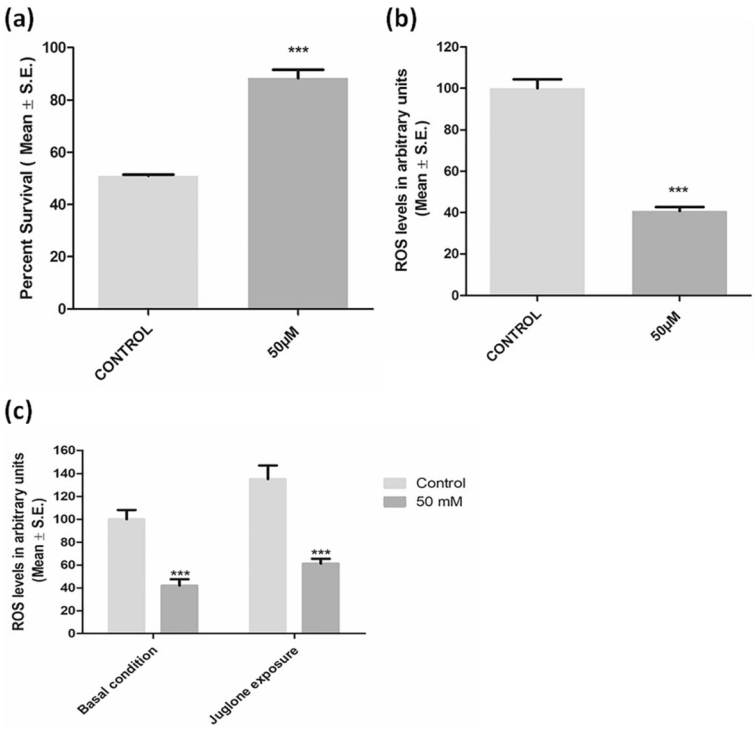

Fig. 2 5-HPF modulates oxidative stress resistance and reduces ROS levels in *C. elegans*. **a** Pre-treatment of 50 μM 5-HPF significantly increases resistance against juglone-induced oxidative stress. **b** 50 μM 5-HPF-treated worms show a significant reduction in the intracellular ROS level. **c** Pre-treatment of 50 μM 5-HPF significantly reduces intracellular ROS level against juglone-induced oxidative stress. The data were analyzed using the ANOVA and independent *t* test wherever applicable in GraphPad Prism version 5. Differences between the data were considered significant at $p \leq .05$(***p value < 0.001)

