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# Beta-caryophyllene modulates expression of stress response genes and mediates longevity in *Caenorhabditis elegans*

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ABSTRACT

Beta-caryophyllene (BCP) is a natural bicyclic sesquiterpene and is a FDA approved food additive, found as an active ingredient in essential oils of numerous edible plants. It possesses a wide range of biological activities including anti-oxidant, anti-inflammatory, anti-cancerous and local anesthetic actions. We used the well established *Caenorhabditis elegans* model system to elucidate the stress modulatory and lifespan prolonging action of BCP. The present study for the first time reports the lifespan extension and stress modulation potential of BCP in *C. elegans*. Upon evaluation, it was found that 50 μM dose of BCP increased the lifespan of *C. elegans* by over 22% ($P \leq 0.0001$) and significantly reduced intracellular free radical levels, maintaining cellular redox homeostasis. Moreover, the results suggest that BCP modulates feeding behavior, pharyngeal pumping and body size effectively. Further, this compound also exhibited significant reduction in intestinal lipofuscin levels. In the present investigation, we have predicted possible biological molecular targets for BCP using molecular docking approaches and BCP was found to have interaction with SIR-2.1, SKN-1 and DAF-16. The prediction was further validated in vivo using mutants and transgenic strains unraveling underlying genetic mechanism. It was observed that BCP increased lifespan of *mev-1* and *daf-16* but failed to augment lifespan in *eat-2*, *sir-2.1* and *skn-1* mutants. Relative quantification of mRNA demonstrated that several genes regulating oxidative stress, xenobiotic detoxification and longevity were modulated by BCP treatment. The study unravels the involvement of multiple signaling pathways in BCP mediated lifespan extension.



## 1. Introduction

The prime importance of phytomolecules in therapeutics and the immense desire of mankind for rejuvenation have centralized the current attention towards the discovery of novel anti-aging drugs (Zhang, 2004). The health beneficial effects of medicinal plants are attributed to various phytomolecules. A number of earlier reports have established that active constituents of different plant extracts are efficient in reversing aging, extending lifespan and improving stress tolerance (Wiegant et al., 2009). Beta-caryophyllene (BCP), a natural bicyclic sesquiterpene is a common constituent of the essential oils of numerous spices and food plants. Several biological activities such as anti-inflammatory, antibiotic, antioxidant, anti-carcinogenic and local anesthetic are attributed to BCP. The plant volatile BCP selectively binds to the CB2 receptor and it is a functional CB2 agonist (Gertsch et al., 2008). Earlier studies suggest that BCP can serve as functional food and dietary supplement owing to its antioxidative effects (Calleja et al., 2013). Further, BCP is also well reported for its potent anti-cancerous and analgesic nature (Klauke et al., 2013; Legault and Pichette, 2007). Despite of the various established potentials of BCP, the anti-aging activity of BCP is yet to be explored and serious efforts are required to detect the possible roles and underlying genetic mechanisms.

Aging is a time dependent debilitating process associated with several chronic disorders like neurodegeneration, cancer, diabetes and cardiovascular disorders imposing huge burden to the society. Previous studies suggest that phytomolecules can effectively modulate the life prolonging mechanistic pathways, thereby extending lifespan significantly (Shukla et al., 2012; Sunagawa et al., 2011). *Caenorhabditis elegans*, a free living soil nematode is a well established model for aging studies. Since the systematic evaluation of efficacy, safety and mechanism of bioactive molecules in mammalian model is expensive and time consuming, *C. elegans* is the most preferred model owing to its several important characteristics viz. short lifespan and life-cycle, easy laboratory maintenance and high homology to mammalian especially human biochemical and genetic pathways (Kenyon, 2010). Furthermore, genetic and pharmacological modification of lifespan mechanism has been well elucidated in *C. elegans* (Lithgow et al., 2005).

Most of knowledge about BCP has originated from in vitro experiments while the in vivo effects have a limited insight for mechanisms such as uptake, metabolism and organ/tissue interactions. Therefore, we evaluated different pharmacological doses of BCP (25, 50, 100 μM) for studying the life prolongation and stress modulatory effects using the *in vivo* model system, *C. elegans*. Results revealed the involvement

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of multiple possible interactions viz. insulin signaling, caloric restriction, oxidative stress and xenobiotic detoxification pathways in BCP mediated lifespan extension which was also validated through mutant and transgenic strains. The study is a frontier step for elucidation of BCP's role in defying aging. Due to higher homology to mammalian genes, the study would be helpful in establishing BCP as a novel natural therapy for aging and age related disorders.

## 2. Materials and methods

### 2.1. C. elegans: strains and maintenance

*C. elegans* strains viz. N2-Bristol (wild type); TK22, *mev-1* (*kn1*); DA1116, *eat-2* (*ad1116*); GR1307, *daf-16* (*mgDf50*); VC-199, *sir-2.1* (*ok434*); EU1, *skn-1* (*zu67*); TJ356, *daf-16*::gfp (*zls356*); CF1553, *muIs84* [*pAD76*(*sod-3*::gfp)]; CL2166 *gst-4*::gfp (*dvls19*); ldIs7 [skn-1B/C::GFP+pRF4(rol-6(su1006))] and *Escherichia coli* OP50 were procured from the *Caenorhabditis* Genetics Centre (CGC), University of Minnesota (Minneapolis, MN, USA). The strains were cultured and maintained at 20 °C on nematode growth medium (NGM) seeded with *E. coli* OP50 bacteria using the standard protocol (Brenner, 1974). A synchronized culture was obtained by sodium hypochlorite treatment, which kills adult worms but not their eggs (Fabian and Johnson, 1994).

### 2.2. Test compound

BCP (Sigma-Aldrich) was dissolved in 10% dimethyl sulfoxide (DMSO) to prepare a 10 mM stock. Toxicity assay was performed with 25 μM, 50 μM, 100 μM and 500 μM to assess toxic effect of BCP. No obvious toxic effect of BCP was observed at 25 μM, 50 μM and 100 μM but 500 μM was found to be toxic to the worm (Fig. 1). Hence, 25 μM, 50 μM and 100 μM were taken as test concentrations. Worms from the L1 stage were treated with the indicated BCP concentrations (25 μM, 50 μM and 100 μM) and 0.1% DMSO was used as control. Each BCP concentration or 0.1% DMSO as a control was spotted directly onto a 35 mM or 60 mM Petri dish containing 2 mL/5 mL of NGM. Each NGM plate was freshly spotted with indicated doses of BCP (25–100 μM final concentration) or 0.1% DMSO. All BCP/Control plates were freshly prepared 2 h before use and seeded with *E. coli* OP50 one day before use (Buchter et al., 2013; Zhang et al., 2009).

