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Specioside ameliorates oxidative stress and promotes longevity in *Caenorhabditis elegans*

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ABSTRACT

Specioside (6-O-coumaroylcatalpol) is an iridoid glucoside which possesses multifunctional activities viz. analgesic, antidyspeptic, astringent, liver stimulating and wound healing properties. The present study for the first time delineates stress alleviating and lifespan prolonging action of specioside (SPC), isolated from *Stereospermum suaveolens* in the free living, multicellular nematode model *Caenorhabditis elegans*. A strong correlation between lifespan extension and stress modulation in adult worms was established in a dose dependent manner. The dietary intake of this phytomolecule elevated juglone induced oxidative and heat induced thermal stress tolerance in *C. elegans*. On evaluation, it was found that 25 μM dose of SPC significantly extended lifespan by 15.47% ($P \leq 0.0001$) with reduction in stress level. Furthermore, SPC enhanced mean survival in *mev-1* mutant suggesting its oxidative stress reducing potential. Furthermore, SPC augmented stress modulatory enzymes superoxide dismutase (SOD) and catalase (CAT) level in *C. elegans*. Altogether, these findings broaden current perspectives concerning stress alleviating potentials of SPC and have implications in development of therapeutics for curing age related disorders.

1. Introduction

Specioside (6-O-coumaroylcatalpol) is an iridoid glucoside isolated from the plant *Stereospermum suaveolens* (Bignoniaceae), commonly known as 'patala'. The plant contains apigenin, lapachol, dianatin-7-glucuroniside, dinatin, β-sitosterol, saponins, palmitic, stearic and oleic acids (Chattarjee and Chandra, 2000). In the previous studies this plant is also validated for hepatoprotective (Chandrashekhar et al., 2010), anti-hyperglycemic (Rao et al., 2010), anti-inflammatory, anticancer and antioxidant activities (Chandrashekhar et al., 2009). The iridoids are groups of natural molecules with a monoterpene cyclic ring, found in a wide variety of plants (Tietze, 1983). The previous phytochemical studies reported that iridoid glucoside possesses a number of activities such as antitumoral, hypotensive, sedative and hepatoprotective activities (Garg et al., 1994; Ghisalberti, 1998). The in vitro effects of SPC have been reported earlier while the limited in vivo effects have been studied. For addressing this aspect, we isolated and characterized SPC (6-O-coumaroylcatalpol) from *S. suaveolens* and evaluated different pharmacological doses of SPC (5, 25 and 50 μM) for lifespan prolongation and stress modulation in *Caenorhabditis elegans*.

Aging is a progressive deterioration of tissue integrity with age, which is universal to all living organisms characterized with decreased stress survival ability and increased functional impairment owing to developmental, genetic or environmental factors (Kenyon, 2005). Aging is one of the most crucial menace factors for society causing huge socioeconomic burden. Hence, discovery of novel molecules which modulate aging, could lead to a new strategy for working upon age related diseases like diabetes, cancer and neuro-degenerative disorders (Brown et al., 2006). Previous studies suggest that phytomolecules can effectively modulate stress and life prolonging mechanistic pathways, thereby extending lifespan significantly (Zhang et al., 2009). Multiple cellular signaling pathways promoting longevity are well established in *C. elegans*. The oxidative stress pathways are well known to alter lifespan in *C. elegans* (Guarente and Kenyon, 2000). *C. elegans* is an excellent model for pharmacological and toxicological studies due to its amenable features like small size, transparent body, short life cycle and well defined genetic factors influencing aging (Guarente and Kenyon, 2000) making it an informative and convenient model for aging and stress studies (Blagosklonny et al., 2009). Despite the various deciphered pharmacological roles of SPC, its antiaging potential is still undetermined. Therefore, the aim of the present study is to evaluate the role of this iridoid glucoside (SPC) in stress modulation and longevity promotion in *C. elegans*.

2. Material and methods

2.1. Plant material

The aerial part of *S. suaveolens* (2.7 kg) was collected in November 2011 from CSIR — Central Institute of Medicinal and Aromatic Plants,

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Lucknow. Authentication of the plant was established by the Taxonomy and Pharmacognosy Department of our institute. A specimen voucher (no. 13545) has been deposited to the herbarium of the institute. The shade dried plant material was stored at room temperature following good storage practices.

2.2. Instrumentation

Semi-preparative LC-8A (Shimadzu) using a reverse phase column (RP-18, Supelcosil, 250 mm × 21.2 mm, 12 μm) was used for the purification and isolation. ^{1}H & ^{13}C NMR spectra were recorded in deuterated methanol on a Bruker Avance 300 MHz spectrometer. ^{1}H–^{1}H correlation spectroscopy (COSY), ^{1}H–^{13}C heteronuclear single quantum coherence (HSQC), and ^{1}H–^{13}C heteronuclear multiple bond correlation (HMBC) experiments were recorded using the standard pulse sequences. The chemical shift referencing was carried out with respect to internal TMS at 0.00 ppm. The ESI-MS was obtained on a MS-2010EV (Shimadzu, Japan) at 70 eV by flow injection into the electrospray source. HPLC equipment (Shimadzu, Japan), consisting of analytical column (Waters Spherisorb ODS2, 250 mm × 4.6 mm, 10 μm), pumps (LC-10AT) and PDA (SPD-M10A) was used for HPLC fingerprint analysis. The column chromatography was performed on silica gel 60–120 mesh.

2.3. Extraction and isolation

The dried and powdered plant material (2.7 kg) was extracted with methanol (6 L × 3) by cold percolation at room temperature for 24 h. The methanol extract was concentrated under reduced pressure to obtain a crude methanolic extract (410 g). The crude methanolic extract was dissolved in water (1.5 L) and fractionated with hexane (66 g), ethyl acetate (90 g) and *n*-butanol (140 g). The ethyl acetate (30 g) extract was subjected to vacuum liquid chromatography, eluting it with varying compositions of chloroform–methanol. Various fractions (250 mL each), eluted with different compositions of chloroform–methanol mixture were monitored on TLC and pooled together on the basis of similar TLC profiles. The pooled fraction A1 (1 g, fraction no. 8–10, chloroform–methanol, 97:3, v/v) was further purified via semi-preparative HPLC [mobile solvent: ACN–H_2O (20:80, v/v), flow rate: 15 mL min^{-1}, detection: 330 nm] which resulted in the separation and purification of SPC (1) (25 mg) [Rt 22.63 min] (Kwak et al., 2009).

2.4. Identification

SPC (6-O-coumaroylcatalpol), brown amorphous powder (25 mg), ESIMS for $C_{24}H_{28}O_{12}$ *m/z*: 531 $[M + Na]^{+}$. ^{1}H NMR 300 MHz (MeOD): δ 7.72 (1H, d, J = 16.2 Hz, H-7″), 7.52 (2H, d, J = 8.4 Hz, H-2″, 6″), 6.86 (2H, d, J = 8.1, H-3″, 5″), 6.40 (1H, d, J = 16.2 Hz, H-8″), 6.38 (1H, dd, J = 6.0, 1.5 Hz, H-3), 5.19 (1H, d, J = 8.7 Hz, H-1), 5.04 (1H, dd, J = 7.5, 1.5 Hz, H-6), 5.01 (1H, dd, J = 6.0, 3.9 Hz, H-4), 4.85 (1H, d, J = 8.4 Hz, H-1′), 4.18 (1H, d, J = 12.9 Hz, H-10a), 3.92 (1H, dd, J = 12.0, 1.8 Hz, H-6′a), 3.84 (1H, d, J = 13.2 Hz, H-10b), 3.78 (1H, s, H-7), 3.68 (dd, J = 11.7, 6.3 Hz, H-6′b), 3.44 (1H, m, H-5′), 3.40 (1H, m, H-3′), 3.34 (2H, m, H-2′, 4′), and 2.65 (2H, m, H-5, 9); ^{13}C NMR (MeOD): δ 169.2 (C-9″), 162.9 (C-4″), 147.3 (C-8″), 142.3 (C-3), 131.3 (C-2″, 6″), 127.08 (C-1″), 116.9 (C-3″, 5″), 114.5 (C-7″), 102.9 (C-4), 99.7 (C-1″), 95.1 (C-1), 81.3 (C-6), 78.5 (C-3′), 77.6 (C-5′), 74.8 (C-2′), 71.7 (C-4′), 66.9 (C-8), 62.8 (C-6′), 61.2 (C-10), 60.3 (C-7), 43.1 (C-9), and 36.6 (C-5). ^{1}H and ^{13}C. The data was consistent with the previously published data (Su et al., 2005).

2.5. HPLC fingerprint

The bioactive marker SPC was separated and identified in the plant crude extract using HPLC-PDA. Well resolved separation of biomarker using binary mobile phase consisting of acetonitrile and water containing 0.1% TFA (20:80, v/v) on Waters Spherisorb ODS2 column (250 mm × 4.6 mm, 10 μm) was achieved (Fig. 1). The flow rate of 1.0 mL/min with column temperature 30 °C was used throughout the analysis. A photodiode array detector was set to measure spectra from 200 to 400 nm. The analysis was performed at the wavelength of 330 nm with a run time of 25 min (Tepe et al., 2007).