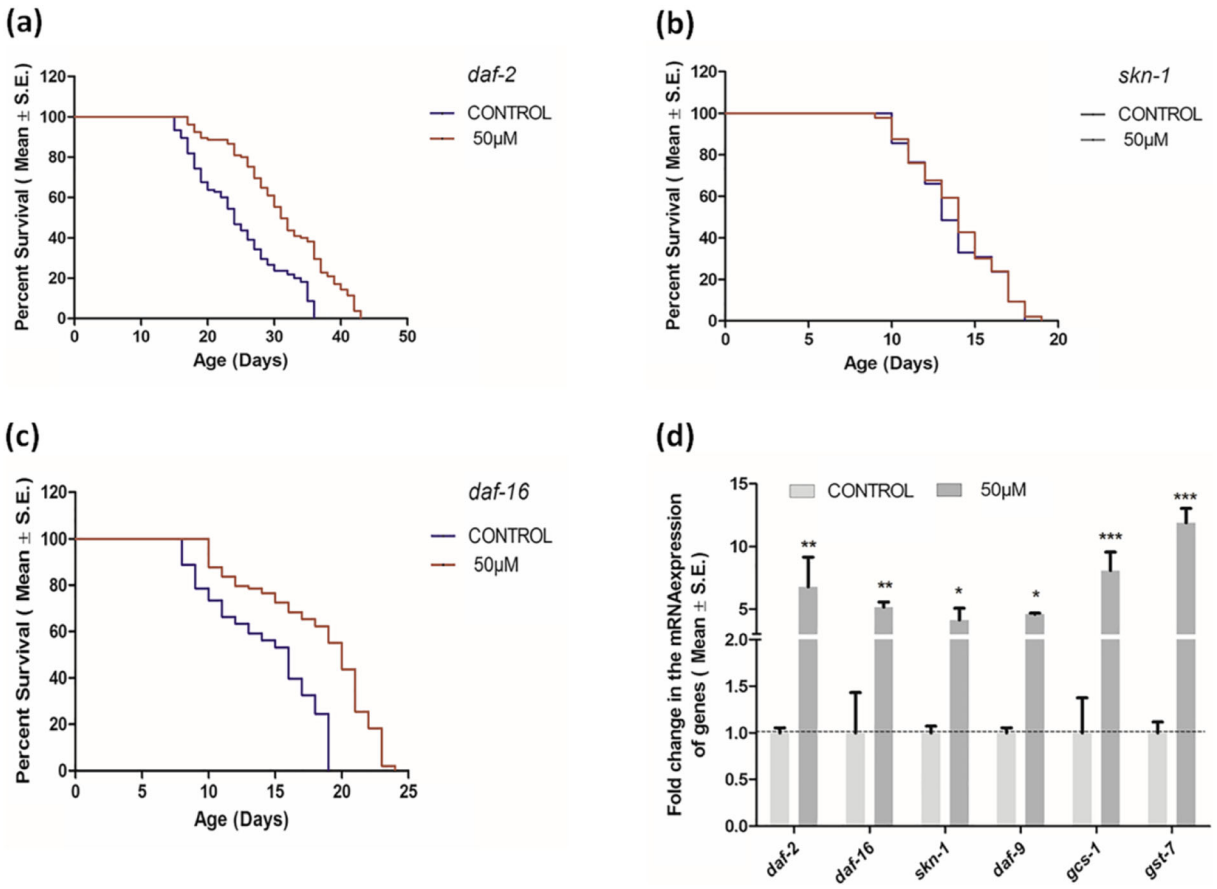

Fig. 3 5-HPF does not modulate IS/ILS pathway. **a** Survival curves of *the daf-2* mutant in the presence and absence of 50 μM 5-HPF. **b** Survival curves of the *skn-1* mutant in the presence and absence of 50 μM 5-HPF. No significant augmentation in the mean lifespan of the mutant was observed in *skn-1* (EU-31) mutant (1.74%, $p = 0.528$). **c** Survival curves of the *daf-16* mutant in the presence and absence of 50 μM 5-HPF. **d** 5-HPF treatment significantly upregulated the fold change in the mRNA expression level of *daf-2*, *daf-16*, and *skn-1* and its downstream target genes *daf-9*, *gcs-1*, and *gst-7*. The data were analyzed using the Kaplan–Meier survival analysis and two-way ANOVA wherever applicable in GraphPad Prism version 5. Differences between the data were considered significant at $p \leq .05$(*p value < 0.05, **p value < 0.01, and ***p value < 0.001)

of *pha-4* in intestinal cells of *pha-4::gfp*-tagged worms upon treatment with 50 μM 5-HPF (2.17 ± 0.10, $p \leq 0.001$) in comparison to the normalized value of control (0.94 ± 0.068) (Fig. 5 b and c). Moreover, we have also evaluated the 5-HPF treatment on *let-363* (ortholog of human MTOR (mechanistic target of rapamycin kinase)) mutant worms. Fifty micromolar 5-HPF failed to extend the mean lifespan of *let-363* (16.07 ± 0.25, $p = 0.89$) compared to the control (16.45 ± 0.46). Besides *pha-4*, SKN-1/NRF2 transcription factor also regulates DR-induced longevity in *C. elegans*. So next, we studied the role of *skn-1* in 5-HPF-mediated longevity. It was found that 50 μM 5-HPF treatment failed to enhance the lifespan of the *skn-1* mutant (13.97 ± 0.27) compared to control (13.73 ± 0.26) (Fig. 3b). In order to further validate our results, we performed drug uptake studies in various genetic backgrounds (N2, *eat-2*, *skn-1*, *pha-4*) by using the absorption spectrometry. Insignificant alterations were observed in the drug uptake studies in mutants (*eat-2*, *skn-1*, *pha-4*) as compared to wild type (Fig. S6a-d; Table S4). We further cross-checked our results by calculating bioconcentration factor and insignificant changes were observed in different genetic backgrounds (Fig. S6e; Table S4).