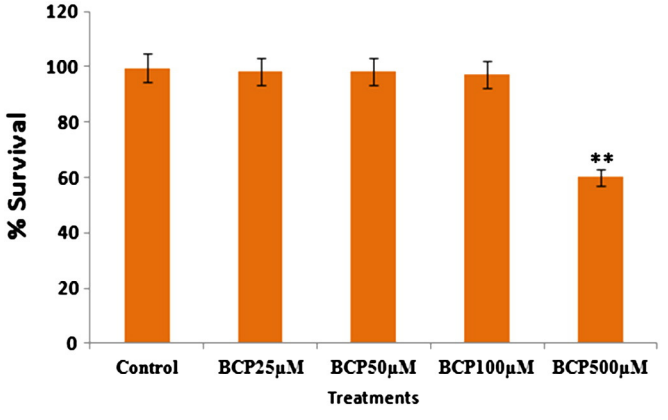


**Fig. 1.** Toxicity assay with different doses of BCP (25 μM, 50 μM, 100 μM and 500 μM). N2 wild type worms were synchronized on NGM plates and on day 2 of adulthood the worms (n = 50) were transferred to 24 well plate containing respective doses of BCP (25 μM, 50 μM 100 μM and 500 μM) in liquid NGM whereas 0.1% DMSO served as control. No obvious toxic effect of BCP was observed at 25 μM, 50 μM, and 100 μM but at 500 μM, BCP was found to be toxic to worm. Hence, 25 μM, 50 μM and 100 μM were taken as test concentrations. The data is statistically analyzed using ANOVA (Duncan test) in ASSISTAT 7.6 beta statistical assistance software. Differences between the data were considered significant at $P \leq 0.05$. Error bars represent mean ± S.E.M. $^{**}P \leq 0.01$.

### 2.3. Lifespan analysis

Age synchronized N2 worms were used for lifespan assay. The isolated eggs were allowed to hatch on NGM plates previously spotted with or without different concentrations of BCP (25 μM, 50 μM and 100 μM) and DMSO (0.1%) served as solvent control till L4 stage. 25–30 L4 molts were then transferred to fresh NGM plates previously spotted with corresponding test concentrations and 50 μM FUdR (Sigma-Aldrich) to block the progeny development (Hosono, 1978). For assessing effect of BCP only during adulthood, 40–50 day 1 adults were transferred to NGM plate plates previously spotted with indicated concentration of BCP/DMSO and OP50 and 50 μM 5-Fluoro-2′-deoxyuridine (FUdR) to inhibit the growth of progeny. Worms were then observed daily for survival and transferred to fresh plates after every 3–4 days to avoid contamination and to assure the presence of the compound throughout the experiment. The experiment was terminated when all worms were scored as dead or censored (Zhang et al., 2009). To test the antibacterial effect of BCP, antimicrobial activity with BCP was performed (Suryanto et al., 2012). Additionally lifespan assays were conducted with heat-killed *E. coli* OP50 (30 min at 65 °C) according to Gruber et al. (2007). Two or three independent trials were performed for all the treatments, and the data shown represents the mean lifespan per trial with similar effect on longevity (Table 1).

### 2.4. Reproduction assay

Age synchronized N2 worms were raised from L1 larvae as mentioned in the lifespan assay. L4 larvae were individually transferred to a fresh plate with or without treatment each day until reproduction ceased. The offspring of each animal was counted at the L2 or L3 stage. The test was performed at least three times.

### 2.5. Measurement of body size

L1 worms were exposed to BCP (50 μM) and control (without BCP) plates were incubated for 72 h. After proper exposure to the test compound, the day 1, day 2, day 5 and day 10 worms were directly picked for body size measurement. The body size of more than 20 randomly selected worms (Saul et al., 2009) was measured on an automated scale using the Leica Application Suite V3 software (version 3.4.0). The experiment was performed on three independent trials.

### 2.6. Measurement of pharyngeal pumping

To investigate the effect of BCP on pharyngeal pumping, the movement of the pharynx terminal bulb was recorded. Worms were grown on NGM plates, previously treated with or without BCP (50 μM) concentration and seeded with *E. coli* OP50. Pumping rate for 20 randomly selected day 5 and day 10 worms was recorded at regular intervals of 20 s at room temperature (Brown et al., 2006). The experiment was performed independently thrice.

### 2.7. Stress resistance assay

Synchronized L1 larvae (as described for the lifespan assay) were transferred to *E. coli* OP50 lawns, with or without BCP (50 μM) at 20 °C. Subsequently, adult worms were subjected to oxidative and thermal stress. Oxidative stress was induced by an acute, lethal concentration of juglone (5-Hydroxy-1,4-napthoquinone, Sigma-Aldrich). For oxidative stress, BCP (50 μM) treated and untreated wild type adult day 2 worms were transferred to NGM plates containing 250 μM juglone. Survival of the worms was scored after 5 h (Zhang et al., 2009).

To assess thermotolerance, BCP treated and control adult worms were placed on NGM plates at 35 °C for 4 h and then scored for viability. Survival of the worms was determined by touch-provoked movement (Zhang et al., 2009). To assess thermotolerance under constant heat