2.6. C. elegans strains, culture and maintenance

C. elegans strains viz. N2-Bristol (wild type); TK22, *mev-1(kn1)*; CF1553, *muIs84* [*pAD76*(*sod-3*::gfp)]; CL2166, *gst-4*::gfp (*dvls19*) and *Escherichia coli* OP-50 strain used in this study were obtained from the *Caenorhabditis* Genetics Center (CGC), University of Minnesota, MN, USA. The strains were grown and maintained under standard laboratory conditions at 20 °C on nematode growth medium (NGM) agar plates seeded with a lawn of *E. coli* OP50 (Brenner, 1974). The synchronized culture was obtained by sodium hypochlorite treatment of gravid hermaphrodites (Fabian and Johnson, 1994).

2.7. Test compound

SPC was dissolved in 10% dimethyl sulfoxide (DMSO) to prepare a 10 mM stock. Toxicity assay was performed with 5, 25, 50, 100, 250, 500 μM and 1 mM to assess toxic effect of SPC. The 5, 25 and 50 μM are found to be non-toxic among all of these concentrations (Fig. 2). Hence, 5, 25 and 50 μM were taken as test concentration. The day 2 adult worms were transferred to 24 well plate containing 300 μL total volume of water with indicated doses of SPC (5 μM–1 mM concentration) or 0.05% DMSO as control. The wild type N2 plates were freshly prepared 2 h before use and seeded with *E. coli* OP50 one day before use (Büchter et al., 2013). The survival of the worms was scored after every 1 h interval and data shown represents the survival of worms after 24 h.

2.8. Lifespan assay

The age synchronized culture was used for lifespan assay; worms were allowed to hatch on NGM plates in the presence or absence of various concentrations of SPC (5, 25 and 50 μM) till their adulthood. L4 molts were then transferred to fresh seeded NGM plates previously spotted with the corresponding test concentration and 50 μM, 5-fluoro-2′-deoxyuridine (FUdR; Sigma, St. Louis, MO, USA) was added to plate to prevent progeny development (Hosono, 1978; Büchter et al., 2013). The worms were scored daily by touch provoke method and transferred onto fresh plates. The experiment was terminated when all worms were scored as dead or censored. The experiment was repeated in three independent trials.

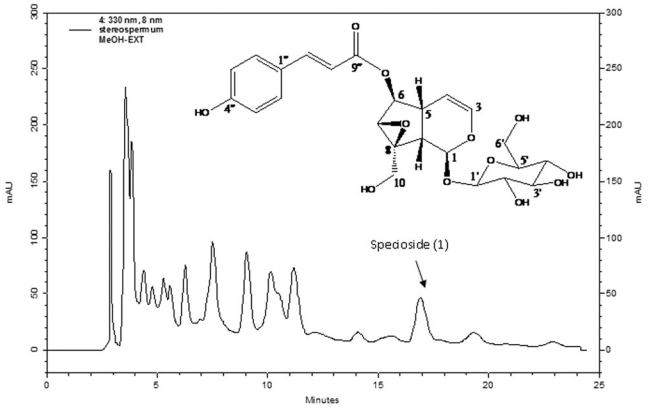

Fig. 1. Representative HPLC chromatogram of methanol extract of *S. suaveolens* and chemical structure of Specioside.

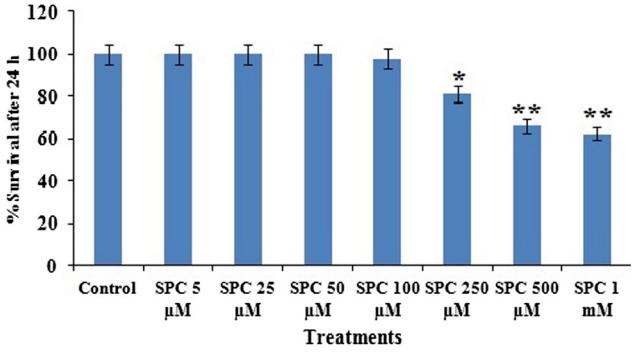

Fig. 2. Toxicity profile of different doses of SPC (5 μM, 25 μM, 50 μM, 100 μM, 250 μM, 500 μM and 1 mM). The N2 wild type worms were synchronized on NGM plates and on day 2 of adulthood the worms (n = 50) were transferred to 24 well plate containing respective doses of SPC (5 μM, 25 μM, 50 μM 100 μM, 250 μM, 500 μM and 1 mM) whereas 0.05% DMSO served as control. No obvious toxic effect of SPC was observed at 5 μM, 25 μM, and 50 μM but 100, 250, 500 μM and 1 mM were found to be toxic to the worms. Hence, 5 μM, 25 μM and 50 μM were taken as test concentrations. The statistical differences between the control and treated worms were determined with the aid of the parametric t-test. The data is statistically analyzed using ANOVA in ASSISTAT 7.7 beta statistical assistance software. Differences between the data were considered significant at $P \leq 0.05$. Error bars represent means ± SEM. $^{*}P \leq 0.05$, $^{**}P \leq 0.001$.

2.9. Pharyngeal pumping analysis

The pharyngeal pumping rate is defined as the number of contraction and relaxation of the pharynx over a one-minute interval. The L1 worms were treated with different concentrations of SPC (5, 25 and 50 μM) and the pharyngeal pumping rate of day 5 and day 10 adult worms was recorded for 20 s using a Leica S8AP0 stereoscopic microscope (Büchter et al., 2013). The experiment was performed in three independent trials.

2.10. Stress resistance assay

To assess the effect of SPC on oxidative stress resistance in worms, juglone (5-hydroxy-1,4-napthoquinone, Sigma-Aldrich), an intracellular ROS generator was used. The age synchronized worms were raised from L1 to adult in the presence/absence of SPC (5, 25 and 50 μM) as described in Lifespan assay. The treated or untreated day 2 adult worms were transferred to 24-well plate containing liquid NGM and oxidative stress was induced by an acute, lethal concentration of juglone (250 μM) in a total volume of 300 μL per well. The worms were incubated at 20 °C and scored for viability per hour of continuous exposure (Pant et al., 2014).

For thermotolerance assay, SPC (5, 25 and 50 μM) treated and control worms were exposed to continuous heat shock at 37 °C for 4 h onto NGM plates and then scored for viability. Worms were scored as dead when they failed to respond to repeated touch with a platinum loop (Lithgow et al., 1995). The experiments were performed in three independent trials.

2.11. Measurement of intracellular ROS in C. elegans

The age synchronized L1 larvae (as described above in Lifespan assay) were transferred to NGM plates seeded with *E. coli* lawns in the presence or absence of SPC (5, 25 & 50 μM) at 20 °C. The intracellular reactive oxygen species levels were measured in *C. elegans* using 2,7-dichlorofluorescein diacetate (H2DCF-DA: Sigma). H2DCF-DA is a non-fluorescent cell permeable dye which gets converted intracellularly into fluorescent 2,7-dichlorofluorescein (DCF) in the presence of ROS. The day 4 of adult worms were collected in 300 μL of PBS (phosphate buffer saline) with 0.1% Tween-20 and washed three times with PBST. The adult worms were transferred into 96 well plate in the presence of 50 μM H2DCF-DA in PBS. The fluorescence was recorded after every 20 min for 2 h 30 min at 37 °C using a fluorescence microplate reader (Spectramax M2: Molecular Devices) at excitation 485 nm and emission 530 nm (Büchter et al., 2013). The assay was performed in three independent trials.

2.12. Analysis of intracellular lipofuscin accumulation

To investigate the effect of SPC on the accumulation of intestinal lipofuscin level, worms were grown on NGM plates in the presence or absence of SPC (25 μM) from L1 stage. Lipofuscin is a biomarker of aging and exhibits auto-fluorescence (Herndon et al., 2002). On the 5th day of adulthood, randomly selected worms were mounted on slides coated with 3% agarose pad, anesthetized with 2% sodium azide. The auto-fluorescence of lipofuscin was visualized with a Leica fluorescence microscope DMI 3000 B (Leica, Wetzlar, Germany) using the DAPI filter set (with excitation at 340–380 nm and emission at 435–485 nm) at 20× objective. The lipofuscin level for each worm was quantified by determining the average pixel intensity in each worm's intestine. The fluorescence was quantified as the average pixel intensity by Image-J software (NIH). The experiment was repeated thrice.