These evidences suggest that 5-HPF treatment exhibits longevity effects similar to DR. We also examined *sir-2.1* for its role in 5-HPF-mediated longevity and observed that 50 μM 5-HPF significantly enhances the mean lifespan by 18.61% (20.01 ± 0.501, $p \leq 0.001$) (Fig. S1b-c) as compared with control (16.87 ± 0.47).

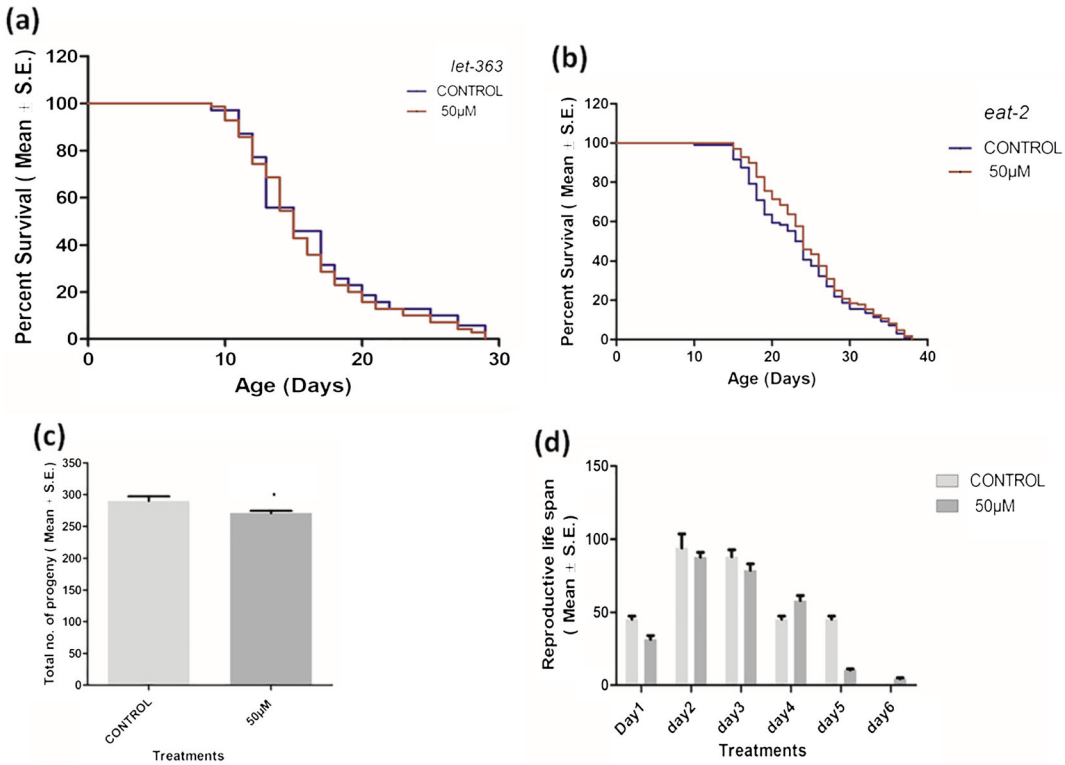

Fig. 4 Effect of 5-HPF on dietary restriction. **a** Survival curves of the *let-363* mutant in the presence and absence of 50 μM 5-HPF; 5-HPF significantly failed to extend the mean lifespan. **b** 50 μM 5-HPF-treated *eat-2* (DA1116) mutant (− 0.12%, $p = 0.660$) failed to extend the mean lifespan of worm in comparison with the control group. **c** 50 μM 5-HPF treatment exhibits a substantial reduction in brood size and also **d** prolongs reproductive span. The data were analyzed using the Kaplan–Meier survival analysis and independent *t* test wherever applicable in GraphPad Prism version 5. Differences between the data were considered significant at $p \leq .05$(*p value < 0.05)

SIR-2.1 stimulates activation of AMPK pathway member AAK-2 that contributes to longevity (Mansfeld 2015). So next, we evaluated the effect of 5-HPF treatment on *aak-2* (an ortholog of human PRKAA1 (protein kinase AMP-activated catalytic subunit alpha 1) and PRKAA2 (protein kinase AMP-activated catalytic subunit alpha 2) mutant worms. Fifty micromolars 5-HPF significantly enhanced the mean lifespan of *aak-2* by 22% (18.32 ± 0.68, $p \leq 0.001$) compared to the control (14.96 ± 0.39) (Fig. S1a-c). Although 5-HPF significantly increases the lifespan in both *sir-2.1* and *aak-2* mutants (Fig. S1a-c), it is not to the same level as wild type, i.e., the increased lifespan of *sir-2.1* and *aak-2* mutants treated with 5-HPF was found significantly less than that of wild-type worms (Fig. S1d) Therefore, this result indicates that both *sir-2.1* and *aak-2* are partially involved in 5-HPF-mediated longevity.