**Table 1**
Lifespan assay analysis: effect of different concentrations of BCP on mean lifespan of wild type (N2).

| Genotype | Treatment (μM) | Mean | ±S.D. | ±S.E. | Sample size | N (trial no.) | % change | *P*-value |
|---|---|---|---|---|---|---|---|---|
| N2 wild type | Control | 17.88 | 3.95 | 0.274 | 207 | 1 | | |
| | BCP (25) | 19.7 | 4.75 | 0.345 | 189 | 1 | 10.17 | ≤0.0001** |
| | BCP (50) | 21.84 | 5.68 | 0.429 | 174 | 1 | 22.7 | ≤0.0001** |
| | BCP (100) | 20.69 | 5.20 | 0.369 | 198 | 1 | 15.7 | ≤0.0001** |
| | Control | 16.99 | 5.96 | 0.497 | 144 | 2 | | |
| | BCP (25) | 18.97 | 4.87 | 0.411 | 142 | 2 | 11.12 | 0.02* |
| | BCP (50) | 20.82 | 4.69 | 0.391 | 141 | 2 | 22.54 | ≤0.0001** |
| | BCP (100) | 19.1 | 7.69 | 0.608 | 159 | 2 | 12.41 | 0.0004* |
| | Control | 17.47 | 5.86 | 0.505 | 134 | 3 | | |
| | BCP (25) | 19.91 | 5.44 | 0.452 | 144 | 3 | 12.20 | 0.0001** |
| | BCP (50) | 21.2 | 4.61 | 0.411 | 125 | 3 | 21.35 | ≤0.0001** |
| | BCP (100) | 20.35 | 5.27 | 0.421 | 156 | 3 | 16.48 | ≤0.0001** |
| N2 wild type (adult) | Control | 16.27 | 3.54 | 0.298 | 140 | 1 | | |
| | BCP (50) | 18.17 | 3.91 | 0.327 | 142 | 1 | 11.85 | ≤0.0001** |
| | Control | 16.6 | 3.52 | 0.297 | 140 | 2 | | |
| | BCP (50) | 18.68 | 4.15 | 0.342 | 147 | 2 | 12.53 | ≤0.0001** |
| N2 wild type | N2 (live OP50) | 18.01 | 4.13 | 0.396 | 108 | 1 | | |
| | Control (dead OP50) | 28.45 | 8.25 | 0.745 | 123 | 1 | | |
| | BCP (dead OP50) | 30.65 | 6.85 | 0.56 | 149 | 1 | 7.73 | ≤0.0001** |
| | N2 (live OP50) | 17.74 | 3.84 | 0.379 | 102 | 2 | | |
| | Control (dead OP50) | 27.2 | 7.00 | 0.667 | 108 | 2 | | |
| | BCP (dead OP50) | 29 | 7.61 | 0.721 | 111 | 2 | 6.61 | ≤0.0001** |

The N2 wild type worms were treated with different test concentrations of BCP from L1 stage until the completion of lifespan. The BCP 50 μM dose was found to be the most effective which was further tested on young adult worm and it was also found to enhance mean lifespan significantly. The antimicrobial effect of the effective dose of BCP i.e. 50 μM was evaluated using heat killed *E. coli* OP-50 bacteria. The mean lifespan was calculated as the average number of days that the worms survived in each test concentration. The data were processed using the Kaplan–Meir survival analysis in Medcalc 12.7.7.0 software.
Mean ± S.E.M.
* $P < 0.05$.
** $P < 0.001$.

stress, BCP treated and control adult worms were placed on NGM plates at 35 °C for 7 h and then scored for viability. Survival of the worms was determined by touch-provoked movement (Lithgow et al., 1995). Worms were scored as dead when they failed to respond to repeated touch with platinum loop. The test was performed three times independently.

### 2.8. Measurement of intracellular ROS in C. elegans

Intracellular ROS levels were quantified using $H_2$DCF-DA as the molecular probe in *C. elegans* (H.C. Yang et al., 2013; J. Yang et al., 2013). Adult day-4 worms previously treated with or without BCP (50 μM) were used for intracellular ROS determination. Worms were collected in 300 μl of 0.1% PBST buffer, equally homogenized and sonicated for measuring of intracellular ROS in dead worms. The homogenized samples were transferred to a 96 well plate and prior to reading, 1.5 μl of 10 mM $H_2$DCF-DA was added to each well. A similar reaction mixture was prepared for detecting intracellular ROS in live worms. Worms were transferred to a 96 well plate and prior to reading, 1.5 μl of 10 mM $H_2$DCF-DA was added to each well. Fluorescent readings were measured using Spectra Max M2 multimode micro plate reader (Molecular Devices), at 485 nm excitation and 530 nm emission. Observations were recorded for 90 min at intervals of 20 min at 37 °C. The experiment was performed three times independently.

### 2.9. Lipofuscin assay

N2 wild-type worms were synchronized and raised from L1 to adult as described in lifespan assay. On the 4th day of adulthood, intestinal autofluorescence due to lipofuscin was analyzed (Zuckerman and Geist, 1983). 20 randomly selected worms from each treatment were mounted onto a 3% agarose pad on a glass slide anesthetized with 2% sodium azide and sealed with a coverslip. The autofluorescence of lipofuscin was captured with a DAPI filter (with excitation at 340–380 nm and emission at 435–485 nm) using a DMI 3000 B (Leica, Wetzlar, Germany) fluorescence microscope at 20×. The lipofuscin levels were quantified by determining the average pixel intensity in each worm using Image-J software (NIH).

### 2.10. In-silico analysis

In the absence of X-ray structures, we performed the computational modeling of the SIR-2.1, SKN-1 and DAF-16 proteins. The amino acid sequences of SIR-2.1 (GI: 74965708), SKN-1 (GI: 21264514) and DAF-16 (GI: 374095504) were retrieved from the Entrez protein database available at NCBI (http://www.ncbi.nlm.nih.gov). The composite modeling which combines various techniques such as threading, ab initio modeling and atomic-level structure refinement approaches implemented in the iterative threading assembly refinement (I-TASSER) server was used to build the full-length protein structure of all the selected proteins (Das et al., 2007; Zhang, 2007, 2008; Zhou et al., 2007). The template modeling score (TM-score) calculation was taken into consideration to assess the structural similarity of the model and template protein structures and to determine the accuracy of the predicted structure (Roy et al., 2010). The resultant models were further improved by their subjugation to the KoBaMIN refinement protocol. The KoBaMIN refinement protocol is described in detail elsewhere (Rodrigues et al., 2012). Briefly, the method is a two-step process. First, a highly convergent energy minimization with an all-atom knowledge-based potential of mean force that implicitly includes the effect of solvent, KB01 is performed by ENCAD program. Second, side chain rotamer positions and other stereochemistry of the models are corrected by performing a restrained energy minimization using MESHI software. The refined models were used for the active site prediction and docking experiments. The ligand-binding site (LBS) of the modeled proteins was identified using the COACH program (H.C. Yang et al., 2013; J. Yang et al., 2013). The program makes use of consensus approach for LBS prediction by combining the multiple prediction results of algorithms from TM-SITE, S-SITE, COFACTOR, FINDSITE and ConCavity. For docking analysis, the chemical structure of beta-caryophyllene (BCP) was taken from the PubChem database (http://pubchem.ncbi.nlm.nih.gov/) and the docking calculations were performed using LibDock program of Accelrys Discovery