2.13. Quantification of stress response gene expression

For visualization and quantification of *sod-3*::GFP and *gst-4*::GFP expressions, transgenic strains CF1553 and CL2166 tagged with GFP were grown on NGM plates spotted with *E. coli* OP-50 as a food source. Worms were treated with SPC (25 μM) and 0.05% DMSO served as control for 72 h at 20 °C. Photographs were captured directly on day 2 for CF1553 (*sod-3*::gfp) and CL2166 (*gst-4*::gfp) worms in comparison to control (Pant et al., 2014). For quantifying the GFP expression, worms were mounted on glass slides coated with 3% agarose pad, anesthetized with 2% sodium azide and visualized under a Leica fluorescence microscope DMI 3000 B (Leica, Wetzlar, Germany) using GFP filter set (with excitation at 365 nm and emission at 420 nm). GFP fluorescence in the worms was measured by digital imaging using 20× objective followed by analysis with Image-J software. The SOD-3 level for each worm was quantified by determining the average pixel intensity in each worm's terminal bulb and the anterior portion of the pharynx. Furthermore, the GST-4 level was quantified by calculating the average pixel intensity of the worm's body wall muscles. The experiment was repeated in three independent trials. The comparison of all images was carried out on the same day with the same microscope settings.

2.14. mRNA quantification of stress modulating gene

The wild type N2 worms were treated with DMSO (0.05%) or 25 μM SPC for 72 h and grown under standard laboratory condition at 20 °C as described in Lifespan assay (Pant et al., 2014). Total RNA was extracted from day 2 adult worms using a Trizol reagent (Invitrogen). The first strand cDNA was synthesized using the cDNA synthesis kit (Invitrogen) according to the manual's instruction. The expression level of genes *sod-1*, *sod-2*, *sod-3*, *gst-4*, *gst-7*, *hsp-16.2*, *hsp-70* and *ctl-1* with respect to housekeeping gene *β-actin* (*act-1*) used as an endogenous control was quantified by using the SYBR green detection method on an Applied Bio-systems 7900HT fast real time PCR system. Relative-fold changes of expressed gene were calculated using the comparative C_t ($\Delta\Delta C_t$) method. The experiment was repeated in three independent trials.

2.15. In vivo antioxidant assay

2.15.1. Measurement of antioxidant enzyme level

The antioxidant enzyme level was analyzed using SPC treated and untreated day 2 adult worms (as described in Lifespan assay above). The treated/untreated worms were washed three times with M9 buffer,

homogenized in the extraction buffer (pH 7.4), and centrifuged at 10,000 rpm for 15 min and the supernatant was used for bio-chemical analysis.

2.15.2. Determination of superoxide dismutase (SOD) level

SOD activity was measured by monitoring the decrease in absorbance at 406 nm to evaluate the auto-oxidation of riboflavin by the enzyme at pH 10 (Misra and Fridovich, 1972). The reaction mixture containing 100 mM phosphate buffer (pH 7.8), 2.25 mM NBT (nitroblue tetrazolium), 3 mM EDTA and 1.5 mM Na_2CO_3 in a total volume of 3.0 mL was used and the reaction was initiated by adding 0.4 mL of riboflavin solution (Leelaja and Rajini, 2012; Ahn et al., 2014). The suitable aliquot of the supernatant was added to the reaction mixture and the inhibition of riboflavin auto-oxidation was monitored. The results are expressed as % SOD activity w.r.t. control. The experiment was repeated in three independent trials.

2.15.3. Measurement of catalase (CAT) level

The supernatant obtained after homogenization of treated or untreated worms is equivalent to approximately 260 μg protein along with 25 μL of H_2O_2 (3%) and was added to 3.0 mL of phosphate buffer (100 mM, pH 7.8) and due to H_2O_2 degradation the decrease in absorbance was measured at 240 nm for 5 min (Claiborne, 1985; Leelaja and Rajini, 2012). The results are expressed as % CAT activity w.r.t. control. The experiment was repeated in three independent trials.

2.16. Data analysis

The statistical differences between the control and treated worms were determined with the aid of the parametric t-test. The significant difference between the lifespan of treated and control worms was determined using the Kaplan–Meier survival assay in MedCalc software. The results are presented as mean lifespan ± standard error (SE). Data other than lifespan were statistically analyzed using ANOVA in ASSISTAT statistical assistance software. All bar graphs show the mean of biologically independent samples and error bars demonstrate ± SEM. Difference between the data was considered as significant at $P \leq 0.05$.

3. Results

3.1. SPC extends lifespan of wild type C. elegans

To evaluate the effect of SPC in the modulation of lifespan, wild type worms were exposed to various concentrations (5, 25 and 50 μM) of SPC from L1 stage till death. It was observed that SPC (25 μM) dose significantly extended the mean lifespan (19.92 ± 0.030; n = 188) of worms by 15.47% ($P \leq 0.0001$). The worms exposed to 5 and 50 μM doses of SPC also demonstrated extension in mean lifespan by 8.9% ($P \leq 0.0001$) and 11.88% ($P \leq 0.0001$) respectively (Table 1, Fig. 3A).

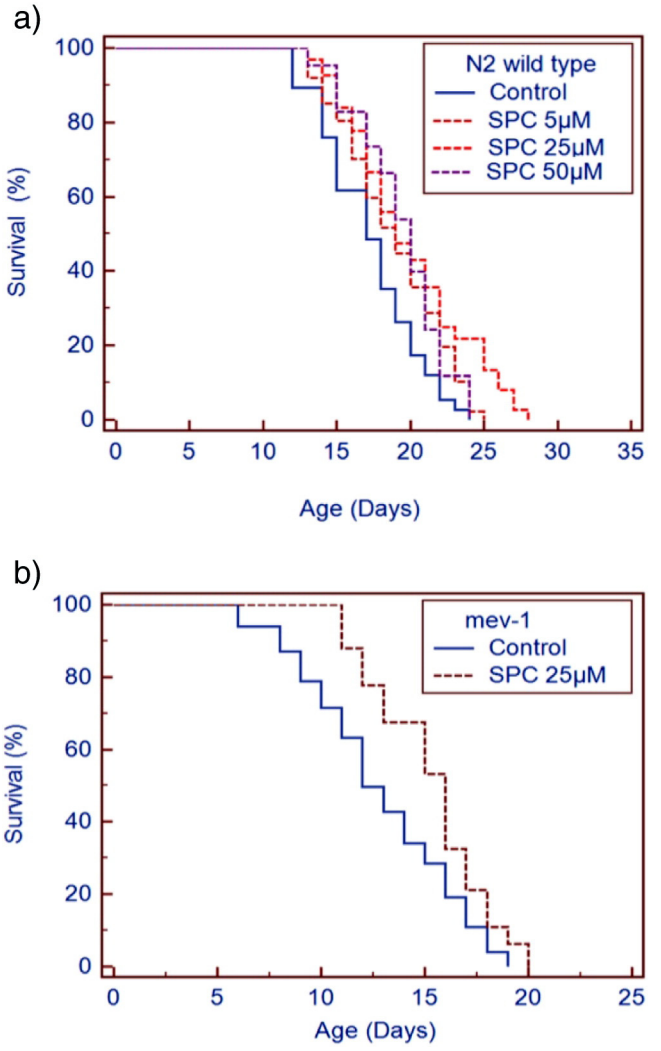

Fig. 3. (a) Effects of SPC on lifespan extension in N2 wild type *C. elegans* at 20 °C. Treatment plates were prepared by standard NGM and SPC at various concentrations (5, 25, 50 μM) and worms were grown on *E. coli* OP50 lawn and allowed to grow till L4 stage. L4 molts were then transferred to fresh NGM plates of the corresponding test concentration with 50 μM FUdR, worms were scored daily for survival. SPC (5 μM, 25 μM and 50 μM) demonstrated significant increase in lifespan of wild type N2 worms. Whereas, SPC 25 μM is the most effective concentration which enhanced lifespan by 15.47% ($P \leq 0.0001$) in comparison to control worms. (b) The % mean survival in 25 μM SPC treated *mev-1(kn1)* mutants is 19.34% in comparison to normal control group ($P \leq 0.0001$). The data were processed using the Kaplan–Meir survival analysis in MedCalc 12.7.7.0 software.

These results suggest that among the various tested concentrations, 25 μM dose of SPC exhibits the most prominent effect on the lifespan of wild type worms.

Table 1
Lifespan assay: Effect of different concentrations of SPC on mean lifespan of wild type (N2) and *mev-1(kn-1)* mutant.
The N2 wild type and *mev-1* mutant worms were treated with different test concentrations of SPC from L1 stage until the completion of lifespan. SPC 25 μM was found to be the most effective dose and enhanced mean lifespan significantly. The mean lifespan was calculated as the average number of days the worms survived in each test concentration. The data were processed using log rank test using the Kaplan–Meir survival analysis in MedCalc 12.7.7.0 software.

Genotype	Treatments (μM)	Mean lifespan ± SE	Sample size (N)	% change	*P* value (log rank significance)
N2 wild type	Control DMSO	17.25 ± 0.21	225		
N2 wild type	SPC 5 μM	18.8 ± 0.26	174	8.9	≤0.0001**
N2 wild type	SPC 25 μM	19.92 ± 0.30	188	15.47	≤0.0001**
N2 wild type	SPC 50 μM	19.3 ± 0.26	128	11.88	≤0.0001**
mev-1(kn1)	Control DMSO	12.77 ± 0.29	147		
mev-1(kn1)	SPC 25 μM	15.24 ± 0.20	175	19.34	≤0.0001**

Means ± SEM.
** $P < 0.0001$.