Further, the role of autophagy genes *bec-1* and *unc-51* was also studied. We observed that 5-HPF treatment (50 μM) failed to extend the mean lifespan of *bec-1* mutant (18.88 ± 0.25, $p = 0.864$) as well as *unc-51* mutant (10.79 ± 0.37, $p = 0.446$) as compared to respective control (18.95 ± 0.23; 10.56 ± 0.34) (Fig. 6 a and b). Besides, we also found increased levels of autophagy puncta in seam cells of *lgg-1::gfp* in 50 μM 5-HPF-treated worms (1.94 ± 0.13, $p \leq 0.001$) over the normalized value of control (1.00 ± 0.18) (Fig. 6 c and d).

5-HPF regulates the expression of *skn-1* and *pha-4* target genes

5-HPF treatment significantly upregulated the fold change in the mRNA expression level of insulin signaling pathway genes *daf-2* (6.77-fold) and *daf-16* (5.15-fold) (Fig. 3d). Downstream targets of SKN-1 coordinately affect the phenomena of longevity in the entire cellular system. We observed a

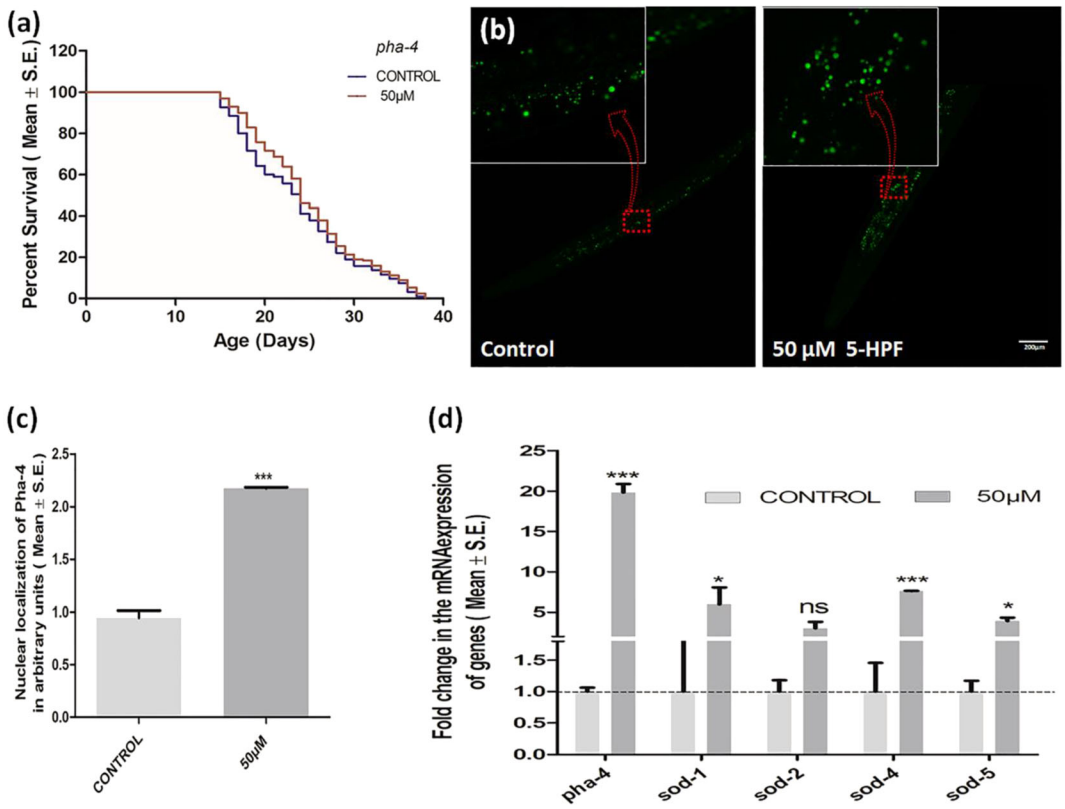

Fig. 5 5-HPF mimics dietary restriction in the regulation of *pha-4*. **a** Survival curves of the *pha-4* mutant in the presence and absence of 50 μM 5-HPF. No significant augmentation in the mean lifespan of the mutant was observed in *pha-4* (SM190) mutant (0.71%, $p = 0.761$). **b** Representative image of nuclear localization of *pha-4* using PHA-4::GFP in intestinal cells treated with 50 μM 5-HPF and control. **c** Graphical representation of nuclear localization of *pha-4* using PHA-4::GFP in intestinal cells treated with 50 μM 5-HPFand control. **d** 5-HPF treatment significantly upregulated the fold change in the mRNA expression level of *pha-4* and its downstream target genes *sod-1*, *sod-4*, and *sod-5*. The images were quantified by determining the average