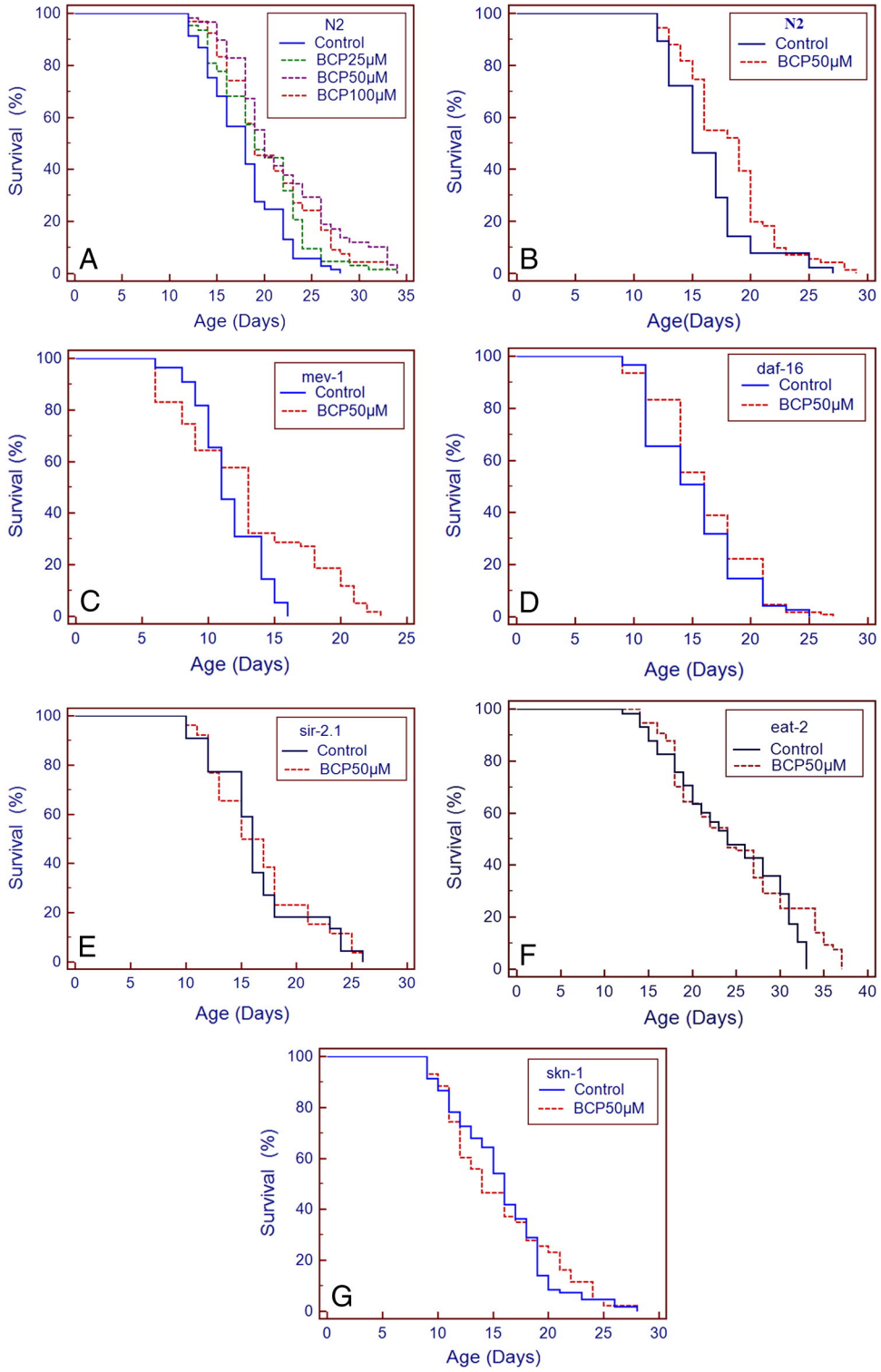


**Fig. 2.** (A) Effects of BCP on lifespan extension in N2 wild type *C. elegans* at 20 °C. Treatment plates were prepared by standard NGM and BCP at various concentrations (25, 50,100 μM) and worms were grown on *E. coli* OP50 lawn and allowed to grow till L4 stage. L4 molts were then transferred to fresh NGM plates of corresponding test concentrations with 50 μM FUdR. The significance of differences from normal control group is at $P \leq 0.0001$. Worms were scored daily for survival. The 50 μM BCP concentration showed a significant increase in lifespan of wild type N2 worms by 22% ($P \leq 0.0001$). B) BCP was also able to enhance lifespan significantly by 11.85% ($P \leq 0.0001$) when administered from start of young adult hood instead of during the L1 stage. The day 1 adults were transferred to seeded NGM plates previously spotted with indicated concentration of BCP/DMSO and 50 μM 5-Fluoro-2′deoxyuridine (FUdR) to inhibit the growth of progeny. C) The % mean survival in 50 μM BCP treated *mev-1* (*kn1*) mutants is 11.31% in comparison to normal control group ($P < 0.0001$). D) Effect of 50 μM BCP on *daf-16* (*mgDf50*) mutant. The 50 μM BCP treated *daf*-16 mutant showed marginal increase in mean lifespan by 6.3% ($P = 0.006$) in comparison to normal control group. Whereas, no significant augmentation in mean lifespan was observed in E) *sir-2.1* (*ok434*) mutant ($P = 0.58$) and G) *skn-1* (*zu67*) ($P = 0.5001$), a marginal increase in mean lifespan of F) *eat-2* (*ad1116*) mutant ($P = 0.65$) was observed by approximately 2% in comparison to control. Two or three independent trials were performed for all treatments, and the data shown represents the mean lifespan per trial with a similar effect on longevity. The data were processed using the Kaplan–Meir survival analysis in Medcalc 12.7.7.0 software.

Studio (DS) 3.5. The size of the docking grid was monitored to encompass the entire active site of SIR-2.1, SKN-1 and DAF-16 proteins and the docking parameters were kept to their default values, however, to ensure the best coverage of the compound's conformational space, the Conformation Method was set to BEST value. Finally, the docked poses of BCP by LibDock were ranked using PLP1 scoring function of DS (Cheng et al., 2009).

### 2.11. Green fluorescent protein (GFP) visualization and quantification

The TJ356 mutant (GFP tagged *daf-16*) was observed for intracellular localization of DAF-16. SOD-3 expression was observed using inducible GFP tagged CF1553 strain, GST-4 expression was observed using inducible GFP tagged *gst-4* strain CL2166 and SKN-1 expression was observed using inducible GFP tagged strain ldIs7. Age synchronized cultures of transgenic strains stably expressing DAF16: :GFP, SOD-3: :GFP, GST4: :GFP and SKN-1: :GFP as a reporter were incubated on NGM plates previously treated with BCP (50 μM) and 0.1% DMSO serving as control for 72 h at 20 °C. Thereafter, the adult live worms were mounted on 3% agarose pad and anesthetized with 2% sodium azide and sealed with a cover slip.