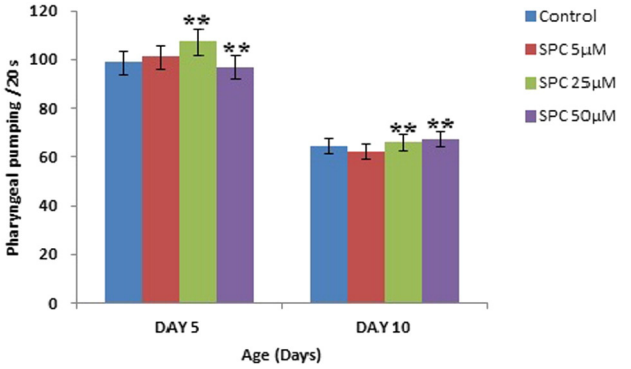

Fig. 4. Effect of SPC on the pharyngeal pumping rate of N2 wild-type *Caenorhabditis elegans*. N2 wild-type worm was raised from L1 to adult as in the lifespan assays. On the 5th and 10th days of adulthood, the pharyngeal pumping rates of individuals (n = 20) were measured by transferring a single worm to a seeded plate and pharyngeal pumping was scored under a dissecting microscope for 20 s at room temperature. The statistical differences between the control and treated worms were determined with the aid of the parametric t-test. The data is statistically analyzed using ANOVA in ASSISTAT 7.7 beta statistical assistance software. Differences between the data were considered significant at $P \leq 0.05$. Error bars represent means ± SEM. $^{**}P \leq 0.001$.

3.2. SPC enhanced the pharyngeal pumping of C. elegans

The pharyngeal pumping is the movement of the pharynx muscles which normally deteriorates with age (Pant et al., 2014). In order to investigate whether SPC affects this physiological behavior, pharyngeal pumping was recorded in *C. elegans* fed with different concentrations of SPC (5, 25 and 50 μM). The worms treated with 25 μM dose of SPC displayed significant rapid pharyngeal pumping at day 5 adult ($P \leq 0.001$) and older stages i.e. day 10 ($P = 0.0017$) than the control group (Fig. 4).

3.3. SPC enhanced the stress resistance in wild type C. elegans

The longevity is closely linked with the oxidative stress resistance and thermotolerance (Büchter et al., 2013). To investigate the effect of SPC on oxidative and thermal stress resistance in *C. elegans*, SPC (5, 25 and 50 μM) treated/control worms were exposed to oxidative and thermal stress condition. Age synchronized worms, pre-treated with various concentrations of SPC for 72 h at 20 °C were exposed to 250 μM juglone (a redox cycler) that generates intracellular ROS causing oxidative stress and incubated for 6 h (De Castro et al., 2004). The results indicate that percentage (%) survival in SPC treated worms was 36.66% ($P = 0.0223$) in 5 μM, 65% ($P \leq 0.001$) in 25 μM and 40% ($P = 0.0113$) in 50 μM as compared to untreated control worms (Fig. 5A).

To assess the effect of SPC on thermotolerance, worms were exposed to thermal stress at 37 °C for 4 h in the presence or absence of SPC. It was observed that 25 μM treatment enhanced mean survival by 23.89% ($P \leq 0.0001$) followed by 6.64% ($P \leq 0.0001$) and 6.34% ($P = 0.0001$) in 5 μM and 50 μM treatments respectively (Fig. 5B and C). The highest mean survival was observed in worms treated with 25 μM SPC.

3.4. SPC reduced intracellular ROS accumulation in C. elegans

To determine whether SPC treatment could delay the age associated cellular damage in *C. elegans*, we evaluated the level of ROS in treated (SPC 5, 25, 50 μM) and untreated day 5 worms. Results suggest that a significant ($P \leq 0.001$) reduction in intracellular ROS level was found in 25 μM (33.7%) treated worms in comparison to untreated control worms followed by SPC 5 μM (58.9%) and SPC 50 μM (49.15%). This indicates that SPC can regulate aging by reducing oxidative stress (ROS) in *C. elegans* (Fig. 6).

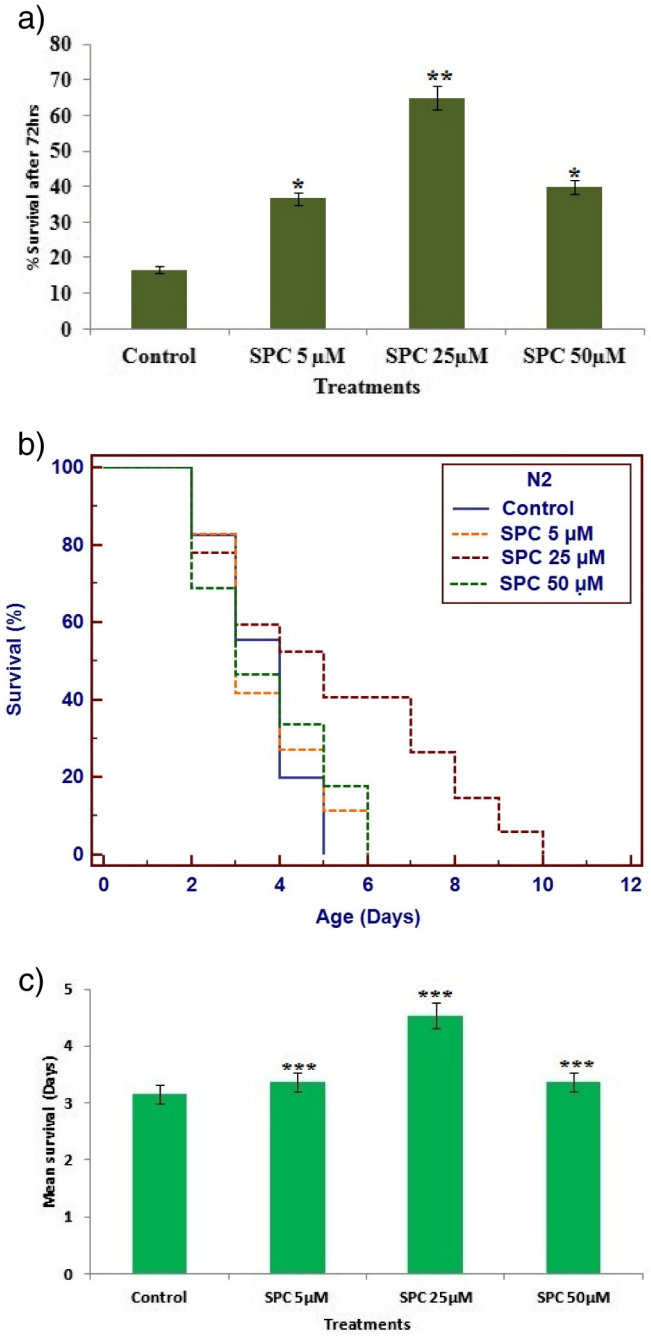

Fig. 5. (a) Effect of SPC on juglone (5-hydroxy-1,4-naphthoquinone) induced oxidative stress in N2 wild type worms. Worms were synchronized on NGM plates supplemented with 5, 25 and 50 μM SPC (n = 120) or without SPC (control = 120). The day 2 adult worms were transferred to 24-well plate containing liquid NGM and juglone (250 μM) in a total volume of 300 μL per well and survival of the worms was measured. SPC 25 μM ($P \leq 0.001$) significantly reduced the juglone sensitivity in pre-treated worms compared to untreated control worms. (b) For assessing thermotolerance under heat stress SPC treated and control adult worms were placed on NGM plates at 37 °C for 4 h and then scored for viability. (c) Mean survival of SPC treated and untreated worms under thermal stress condition. The statistical differences between the control and treated worms were determined with the aid of the parametric t-test. The data is statistically analyzed using ANOVA in ASSISTAT 7.7 beta statistical assistance software and Kaplan–Meir survival analysis in MedCalc 12.7.7.0 software. Differences between the data were considered significant at $P \leq 0.05$. Error bars represent means ± SEM. $^{*}P \leq 0.05$, $^{**}P \leq 0.001$, $^{***}P \leq 0.0001$.