pixel intensity in each worm using ImageJ software (NIH). The data were analyzed using the Kaplan–Meier survival analysis, two-way ANOVA, and independent *t* test wherever applicable in GraphPad Prism version 5. Differences between the data were considered significant at $p \leq .05$(*p value < 0.05 and ***p value < 0.001; scale bar = 200 μm, $N = 30$/group)

significant upregulation of *skn-1* (4.12-fold), *gst-7* (11.91-fold), and *gcs-1* (8.08-fold). This justifies the observed oxidative stress resistance along with the upregulation of *daf-9* (4.59-fold) which is crucial for xenobiotic detoxification (Lindblom and Dodd 2006) (Fig. 3d). In addition to this, we also observed an increase in mRNA transcript levels of *pha-4* (1.34-fold), and its downstream target genes *sod-1* (5.95-fold), *sod-4* (7.59-fold), and *sod-5* (3.94-fold) (Fig. 5d). Moreover, 5-HPF treatment significantly upregulated the fold change in the mRNA expression level of genes *bec-1* (2.34-fold) and *lgg-1* (2.73-fold) confirming the role of 5-HPF in autophagy mediated by DR (Fig. 6e).

Discussion

5-HPF is a polymethoxyflavone, found in *Gardenia lucida*, *Citrus* sp., *Mentha piperita*, and *Thymus* sp. (Akao et al. 2008; Georgiou et al. 2015; Mimica-Dukic and Bozin 2008; Trivedi et al. 2017). 5-HPF exhibits several bioactivities, such as antibacterial, antioxidant, anti-inflammatory, antihypertension, antiobesity, and neuroprotection activities (Maurya et al. 2017; Trivedi et al. 2017). We have previously reported that 5-HPF improves cholinergic function and oxidative stress tolerance in *C. elegans* (Trivedi et al. 2017). In the present study, we showed that 5-HPF enhances the lifespan of nematode *C. elegans* in a dose-dependent