For DAF16: :GFP reporter assay, worms subjugated to short thermal stress at 37 °C for 20 min served as positive control. Worms were divided into three groups (nuclear, cytoplasmic and both) on the basis of localization of the DAF16: :GFP protein. The nuclear category included the worms exhibiting nuclear fluorescence and cytoplasmic category included worms without nuclear fluorescence. Worms that did not fit to any category were classified as both (Shukla et al., 2012). Photomicrographs for *sod-3*: :gfp and *gst-4*: :gfp and *skn-1*: :*gfp* were taken directly after 72 h of treatment (Shukla et al., 2012). The assay was performed using a GFP filter (with excitation at 365 nm and emission at 420 nm) using the DMI 3000 B (Leica, Wetzlar, Germany) fluorescence microscope at 20×. The fluorescence levels were quantified by using Image-J software (NIH).

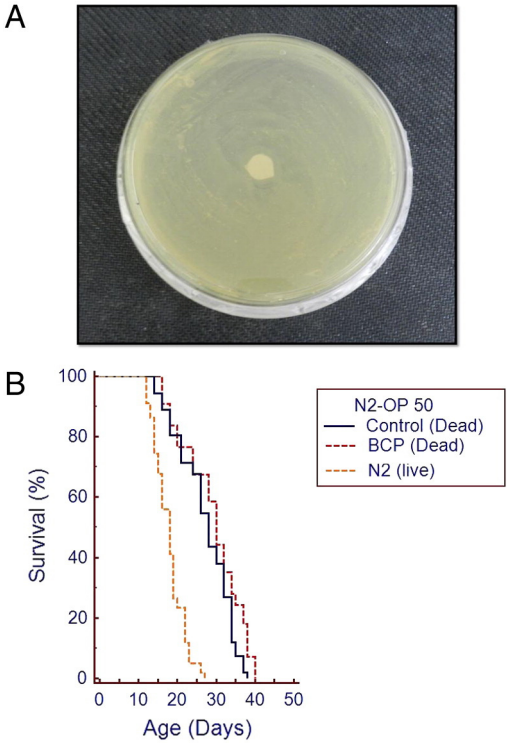


**Fig. 3.** A) Antimicrobial assay for assessing effect of BCP on *E. coli* proliferation. No zone of inhibition was exhibited by 50 μM BCP. B) Lifespan assay was conducted with heat-killed *E. coli* OP50 (30 min at 65 °C). The heat killed *E. coli* OP50 increased the mean lifespan in the absence of BCP treatment, whereas 50 μM BCP further enhanced lifespan significantly by 6–7% ($P \leq 0.0001$). Hence, as per our results the BCP mediated lifespan extension is independent of antimicrobial properties. Two independent trials were performed for all treatments. The data were processed using the Kaplan–Meir survival analysis in Medcalc 12.7.7.0 software.

### 2.12. Gene quantification through real time PCR

Day 2 adult worms (treated/untreated) with 50 μM BCP and total RNA was isolated using Trizol reagent (Invitrogen). cDNA was synthesized with an equal amount of RNA using a cDNA synthesis kit (Invitrogen) according to the manual's instruction. The mRNA expression was quantified

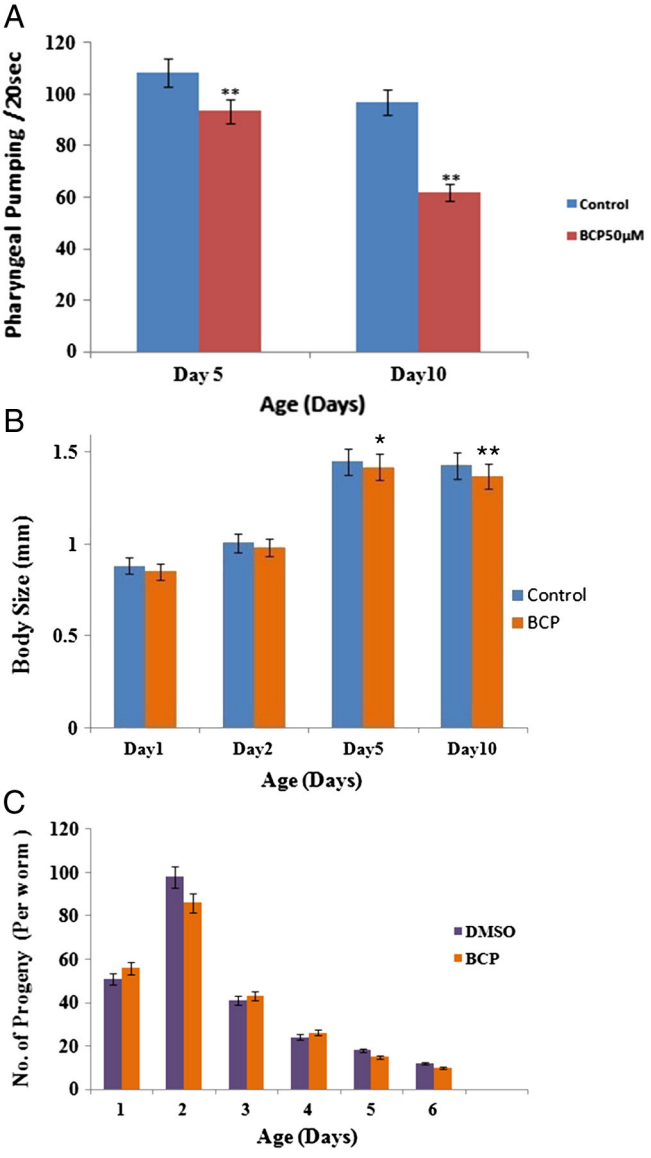


**Fig. 4.** (A) Effect of BCP on the pharyngeal pumping rate and body length of N2 wild-type *Caenorhabditis elegans*. N2 wild-type worms were raised from L1 to adulthood as in the lifespan assays. On the 5th and 10th days of adulthood, the pharyngeal pumping rates of individuals (n = 20) were measured by transferring single worm to a seeded plate and pharyngeal pumping was scored under a dissecting microscope for 20 s at room temperature. (B) For the body size, N2 wild-type worms were raised from L1 to adulthood as in the lifespan assays. On the 1st, 2nd, 5th and 10th days of adulthood, photographs were taken of animals, and the body length of each individual worm (n = 20) was measured with a scale using the Leica Application Suite V3 software (version 3.4.0). C) Effect of BCP treatment on reproduction was evaluated by studying the effect on progeny production. Age synchronized N2 worms were raised from L1 larvae as mentioned in the lifespan assays above. L4 larvae were individually transferred to a fresh plate each day until reproduction ceased. The offspring of each animal was counted at the L2 or L3 stage. The non-significant variation in progeny production between untreated and 50 μM BCP treated worms was observed. The data is statistically analyzed using ANOVA (Duncan test) in ASSISTAT 7.6 beta statistical assistance software. Differences between the data were considered significant at $P \leq 0.05$. Error bars represent mean ± S.E.M. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