3.5. SPC alleviates oxidative stress in oxidative stress sensitive mev-1 mutant

Aging is associated with cellular damage induced by the production of free radicals and lifespan extension is closely related with increased

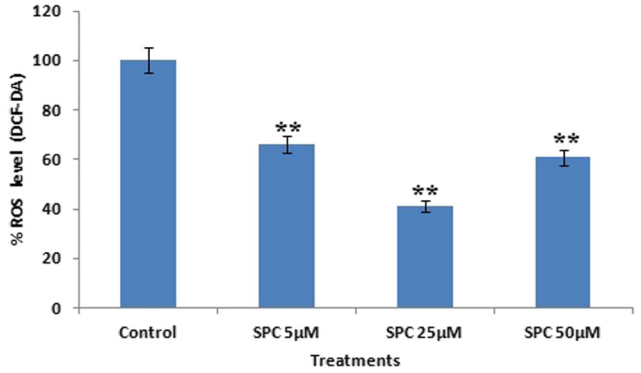

Fig. 6. Effect of SPC (5, 25 and 50 μM) on ROS levels in *C. elegans*. The age synchronized treated and non-treated N2 worms were used to measure intracellular ROS. To measure intracellular ROS levels age-synchronized wild-type N2 worms were treated with SPC (25 μM). The day 4 adult treated and non-treated live worms were subjected to ROS level detection using DCF-DA dye using a 96-well micro-plate reader at 37 °C. The graph was plotted as relative change in ROS compared to control at 100%. The statistical differences between the control and treated worms were determined with the aid of the parametric t-test. The data is statistically analyzed using ANOVA in ASSISTAT 7.7 beta statistical assistance software. Differences between the data were considered significant at $P \leq 0.05$. Error bars represent means ± SEM. $^{**}P \leq 0.001$.

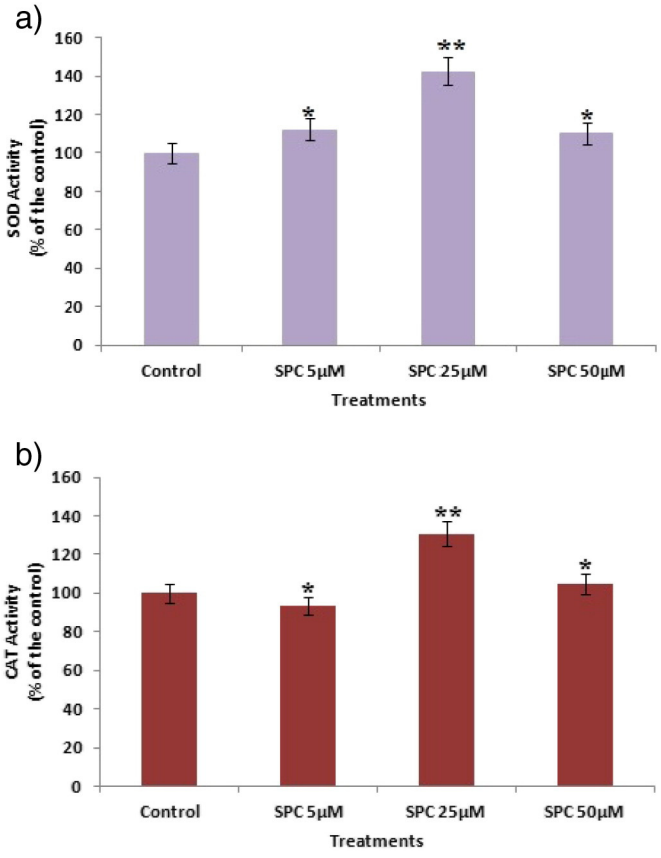

Fig. 7. Effect of SPC on oxidative stress markers. (a) Effect of SPC (5 μM, 25 μM and 50 μM) on SOD (superoxide dismutase) level. (b) Effect of SPC (5 μM, 25 μM and 50 μM) on CAT (catalase) level with respect to control. The statistical differences between the control and treated worms were determined with the aid of the parametric t-test. The data is statistically analyzed using ANOVA in ASSISTAT 7.7 beta statistical assistance software. Error bars represent means ± SEM. $^{*}P \leq 0.05$, $^{**}P \leq 0.001$.

survival under oxidative stress (Blagosklonny et al., 2009). It was found that SPC increased resistance to oxidative and thermal stress in *C. elegans* (Fig. 5A, B and C). Additionally, SPC treatment extended the mean lifespan by 19.34% ($P \leq 0.0001$) in oxidative stress sensitive *mev-1* mutants (Table 1, Fig. 3B). The *mev-1* gene encodes cytochrome b, a large subunit of the complex-II enzyme succinate CoQ reductase of the mitochondria. The *mev-1* deletion mutant is hypersensitive to oxidative stress due to elevated mitochondrial ROS production. Altogether the data suggests that SPC mediated lifespan extension in *C. elegans* can be attributed to reduction in oxidative stress.

3.6. Effect of SPC on oxidative stress markers in worms

To evaluate whether treatment of SPC changes the level of antioxidant enzymes in the worms, we analyzed biochemically SOD and CAT activities. All the observed concentration of SPC (5, 25 and 50 μM) enhanced level of antioxidant enzyme SOD and CAT in vivo when tested biochemically in comparison to control. The 25 μM of SPC exhibited maximum level of SOD and CAT activities in comparison to other doses (Fig. 7A and B).

3.7. SPC reduces accumulation of intracellular lipofuscin in C. elegans

The intracellular level of lipofuscin is an auto-fluorescent molecular marker of cellular damage with progression in age in *C. elegans*. Worms treated with SPC (25 μM) exhibited reduction in intestinal lipofuscin level as compared to untreated control worms (Fig. 8A and B).

3.8. SPC increased the expression of SOD-3 and GST-4 in transgenic strains of C. elegans

In order to validate the findings of our prior results, we examined the effect of SPC on the expression of stress response genes *sod-3* (superoxide dismutase) and *gst-4* (glutathione S transferase). We used GFP tagged transgenic strains CF1553 (*sod-3*::gfp) and CL2166 (*gst-4*::gfp) treated with or without SPC (25 μM). SOD-3 is an antioxidant enzyme which plays an important role in longevity and stress resistance (Guarente and Kenyon, 2000). To evaluate the protective effects of SPC in *C. elegans*, we exposed the *sod-3*::gfp and *gst-4*::gfp transgenic worms to 25 μM SPC for 72 h and observed changes in the expression of SOD-3 and GST-4 in day 2 adult worms. The 25 μM SPC significantly enhanced ($P \leq 0.001$) the expression of SOD-3 in the pharynx and anterior region of treated worms as compared to untreated control (Fig. 9A, B and C), thereby reducing oxidative stress. Furthermore, we investigated the effect of SPC on the expression of GST-4 which plays a main role in phase-II detoxification pathway. SPC (25 μM) was observed to increase the expression of GST-4 in SPC (25 μM) treated worms as compared to untreated control (Fig. 10A, B and C).

3.9. SPC upregulates the expression of stress modulating genes in C. elegans

The ROS scavenging and longevity promoting activities of SPC in *mev-1* mutant can be attributed to its stress alleviating potential in *C. elegans*. To elucidate the mechanism involved in the regulation of lifespan extension and stress modulation, quantitative real time PCR was performed for the antioxidant genes viz., *sod-1*, *sod-2*, *sod-3*, *gst-4*, *gst-7*, *hsp-16.2*, *hsp-70* and *ctl-1*. Our results demonstrate that SPC (25 μM) significantly upregulated mRNA expression of *sod-1* (1.94 fold, $P \leq 0.001$), *sod-2* (3.68 fold, $P \leq 0.001$), *sod-3* (3.75 fold, $P \leq 0.001$), *gst-4* (2.28 fold, $P \leq 0.001$), *gst-7* (2.90 fold, $P \leq 0.001$), *hsp-16.2* (2.32 fold, $P \leq 0.001$), *hsp-70* (2.29 fold, $P \leq 0.001$) and *ctl-1* (2.20 fold, $P \leq 0.001$) with respect to endogenous control *β-actin* (Fig. 11). The upregulation of *sod-1*, *sod-2*, *sod-3*, *hsp-16.2* and *hsp-70* indicates stress modulatory effect of SPC (25 μM) which is consistent with the results observed for oxidative and thermal stress assay (Fig. 5A, B and C).

4. Discussion

After several years of waning enthusiasm for an approach to drug-making that was once called revolutionary, interest in phytomolecule