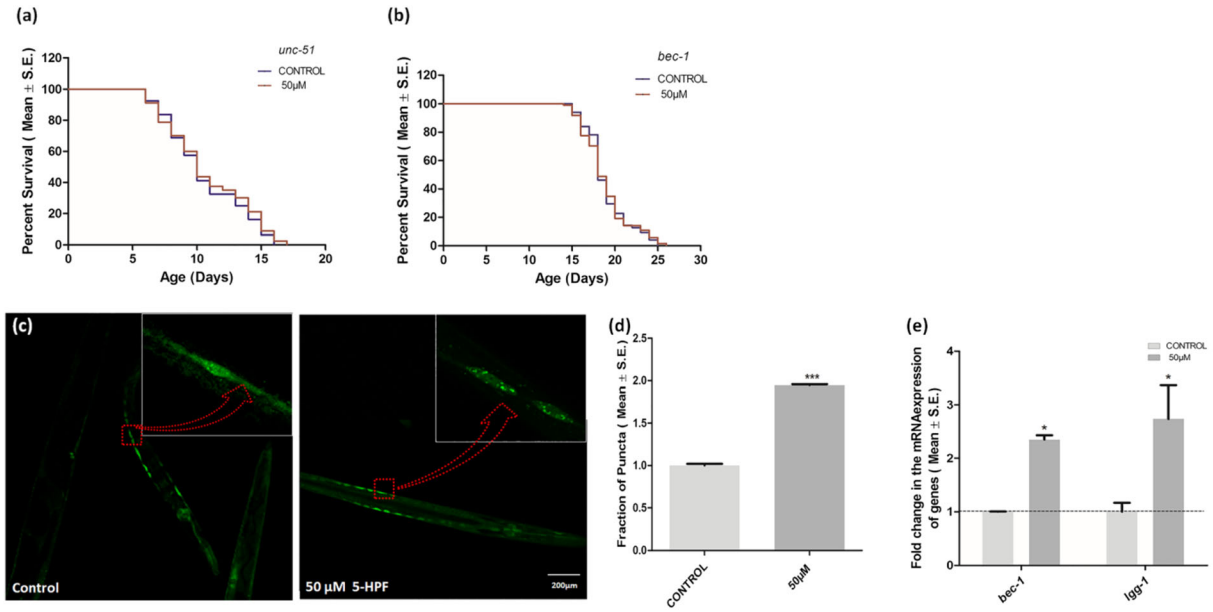

Fig. 6 5-HPF extends the mean lifespan of *C. elegans* by altering the autophagy pathway. **a** No significant augmentation in the mean lifespan of the mutant was observed in *unc-51* (BE150) mutant (2.17%, $p = 0.446$). **b** 50 μM 5-HPF-treated *bec-1* (VC424) mutant (− 0.36%, $p = 0.864$) failed to extend the mean lifespan of worm in comparison with the control group. **c** Representative image of localization of *lgg-1::gfp* puncta in autophagic membranes treated with 50 μM 5-HPF and control. **d** Graphical representation of localization of *lgg-1::gfp* puncta in autophagic membranes treated with 50 μM 5-HPF and control. **e** 5-HPF treatment significantly upregulated the fold change in the mRNA expression level of genes *bec-1* and *lgg-1*. The images were quantified by determining the average pixel intensity in each worm using ImageJ software (NIH). The data were analyzed using the Kaplan–Meier survival analysis, two-way ANOVA, and independent *t* test wherever applicable in GraphPad Prism version 5. Differences between the data were considered significant at $p \leq .05$ (*p value < 0.05 and ***p value < 0.001; scale bar = 200 μm, $N = 30$/group)

manner (Fig. 1 a–c, Table S1). Notably, 50 μM 5-HPF treatment enhanced the mean lifespan by 28% to an extent higher than several phytomolecules within the same chemical group (Kampkötter et al. 2008; Yao et al. 2012; Yang et al. 2020). Lifespan extension by 5-HPF is likely to involve DR-related nutrient-sensing pathways. Our data show that 5-HPF extended the lifespan of *C. elegans*, curtailed the age-related biomarkers, and improved the resistance to oxidative stress. The free radical theory of aging is a widely accepted hypothesis that describes reactive oxygen species (ROS) as tremendously reactive molecules that contribute to aging and age-related manifestations (Gladyshev 2014; Harman 1992). Excess ROS generated during cellular metabolism and damage to DNA, proteins, and lipids contribute to aging (Goh et al. 2019). Polymethoxyflavones exert indirect antioxidant potential by the modulation of antioxidant defense systems (Büchter et al. 2013; Yao et al. 2012). The ROS scavenging ability of polymethoxyflavones has previously been reported (Finkel and Holbrook 2000). The results showed a decrease in total ROS, supporting our hypothesis about the mitigated ROS-mediated lifespan extension (Fig. 2b). Previous reports suggest the role of oxidative stress resistance in the lifespan extension (Grünz et al. 2012; Nohara et al. 2019). Consequently, in the present study, 5-HPF exhibited enhanced oxidative stress resistance against exposure to a ROS inducer, 5-hydroxy-1, 4-naphthalenedione (Fig. 2 a and c).