for *daf-16*, *sir-2.1 skn-1*, *sod-2*, *sod-3*, *hsp-70*, *gst-4*, *gst-7*, *hsf-1*, *pha-4* and *daf-9* genes in comparison to housekeeping gene *β-actin* (*act-1*) using SYBR green detection method on a fast real-time PCR system (Applied Biosystems 7900 HT). Relative quantification for the expressed genes was done using a comparative $C_t$ ($\Delta\Delta C_t$) method (Schmittgen and Livak, 2008).

### 2.13. Statistical analysis

Significant differences between lifespan of treated and control worms under normal and stressed conditions were determined using the Kaplan–Meir survival assay in Med Calc software version 12.7.7.0. Data other than lifespan were statistically analyzed using ANOVA (Duncan test) in ASSISTAT 7.6 beta statistical assistance software. Differences between the data were considered significant at $P \leq 0.05$.

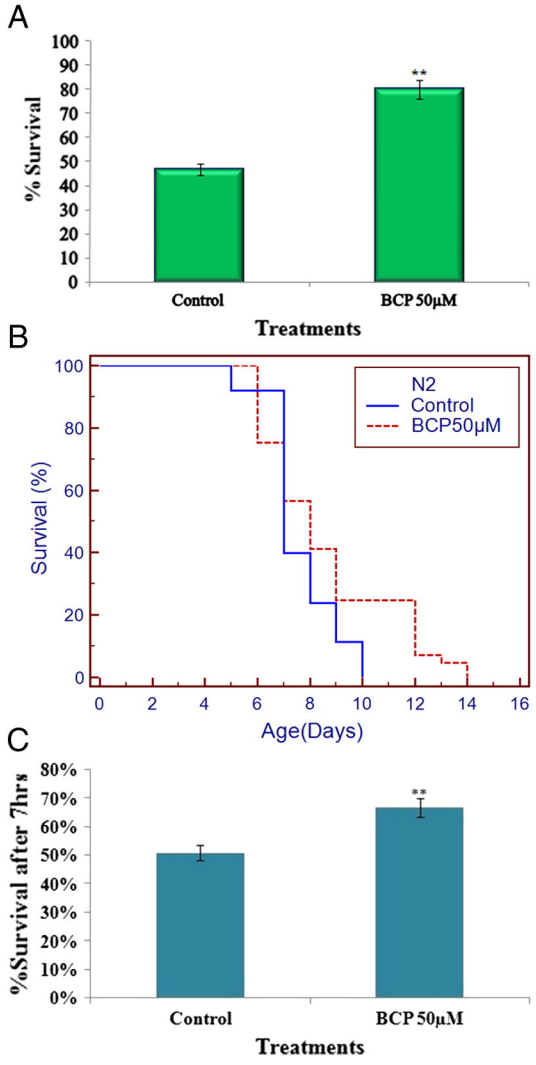


**Fig. 5.** (A) Effect of BCP on juglone (5-Hydroxy-1,4-napthoquinone) induced oxidative stress in N2 wild type worms. Worms were synchronized on NGM plates supplemented with 50 µM BCP (n = 120) or without (control = 120) BCP. On adult day 2 the worms were transferred to juglone (250 µM)-treated plates and survival of the worms was measured after 5 h. BCP 50 µM ($P \leq 0.001$) significantly reduced the juglone sensitivity in pre-treated worms compared to untreated control worms. (B) Mean lifespan of heat-stress exposed worm pre-treated with or without 50 µM of BCP. For thermal tolerance assay, 50 µM BCP treated (n = 255) and control (0 µM, n = 264) day 2 worms were incubated at 35 °C for 4 h, and then their viability was scored. C) For assessing thermotolerance under constant heat stress BCP treated and control adult worms were placed on NGM plates at 35 °C for 7 h and then scored for viability. The data is statistically analyzed using ANOVA (Duncan test) in ASSISTAT 7.6 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 mean ± S.E.M. $^{**}P \leq 0.001$.

## 3. Results

### 3.1. BCP extends the mean lifespan of wild-type N2 worms

To evaluate the effect of BCP on lifespan of N2 wild type *C. elegans*, age-synchronized worms were subjected to different concentrations of BCP viz., 25, 50 and 100 µM at 20 °C. Worms were observed daily for survival by the touch provoked method (Brown et al., 2006) and the survival of BCP treated worms was compared with that of untreated worms. In this study all the tested concentrations extended mean survival of wild-type worms in comparison to untreated control worms at 20 °C (Table 1, Fig. 2A). It was observed that the 50 µM concentration of BCP was the most effective for increasing the mean survival of worms by 22% ($P \leq 0.0001$). Treatment of worms with 50 µM BCP significantly increased mean lifespan by 11.85% ($P \leq 0.0001$) even when treatment is started during early adulthood (Table 1, Fig. 2B).