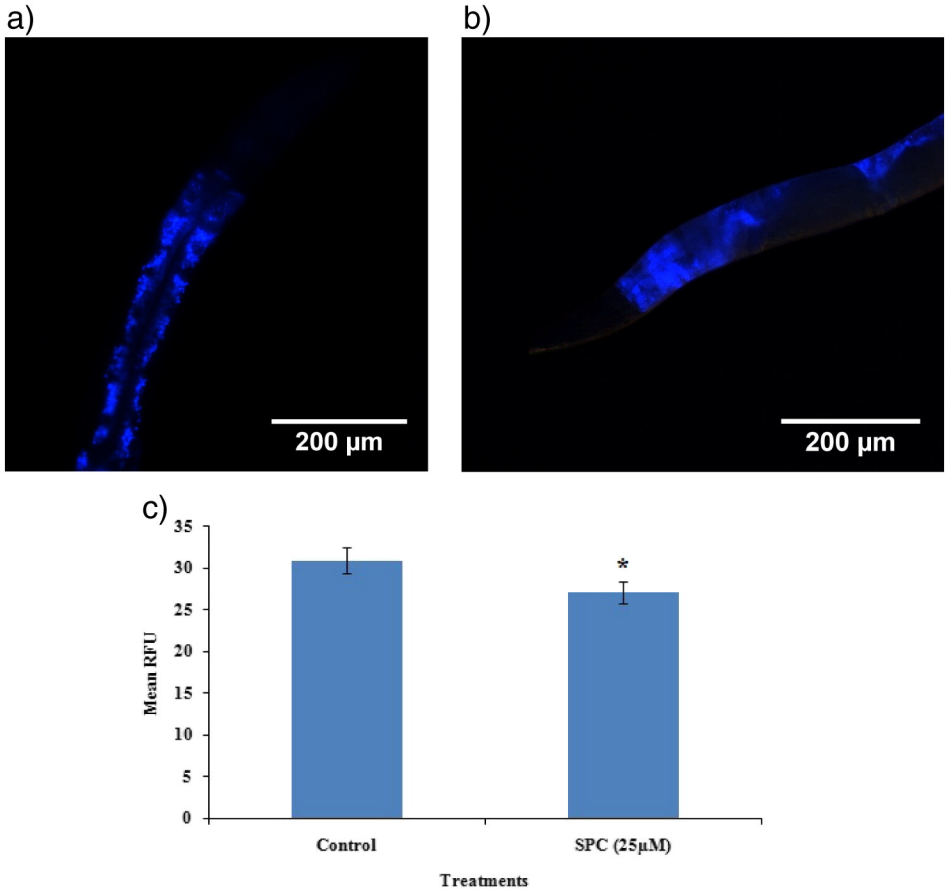

Fig. 8. The SPC (25 µM) treatment significantly reduced intestinal auto-fluorescence due to lipofuscin. The auto-fluorescence of lipofuscin of randomly selected 20 treated/untreated worms was captured using DAPI filter (with excitation at 340–380 nm and emission at 435–485 nm) with a Leica fluorescence microscope DMI 3000 B (Leica, Wetzlar, Germany) at 20× objective. (a) Microphotograph captured on day 4 of untreated control worms. (b) Microphotograph captured on day 4 of SPC (25 µM) treated worms. SPC (25 µM) treated day 4 adult worms demonstrated reduced lipofuscin auto-fluorescence compared to control. (c) Quantification of auto-fluorescence level presented as mean RFU. Scale bar = 200 µm. The lipofuscin level was quantified by determining the average pixel intensity in each worm using Image-J software (NIH). The statistical differences between the control and treated worms were determined with the aid of the parametric t-test. Differences between the data were considered significant at $P \leq 0.05$. Error bars represent means ± SEM. *$P \leq 0.05$.

based medicine curing aging and age related disorders is suddenly on upswing (Lucanic et al., 2013). The field got high-octane boost in the last decade with the discovery of a variety of natural bio-active phytomolecules enhancing stress resistance and longevity (Morimoto, 2006). Worldwide researchers are deploying a range of model organisms in studying complex realm of anti-aging effect of phytomolecules. The ultimate goal of all of these is to battle against age and age related disorders by modulating multiple cell signaling pathways underlying aging phenomena (Guarente and Kenyon, 2000). The longevity prolonging action of several bioactive molecules viz. epigallocatechin-gallate, beta-caryophyllene, resveratrol, iridoids and polyphenols etc. has earlier been reported (Valenzano et al., 2006; Wilson et al., 2006; Zhang et al., 2009; Shukla et al., 2012; Pant et al., 2014). SPC (6-O-coumaroylcatalpol) is an iridoid glucoside isolated from the aerial parts of *S. suaveolens*. The antioxidant activity of SPC has been reported in prior studies (Chandrashekhar et al., 2009). These studies about interaction and effect of this molecule is based on a variety of in vitro work and in vivo work, which give limited insight about the interaction of SPC with living systems and its effect on aging. Therefore, we selected *C. elegans* for studying in vivo interaction of SPC. Previously, this model system has been used for elucidating anti-aging and stress modulating effects of phytomolecules (Valenzano et al., 2006). These studies motivated us to study stress modulation and anti-aging potentials of SPC. To address this possibility we evaluated the effect of SPC on lifespan and stress resistance in *C. elegans* for the first time. In the present study an increment in mean lifespan in wild type SPC treated N2 worms was observed with respect to untreated control under normal culture condition at 20 °C (Table 1, Fig. 3A). There are several studies in which protective actions of flavonoids and iridoids have been attributed to their anti-aging activity in *C. elegans* (Valenzano et al., 2006; Shukla et al., 2012). The progression in age has been associated with decline in feeding behavior and pumping of the pharyngeal bulb is responsible for feeding in *C. elegans* which normally deteriorates with age. The SPC treatment alleviates age related decline as enhancement in the pharyngeal pumping rate was observed in treated worms (Fig. 4). The longevity and stress resistance are interconnected phenomenon (Wilson et al., 2006). The progression in age is associated with elevated accumulation by products of various metabolic processes due to compromised oxidative stress defence system, the aggregation of pigment like molecule lipofuscin due to iron catalyzed oxidation of protein and lipid residues. This age pigment is also known as biomarker of aging and is oftenly associated with various age related degenerative disorders (Herndon et al., 2002). The SPC (25 µM) treatment was able to reduce lipofuscin aggregation suggesting alleviation of age related stress by SPC (Fig. 8A, B and C). The SPC (5, 25 and 50 µM) treatment was able to enhance oxidative and thermal stress tolerance and 25 µM SPC treated worms demonstrated maximum stress resistance in comparison to other tested doses of SPC (Fig. 5A and B). In addition to that SPC (25 µM) treated worms exhibited enhanced mean survival and thermotolerance under continuous exposure to heat induced thermal stress (Fig. 5B). The increment in oxidative and thermal stress tolerance indicates lifespan extension due to stress modulation by SPC. Similar findings suggest that lifespan extension due to stress modulation has been reported previously by various researchers (Sunagawa et al., 2011; Shukla et al.,

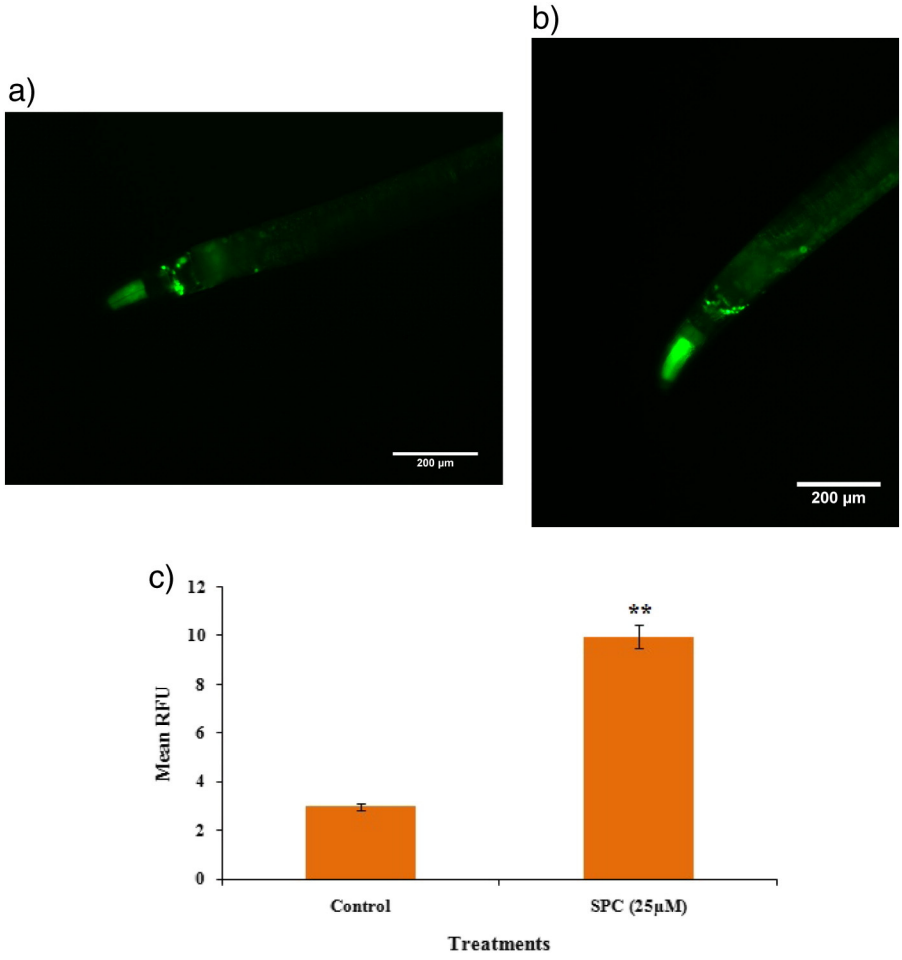

Fig. 9. Effect of SPC 25 μM on SOD-3. The age-synchronized L1 CF1553, *muls84* (pAD76(*sod-3*::gfp)) transgenic strains were continuously exposed to SPC 25 μM for 72 h. Worms were mounted on a 3% agarose pad on a glass slide and analyzed under a fluorescence microscope. SPC supplementation enhanced expression of SOD-3. (a) Control *sod-3*::gfp (n = 92). (b) SPC 25 μM treated *sod-3*::gfp (n = 90). (c) Quantification of GFP expression. The results are presented as means ± SEM of the relative fluorescence intensity. The experiment was repeated three times and images were captured using a 20× objective. n, number of worms imaged and scale bar = 200 μm. The GFP expression was quantified by determining the average pixel intensity in each worm using Image-J software (NIH). The statistical differences between the control and treated worms were determined with the aid of the parametric t-test. Differences between the data were considered significant at $P \leq 0.05$. Error bars represent means ± SEM. $^{**}P \leq 0.001$.