There is an inverse correlation between longevity and fecundity or brood size in *C. elegans* (Berman and Kenyon 2006). Kirkwood has reported that lifespan extension is accompanied by a reduced reproduction rate (Liu et al. 2013). Notably, germline ablation has been reported to increase lifespan in *C. elegans* (Berman and Kenyon 2006). As expected, we observed a decrease in brood size, along with an increase in the reproductive span in treated worms. These results imply that 5-HPF contribute to lifespan extension in a manner similar to DR. Notably, curtailed brood size is a characteristic feature of DR-mediated lifespan extension. Increased reproductive span correlates with the extended

lifespan in treated worms (Fig. 4 c and d). Accumulation of age pigment, lipofuscin, with the advancement in age is pathologically connected with various age-associated disorders (Skoczyńska et al. 2017). It has been reported that an increased level of intracellular ROS contributes to the aggregation of lipofuscin (König et al. 2017). The reduced levels of lipofuscin in 5-HPF treated worms (Fig. 1 d and e) correlate with mitigated levels of free radicals. Previous studies have linked altered lipid metabolism with aging (Dexter et al. 1994; (Ackerman and Gems 2012). Due to their methylation potential, polymethoxyflavones possess a greater degree of lipid-solubilizing activity (Yang et al. 2020). We observed a significant reduction in triglyceride levels upon treatment with 5-HPF (Fig. S5c-e). Besides, a significant decrease in lipid levels (Fig. 1 f and g) suggested the fat-solubilizing potential of 5-HPF.

After the efficacy of 5-HPF against aging endpoints was established, the study was furthered to identify underlying longevity pathways using mutants and transcriptional profiling. The insulin/IGF-1 signaling pathway is highly conserved among species, and its major transcription factor DAF-16 plays a significant role in growth, lifespan, and detoxification mechanism (Sun et al. 2017). Moreover, it regulates aging, stress resistance, and immunity in *C. elegans*. It has been reported that DAF-16 is responsible for the activation of major antioxidant machinery (Klotz et al. 2015). Also, *daf-16* mutants lack resistance to oxidative stress suggesting the vital role of transcriptional targets of DAF-16 in conferring stress resistance (Honda and Honda 1999). In consensus to this, the lifespan-extending property of 5-HPF is independent of the IIS pathway-related genes *daf-16* and *daf-2*. Lifespan extension as a result of 5-HPF treatment remained unaltered in *daf-16* and *daf-2* mutant worms (Fig. 3 a and c). The NAD^+/Sirtuin signaling pathway interacts with the DAF-16 transcription factor to contain oxidative stress. SIR-2.1 and AMPK both are evolutionarily conserved energy sensors that respond against augmented levels of cellular AMP and NAD^+ concentrations. Moreover, SIR-2.1 stimulates the activation of AMPK that has been reported to reduce intracellular ROS level against any ROS generator. The results from the mutant study indicated that 5-HPF significantly increases the lifespan in both *sir-2.1* and *aak-2* mutants (Fig. S1a-c). However, the increased lifespan was significantly lesser in comparison to wild-type worms (Fig. S1d). Thus, the results reveal the partial involvement of *sir-2.1* and *aak-2* in 5-HPF-mediated longevity.