### 3.2. BCP mediated lifespan extension in C. elegans is independent of antibacterial effects

Bacterial proliferation contributes to late age mortality in *C. elegans* due to production of harmful metabolites (Garigan et al., 2002). To investigate the possibility of lifespan extension due to the antimicrobial effect of BCP, an antimicrobial assay was performed in order to study the effect of 50 µM BCP on bacterial proliferation (Suryanto et al., 2012).The indicated dose of BCP does not effect bacterial proliferation as no zone of inhibition was observed on BCP treatment (Fig. 3A), suggesting that lifespan extension by BCP is independent of its antimicrobial activity. In order to validate the absence of antibacterial effects, worms were fed with heat-killed (non-proliferating) *E. coli* OP50 bacteria in absence or presence of BCP. We observed heat killed *E. coli* OP50

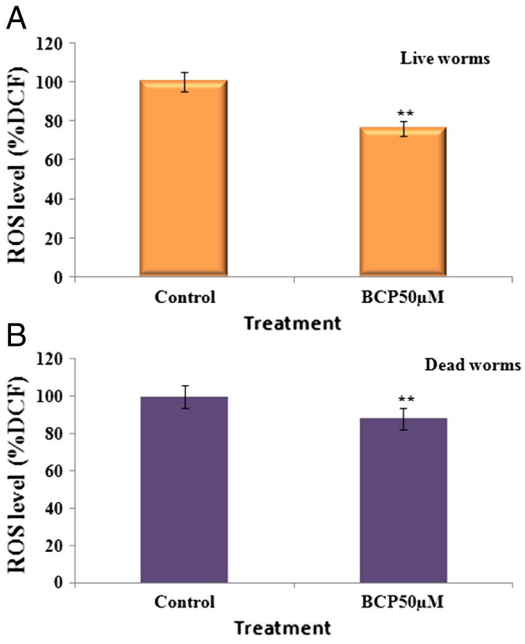


**Fig. 6.** A) Effect of BCP (50 µM) on ROS levels in *C. elegans*. The age synchronized treated and non-treated N2 worms were used to measure intracellular ROS. A) The day 4 adult treated and non-treated live worms were subjected to ROS level detection using DCF-DA dye using a 96-well microplate reader at 37 °C and B) the day 4 adult treated/untreated worms were homogenized and ROS levels were detected using DCF-DA dye using a 96-well microplate reader at 37 °C. The graph was plotted as relative change in ROS compared to control at 100%. The data is statistically analyzed using ANOVA (Duncan test) in ASSISTAT 7.6 beta statistical assistance software. Differences between the data were considered significant at $P \leq 0.05$. Error bars represent mean ± S.E.M. $^{**}P \leq 0.001$.

increased the mean lifespan from 18.01 days to 28.45 days in the absence of BCP, whereas 50 μM BCP further enhanced mean lifespan significantly by 6–7% and mean lifespan of 30.65 days ($P \leq 0.0001$) was observed in presence of BCP (Fig. 3B; Table. 1). Hence, as per our results the BCP mediated lifespan extension is not dependent on the antimicrobial properties of BCP.

### 3.3. BCP alters the pharyngeal pumping rate, and body size but not reproduction in C. elegans

The rate of pharyngeal contraction and relaxation is one of the morphometric parameter in aging studies and decline in pumping rate can induce dietary restriction (DR) like effects in worms (Powolny et al., 2011). To evaluate the possibility of DR, the contraction and relaxation of pharyngeal bulb was recorded for every 20 s in day 5 and day 10 worms. We observed a significant decline in pharyngeal pumping rate in BCP (50 μM) treated worms ($P \leq 0.001$) at day 5 and day 10 in comparison to untreated worms, indicating that BCP likely effects feeding behavior and induces DR like effects in *C. elegans* (Fig. 4A).

An enhancement in lifespan and stress tolerance is often associated with reduction in fecundity and growth (Kirkwood, 1977; Partridge et al., 2005). To study the effect of BCP on growth in *C. elegans*, we measured the body length of day-1, day-2, day-5 and day-10 treated (BCP 50 μM) and untreated N2 worms. Results indicated insignificant differences in the body size of day 1 ($P = 0.14$), day 2 ($P = 0.18$) and significant differences in the body size of day 5 ($P = 0.0213$) and day 10 ($P = 0.0018$) treated and untreated worms (Fig. 4B).

In order to evaluate whether BCP effects *C. elegans* reproduction, we measured effect of BCP on progeny production and reproduction in *C. elegans*. We observed non-significant variation in progeny production between untreated and 50 μM BCP treated worms. Thus, BCP does not likely impact reproduction in *C. elegans* (Fig. 4C). The observations signify that BCP effects feeding behavior, altering growth in *C. elegans* and causing DR like behavior without effecting fecundity.

### 3.4. BCP accelerates stress resistance in C. elegans

To evaluate longevity promoting stress resistance ability of BCP, the worms were individually exposed to oxidative and thermal stressed conditions. Age synchronized L1 wild type worms, pre-treated with 50 μM BCP for 72 h at 20 °C were exposed to 250 μM juglone (a redox cycler) that generates intracellular ROS causing oxidative stress and were incubated for 5 h (De Castro et al., 2004). The results suggested that percentage survival in BCP pre-treated worms was approximately 80% ($P \leq 0.001$) and 46% in untreated control worms (Fig. 5A). Previously, it has been established that longevity is closely associated with increase in oxidative and thermal stress resistance (Lithgow et al., 1995; Muñoz and Riddle, 2003). For the thermotolerance assay, pre-treated adult day 2 wild-type worms were exposed to thermal stress at 35 °C for 4 h and evaluated for percentage survival. It was found that BCP treatment significantly enhanced tolerance against thermal stress and the percentage mean survival was increased by 9.9% ($P \leq 0.0001$) in treated worms as compared to untreated worms (Fig. 5B). Furthermore, for assessing worm survival under constant thermal stress worms were subjected to constant heat stress for 7 h at 35 °C and percentage survival was evaluated. BCP treatment was found to enhance % survival by 15.95% ($P \leq 0.001$) in comparison to untreated control (Fig. 5C).

### 3.5. BCP treatment attenuates intracellular ROS levels in C. elegans

We evaluated the reduction in age-related cellular damage due to ROS scavenging activity of BCP in wild-type N2 worms. The accumulation of intracellular ROS causes decline in cellular health as postulated by free radical theory (Harman, 1956). We observed intracellular ROS levels in BCP (50 μM) treated and untreated day 4 worms. Results indicated that BCP could reduce age-related oxidative damage. A significant reduction ($P \leq 0.001$) in intracellular ROS was observed in treated worms as compared to untreated worms suggesting that BCP can delay aging by reducing intracellular ROS levels (Fig. 6A & B).

### 3.6. BCP reduces lipofuscin level in C. elegans

Intracellular lipofuscin level is one of the prominent markers of aging and age-related cellular damage. Lipofuscin is an endogenous intestinal autofluorescent protein that accumulates with aging in *C. elegans* (Brunk and Terman, 2002). Worms treated with BCP (50 μM) exhibited reduced intestinal lipofuscin approximately by 20% ($P \leq 0.01$) as compared to untreated worms (Fig. 7A & B).