2012; Pant et al., 2014). The accumulation of higher intracellular ROS causes oxidative stress which plays a major role in aging. The Harman's free radical theory suggests that enhanced free radical level is linked with decline in lifespan or vice versa (Harman, 1956). The intracellular free radical level is balanced by range of antioxidative enzyme like superoxide dismutase, glutathione S transferase and catalase. These antioxidative enzymes scavenge free radicals and maintain cellular homeostasis in living system. We evaluated the intracellular ROS level and our results are similar to the previous findings where various phytomolecules have been found to scavenge intracellular ROS and remediate oxidative stress (Shukla et al., 2012). SPC significantly reduced intracellular ROS and the modulation in oxidative stress can be attributed to attenuation in intracellular ROS level. Furthermore, we observed extension in mean lifespan of *mev-1* mutant treated with SPC treatment. *mev-1* deletion mutant is an oxidative stress hypersensitive strain due to loss of function in MEV-1, a subunit of complex II in the electron transport chain which leads to ROS overproduction and reduced lifespan (Ishii et al., 1998). We observed extension in mean lifespan of *mev-1* mutant on SPC treatment (Table 1, Fig. 3B) indicating stress reducing nature of SPC. Additionally, SPC (25 μM) significantly enhanced the level of antioxidant enzymes SOD and CAT when tested biochemically. Altogether increase in mean lifespan of *mev-1* and enhanced levels of SOD and CAT can be attributed to oxidative stress reducing properties of SPC. Aging is marked with an increase in oxidative stress and depletion in antioxidant enzyme level like SOD and CAT. The different concentrations of SPC remarkably enhanced the level of antioxidant enzymes CAT and SOD in comparison to reduced level in untreated worm suggesting its stress alleviating activity (Fig. 7A and B). The 25 μM dose of SPC significantly alleviated the harmful effects of age related oxidative stress revealing the antioxidant activity of the SPC. The imbalance between ROS production and antioxidant enzyme levels results in cellular oxidative stress. The cellular oxidants are generated with standard metabolic process occurring inside the cell. Additionally, oxidative damage is caused by non-metabolic processes viz. exposure to cytotoxic chemicals, ionizing radiation or certain drugs (Harman, 1956). These reactive oxygen species can cause DNA and cellular damage which leads to aging and age related decline. The cellular defence system consists of enzymatic antioxidants such as CAT and SOD which counterbalance the cellular ROS level (Finkel and Holbrook, 2000). The SOD and catalase levels were enhanced on SPC treatment in comparison to untreated control group which indicates the potential antioxidant and stress modulatory properties of the SPC. Additionally, SPC treatment enhanced the expression of antioxidant genes *sod-3* and *gst-4*. SPC mediated overexpression of this stress response gene in transgenic strains *sod-3*::gfp (Fig. 9A, B and C) and *gst-4*::gfp (Fig. 10A, B and C) validates oxidative stress resistance demonstrated by SPC treated worms above (Fig. 5A and B). Furthermore, SPC treatment upregulated the stress responsive genes like *sod-1*, *sod-2*, *sod-3*, *gst-4*, *gst-7*, *hsp-16.2*, *hsp-70* and *ctl-1*

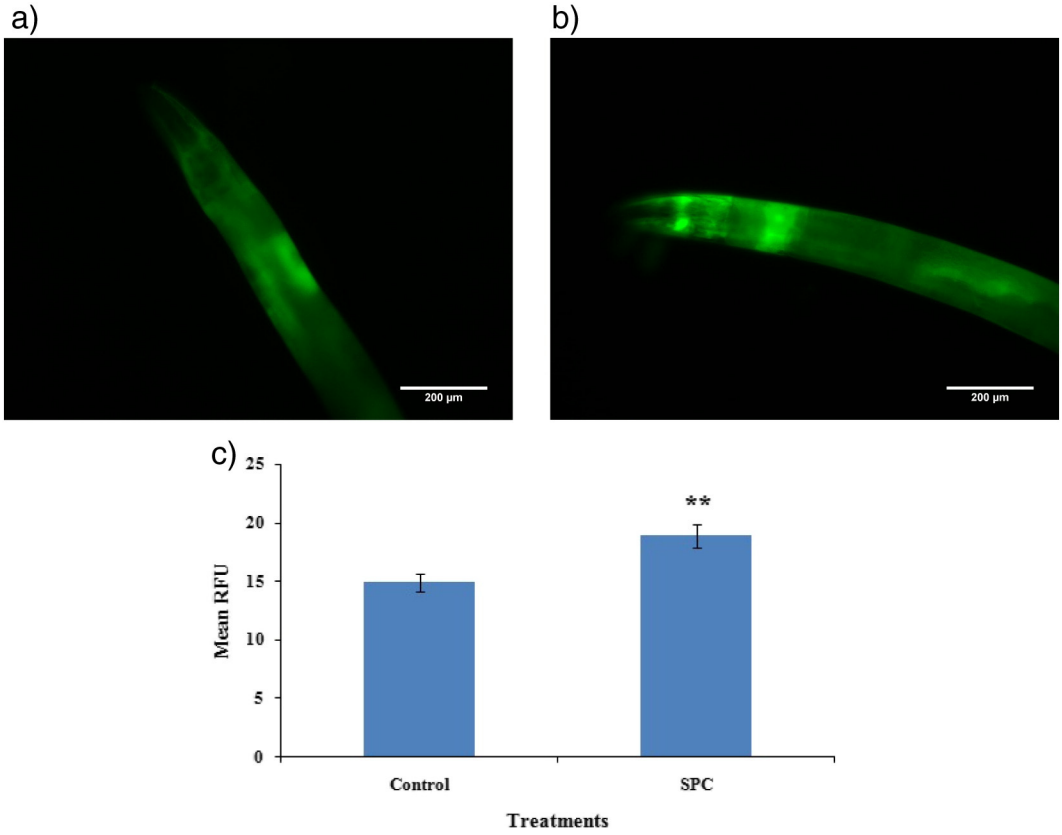

Fig. 10. Effect of SPC 25 μM on GST-4 expression. The age-synchronized L1 CL2166 (*gst-4*::gfp) (*dvls19*) transgenic strains were continuously exposed to SPC 25 μM for 72 h. Worms were mounted on a 3% agarose pad on a glass slide and analyzed under a fluorescence microscope. The SPC supplementation enhanced expression of GST-4. (a) Control *gst-4*::gfp (n = 88). (b) SPC 25 μM treated *gst-4*::gfp transgenic strain (n = 90) of *C. elegans* and scale bar = 200 μm. (c) Quantification of GFP expression. The results are presented as means ± SEM of the relative fluorescence intensity. The experiment was repeated three times and images were captured using a 20× objective. n, number of worms imaged and scale bar = 200 μm. The GFP expression was quantified by determining the average pixel intensity in each worm using Image-J software (NIH). The statistical differences between the control and treated worms were determined with the aid of the parametric t-test. Differences between the data were considered significant at $P \leq 0.05$. Error bars represent means ± SEM. $^{**}P \leq 0.001$.

(Fig. 11). The upregulation of *sod-1*, *sod-2*, *sod-3* and *ctl-1* validates increment in SOD and CAT levels conferring oxidative stress resistance (Fig. 7A and B). The elevated level of *hsp-70* and *hsp-16.2* supports the enhanced thermotolerance by SPC treated worms (Fig. 5B and C). Overall our results suggest that SPC modulates age related oxidative stress and antioxidant enzymatic levels promoting longevity which can be subjected to future investigations.

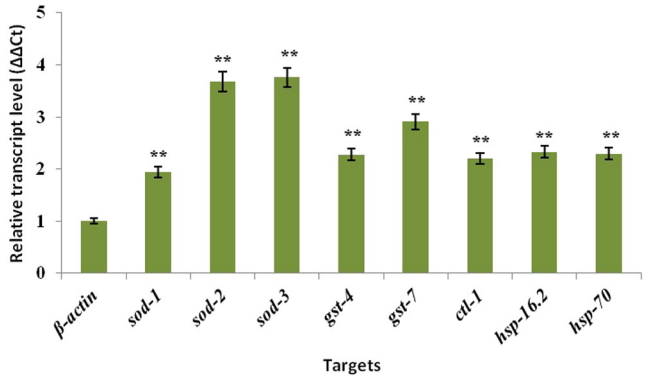

Fig. 11. Effect of SPC (25 μM) treatment on expression of stress responsive genes like *sod-1*, *sod-2*, *sod-3*, *gst-4*, *gst-7*, *hsp-16.2*, *hsp-70* and *ctl-1*. The housekeeping gene *β-actin* (*act-1*) was used as endogenous control and the relative expression level was quantified using real-time PCR using comparative C_t ($\Delta\Delta C_t$) method. The data is statistically analyzed using ANOVA in ASSISTAT 7.7 beta statistical assistance software. Differences between the data were considered significant at $P \leq 0.05$. Error bars represent means ± SEM. $^{**}P \leq 0.001$.