SKN-1 plays a diverse role in the enhancement of life expectancy during oxidative and ER stress, dietary restriction, proteasome action, and lowered translation (Ogawa et al. 2016). In the present study, 5-HPF failed to augment the lifespan of *skn-1* mutants (Fig. 3b). 5-HPF was found to upregulate the mRNA expression of *skn-1* and its downstream target *gcs-1*, *gst-7*, and *daf-9* in wild-type worms (Fig. 3d). Mutant and qPCR studies identified that *skn-1* was critical to 5-HPF-mediated lifespan extension. In *C. elegans*, *daf-9* is equivalent to cytochrome P450 that regulates phase-I–induced longevity that might depend on the dietary restriction (DR) pathway (Baumeister et al. 2006). Various plant-derived molecules have been reported to show antimicrobial activity. Considering the relation between antimicrobial activity, pharyngeal pumping, and DR activity, worms were devoid of any alteration in the pharyngeal pumping of wild-type N2 worm (Fig. S4) and no bacterial zone of inhibition was observed. Hence, we concluded that DR did not result due to any bactericidal activity (Fig. S3 a and b). Previous studies reported that besides SKN-1/NRF2, PHA-4/FOXA is also necessary for DR-induced lifespan extension in *C. elegans* (Smith-Vikos et al. 2014). Reduced TOR signaling triggers autophagy by activating PHA-4/FOXA and reduces translation by downregulating ribosomal S6 kinase 1 (RSKS-1) (Lee et al. 2015b). DR induced by TOR signaling has also been influenced by *eat-2*, under the regulation of PHA-4/FOXA (Hansen et al. 2007; Meissner et al. 2004).The *eat-2* mutant is feeding defective and exhibits DR independent of DAF-16. Surprisingly, we observed that 5-HPF failed to augment the lifespan in *let-363* and *eat-2* mutants (Fig. 4a, b). It also affects the PHA-4 expression (Fig. 5 b and c) in pharyngeal and intestinal cells in PHA-4::GFP reporter strain and failed to enhance the mean lifespan of *pha-4* mutant worms (Fig. 5a). This was further validated by qPCR studies, which exhibited upregulated expression of *pha-4* and its target genes *sod-1*, *sod-4*, and *sod-5*. The *C. elegans* genome has five SOD genes including *sod-1*, *sod-4*, and *sod-5* (Lee et al. 2003). The *sod-1*, *sod-4*, and *sod-5* are cytoplasmic Cu/Zn superoxide dismutases that have consensus PHA-4-binding sites. These genes are known to protect against oxidative damage and are also required for redox maintenance (Fukai and Ushio-Fukai 2011). The response to dietary

restriction involves the PHA-4-dependent expression of *sod-1*, *sod-2*, *sod-4*, and *sod-5* (Panowski et al. 2007). Hence, the 5-HPF-related longevity is majorly dependent on dietary restriction pathway. Moreover, the vital role of autophagy in DR-induced longevity in *C. elegans* has also been reported. In *C. elegans*, *unc-51* is involved in autophagy. ULK-1 (*unc-51* like autophagy activating kinase 1) and ULK-2 (*unc-51* like autophagy activating kinase 2) are human orthologs of *unc-51*(Ogura et al. 1994). Similarly, *bec-1* is the ortholog of the human tumor suppressor gene Beclin1 (Ruck et al. 2011). This gene collaborates with class III PI3 kinase VPS-34 and is critical for autophagy, endocytosis, and membrane trafficking (Gelino et al. 2016; Melendez and Levine 2009). Recent findings suggest that *bec-1* and *daf-16* act in parallel pathways, augmenting lifespan in *daf-2* mutants (Lionaki et al. 2013). Enhanced expression of *bec-1* triggers autophagy, decreases α-synuclein toxicity, and alleviates related neuritic alterations. Involvement of the autophagy was confirmed through studies on *bec-1* and *unc-51* mutants. 5-HPF failed to extend the mean lifespan of *bec-1* and *unc-51* mutants (Fig. 6 a and b), identifying the underlying role of *bec-1* and *unc-51* in 5-HPF-mediated lifespan extension.

Enhanced autophagy was further confirmed through transgenic strain DA2123, expressing *lgg-1::gfp*. *C. elegans*, gene *lgg-1*, is the homolog of yeast Atg8 and mammalian MAP-LC3, which is vital for the degradation of cellular components through autophagy. It has been reported that *lgg-1* enhances normal Dauer formation and lifespan (Alberti et al. 2010). On the other hand, in extended lifespan showing worms, LGG-1 localizes at hypodermal seam cells. It enhances the number of pre-autophagosomal and autophagosomal structures that are observed as punctate structures. The result showed a slight increase in punctate structures of LGG-1 representing augmented autophagy in 5-HPF-treated worm (Fig. 6 d and e). In the present study, 5-HPF significantly augments the mRNA expression of *lgg-1* and *bec-1* in wild-type worms (Fig. 6c) that supports the previous finding performed with DA2123 strain. Hence, this augmented mRNA expression level showed that 5-HPF leads to *lgg-1*-mediated autophagy in wild-type worms.

The above studies for the first time identified the stress-reducing and longevity-promoting potential of 5-HPF in the *C. elegans* model system. The study suggests that 5-HPF extends the lifespan by modulating the DR pathway, through activation of autophagy in *eat-2/let-363/pha-4*-dependent manner. Our study advocates the supplementation of 5-HPF in the development of useful therapeutic interventions that unravel a novel protective strategy for aging and age-linked diseases.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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