5. Conclusion

The present experiment demonstrated stress and lifespan modulating potentials of SPC. SPC modulated the oxidative and thermal stress and enhanced antioxidant enzyme levels attenuating cellular ROS levels. The study demonstrates the involvement of antioxidant enzymes CAT and SOD in stress resistance conferred by SPC. This is the first study to report the lifespan extending and stress modulating potentials of SPC in *C. elegans* model system. The present study opens doors for development of phytomolecule based therapeutics for prolonging lifespan and managing age related severe disorders.

Conflict of interest

There exists no conflict of interest among the authors.

Acknowledgment

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References

Ahn, D., Lee, E.B., Kim, B.J., Lee, S.Y., Lee, T.G., Ahn, M.S., Kim, D.K., 2014. Antioxidant and lifespan extending property of quercetin-3-O-dirhamnoside from *Curcuma longa* L. in *Caenorhabditis elegans*. J. Korean Soc. Appl. Biol. Chem. 57, 709–714.

Blagosklonny, M.V., Campisi, J., Sinclair, D.A., 2009. Aging: past, present and future. Aging 1, 1.

Brenner, S., 1974. The genetics of *Caenorhabditis elegans*. Genetics 77 (1), 71–94.

Brown, M.K., Evans, J.L., Luo, Y., 2006. Beneficial effects of natural antioxidants EGCG and α-lipoic acid on life span and age-dependent behavioral declines in *Caenorhabditis elegans*. Pharmacol. Biochem. Behav. 85, 620–628.

Büchter, C., Ackermann, D., Havermann, S., Honnen, S., Chovolou, Y., Fritz, G., Wätjen, W., 2013. Myricetin-mediated lifespan extension in *Caenorhabditis elegans* is modulated by DAF-16. Int. J. Mol. Sci. 14 (6), 11895–11914.

Chandrashekhar, V.M., Ashok, A.M., Sarasvathi, V.S., Muchandi, I.S., 2009. Free radical scavenging activity of *Stereospermum suaveolens* DC: an *in-vitro* evaluation. Pharmacol. Online 1, 50–56.

Chandrashekhar, V.M., Ashok, A.M., Sarasvathi, V.S., Ganapty, S., 2010. Hepatoprotective activity of *Stereospermum suaveolens* DC. against CCl4-induced liver damage in albino rats. Pharm. Biol. 48, 524–528.

Chattarjee, A., Chandra, P.S., 2000. The Treatise on Indian Medicinal Plants. Vol. 2. National Institute of Science Communication, New Delhi, pp. 10–11.

Claiborne, A., 1985. Catalase activity. In: Greenwald, R.A. (Ed.), CRC Hand Book of Methods for Oxygen Radical Research. CRC Press, Boca Raton, Florida, USA, pp. 283–284.

De Castro, E., Hegi de Castro, S., Johnson, T.E., 2004. Isolation of long-lived mutants in *Caenorhabditis elegans* using selection for resistance to Juglone. Free Radic. Biol. Med. 37, 139–145.

Fabian, T.J., Johnson, T.E., 1994. Production of age-synchronous mass cultures of *Caenorhabditis elegans*. J. Gerontol. 49, B145–B156.

Finkel, T., Holbrook, N.J., 2000. Oxidants, oxidative stress and the biology of ageing. Nature 408, 239–247.

Garg, H.S., Bhandari, S.P.S., Tripathi, S.C., Patnaik, G.K., Puri, A., Saxena, R., Saxena, R.P., 1994. Antihepatotoxic and immunostimulant properties of iridoid glycosides of Scrophularia. Phytother. Res. 8, 224–228.

Ghisalberti, E.L., 1998. Biological and pharmacological activity of naturally occurring Iridoids and Secoiridoids. Phytomedicine 5, 147–163.

Guarente, L., Kenyon, C., 2000. Genetic pathways that regulate ageing in model organisms. Nature 408, 255–262.

Harman, D., 1956. Aging: a theory based on free radical and radiation chemistry. J. Gerontol. 11, 298–300.

Herndon, L.A., Schmeissner, P.J., Dudaronek, J.M., Brown, P.A., Listner, K.M., Sakano, Y., Driscoll, M., 2002. Stochastic and genetic factors influence tissue-specific decline in ageing *C. elegans*. Nature 419 (6909), 808–814.

Hosono, R., 1978. Sterilization and growth inhibition of *Caenorhabditis elegans* by 5-fluorodeoxyuridine. Exp. Gerontol. 13, 369–373.

Ishii, N., Fujii, M., Hartman, P.S., Tsuda, M., Yasuda, K., Senoo-Matsuda, N., Suzuki, K., 1998. A mutation in succinate dehydrogenase cytochrome b causes oxidative stress and ageing in nematodes. Nature 394 (6694), 694–697.

Kenyon, C., 2005. The plasticity of aging: insights from long-lived mutants. Cell 120, 449–460.

Kwak, J.H., Kim, H.J., Lee, K.H., Kang, S.C., Zee, O.P., 2009. Antioxidative iridoid glycosides and phenolic compounds from *Veronica peregrina*. Arch. Pharm. Res. 32, 207–213.

Leelaja, B.C., Rajini, P.S., 2012. Impact of phosphine exposure on development in *Caenorhabditis elegans*: involvement of oxidative stress and the role of glutathione. Pestic. Biochem. Physiol. 104 (1), 38–43.

Lithgow, G.J., White, T.M., Melov, S., Johnson, T.E., 1995. Thermotolerance and extended life-span conferred by single-gene mutations and induced by thermal stress. Proc. Natl. Acad. Sci. 92, 7540–7544.

Lucanic, M., Lithgow, G.J., Alavez, S., 2013. Pharmacological lifespan extension of invertebrates. Ageing Res. Rev 12, 445–458.

Misra, H.P., Fridovich, I., 1972. The role of superoxide anion in the auto-oxidation of epinephrine and a sample assay for superoxide dismutase. J. Biol. Chem. 247, 3170–3175.

Morimoto, R.I., 2006. Stress, aging and neurodegenerative disease. N. Engl. J. Med. 21, 355-225.

Pant, A., Saikia, S.K., Shukla, V., Asthana, J., Akhoon, B.A., Pandey, R., 2014. Beta-caryophyllene modulates expression of stress response genes and mediates longevity in *Caenorhabditis elegans*. Exp. Gerontol. 57, 81–95.

Rao, M.M., Meena, A.K., Yadav, A.K., Panda, P., Preet, K., 2010. Review on *Stereospermum suaveolens* DC: a potential herb. Drug Invent. Today 2 (5).

Shukla, V., Yadav, D., Phulara, S.C., Gupta, M.M., Saikia, S.K., Pandey, R., 2012. Longevity promoting effects of 4-hydroxy-E-globularinin in *Caenorhabditis elegans*. Free Radic. Biol. Med. 53, 1848–1856.

Su, B.N., Pawlus, A.D., Jung, H.A., Keller, W.J., McLaughlin, J.L., Kinghorn, A.D., 2005. Chemical constituents of the fruits of *Morinda citrifolia* (Noni) and their antioxidant activity. J. Nat. Prod. 68, 592–595.

Sunagawa, T., Shimizu, T., Kanda, T., Tagashira, M., Sami, M., Shirasawa, T., 2011. Procyanidins from Apples (*Malus pumila* Mill.). Planta Med. 77, 122–127.

Tepe, B., Daferera, D., Tepe, A.S., Polissiou, M., Sokmen, A., 2007. Antioxidant activity of the essential oil and various extracts of *Nepeta flavida* Hub.-Mor. from Turkey. Food Chem. 103, 1358–1364.

Tietze, L.F., 1983. Secologanin, a biogenetic key compound — synthesis and biogenesis of the Iridoids and Secoiridoidal glycosides. Angew. Chem. Int. Ed. 22, 828–841.

Valenzano, D.R., Terzibasi, E., Genade, T., Cattaneo, A., Domenici, L., Cellerino, A., 2006. Resveratrol prolongs lifespan and retards the onset of age-related markers in a short-lived vertebrate. Curr. Biol. 16, 296–300.

Wilson, M.A., Shukitt, Hale B., Kalt, W., Ingram, D.K., Joseph, J.A., Wolkow, C.A., 2006. Blueberry polyphenols increase lifespan and thermotolerance in *Caenorhabditis elegans*. Aging Cell 5, 59–68.

Zhang, L., Jie, G., Zhang, J., Zhao, B., 2009. Significant longevity-extending effects of EGCG on *Caenorhabditis elegans* under stress. Free Radic. Biol. Med. 46, 414–421.