### 3.7. Molecular docking reveals the highest binding affinity of BCP for SKN-1

Phytomolecules are known to interact with multiple cellular signaling pathways modulating lifespan in *C. elegans*. Thus, in order to predict possible biological molecular targets for BCP, we used molecular docking

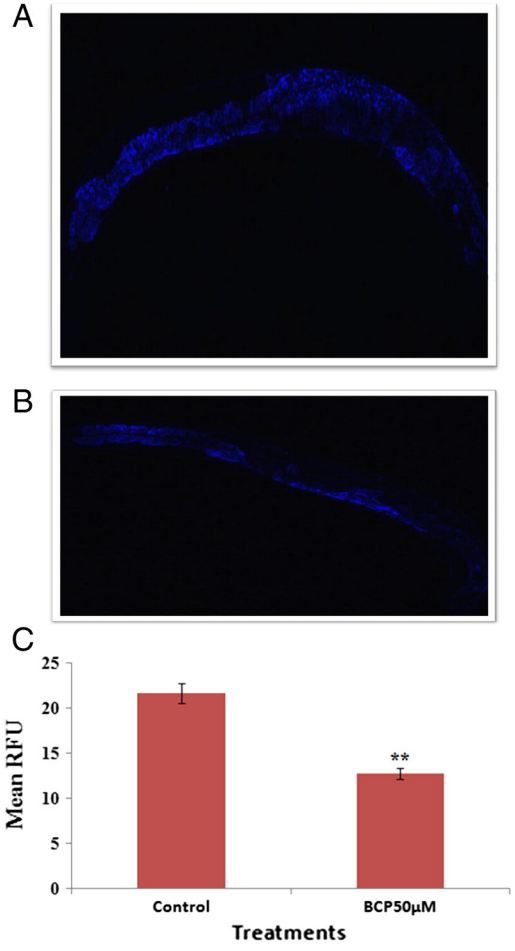


**Fig. 7.** The BCP 50 μM treatment significantly reduced intestinal auto-fluorescence due to lipofuscin. The auto-fluorescence of lipofuscin of randomly selected 20 treated/untreated worms were captured using DAPI filter (with excitation at 340–380 nm and emission at 435–485 nm) with the DMI 3000 B Leica fluorescence microscope (Leica, Wetzlar, Germany). A) Microphotograph captured on day 4 of untreated control worms. B) Microphotograph captured on day 4 of BCP (50 μM) treated worms. BCP (50 μM) treated day 4 adult worms demonstrated reduced lipofuscin auto-fluorescence compared to control. The lipofuscin level was quantified by determining the average pixel intensity in each worm using Image-J software (NIH). The experiment was repeated three times and images were captured using a 20× objective. The data is statistically analyzed using ANOVA (Duncan test) in ASSISTAT 7.6 beta statistical assistance software. Differences between the data were considered significant at $P \leq 0.05$. Error bars represent mean ± S.E.M. $^{**}P \leq 0.001$.

approaches and BCP was found to have interaction with SIR-2.1, SKN-1 and DAF-16. The 3D structure details of proteins provide good insights into their molecular functions. The I-TASSER or Zhang-Server (3D structure modeling server) is ranked as the best server for protein structure prediction in recent CASP7, CASP8, CASP9, and CASP10 experiments. The program uses restraints from the protein templates identified by the multiple threading programs to build the full length protein model using replica-exchange Monte-Carlo simulations. SIR-2.1 protein was modeled by the I-TASSER using restraints from the PDB templates 4I5IA, 1J8FA, 4IF6A, 4KXQA while the SKN-1 used restrains from PDBs 3CHNS, 2OCWA, 3CM9S, 2QZVA, 2OCWA, 2LZ1A, 4BTGA, 1SKNA, 1ZLGA. Similarly, the DAF-16 model was built using the following PDB templates; 3CHNS, 3CM9S, 2OCWA, 2K86A, 1VTNC, 1E17A, 1W0RA, 2K86A. TM-score, a proposed scale for measuring the structural similarity between two structures was taken into consideration to check the topology of the theoretical models (Zhang and Skolnick, 2004). TM-score varies from 0 to 1 and <0.17 means a random similarity. In our study, we observed that all the models showed the TM-scores above this threshold value (SIR-2.1: 0.37 ± 0.13, SKN-1: 0.48 ± 0.15, and DAF-16: 0.68 ± 0.12), reflecting the reliability of the models for further analysis. The KoBaMIN refinement program that uses knowledge-based potential energy function was used for further refinement of I-TASSER produced SKN-1, SIR-2.1 and DAF-16 protein structures. A consensus approach (COACH) that combines the prediction results of other LBS tools including TM-SITE, S-SITE, COFACTOR, FINDSITE and ConCavity by the support vector machine training, was preferred to explore the possible binding sites of the refined structures of the SKN-1, SIR-2.1, and DAF-16 proteins. C-score, confidence score of the predicted binding site was chosen as the criterion for the active site selection. C-score has its range from [0–1], where a higher score indicates a more reliable prediction. The best ranked active site of the SIR-2.1 by COACH with a C-score of 0.53 was used as a binding spot for the docking of BCP. For SKN-1 and DAF-16, S-SITE, a template-based method, which detects protein templates and the LBSs using binding site specific, sequence profile–profile comparisons, was found to perform well than the other methods, resulting in the active site prediction with C-score of 0.33 and 0.36. Moreover, this prediction method also showed the possibility of poly-nucleotide binding within such active sites. Since SKN-1 and DAF-16 are the transcription factors with the ability to bind the DNA, these active sites were therefore chosen as the appropriate sites for BCP docking experiments.

To calculate the binding affinity of BCP with the SKN-1, SIR-2.1, and DAF-16 proteins, we conducted docking studies using the LibDock program and the PLP1 score was used as a scoring function to assess BCP binding. Using the BEST Conformation Method, the program generated 11 lowest energy conformers of BCP and the docking of BCP into the binding sites of SKN-1, SIR-2.1, and DAF-16 resulted in 20, 7 and 5 BCP poses respectively. The PLP1 scores of the observed poses are presented in Table 3 (Fig. 13). It is clear from the data summarized in Table 2 that SKN-1 has better docking score (−PLP1 Score: 48.29) than SIR-2.1 (−PLP1 Score: 46.3), followed by DAF-16 (−PLP1 Score: 45.18).

### 3.8. Lifespan extension by BCP requires multiple cellular pathways

Multiple cellular pathways that extend lifespan are well established in *C. elegans*. Discovery of single gene mutation in insulin growth factor (IIS) in *C. elegans* has led to the identification of various involved genes which modulate lifespan (Kenyon et al., 1993). Apart from the insulin

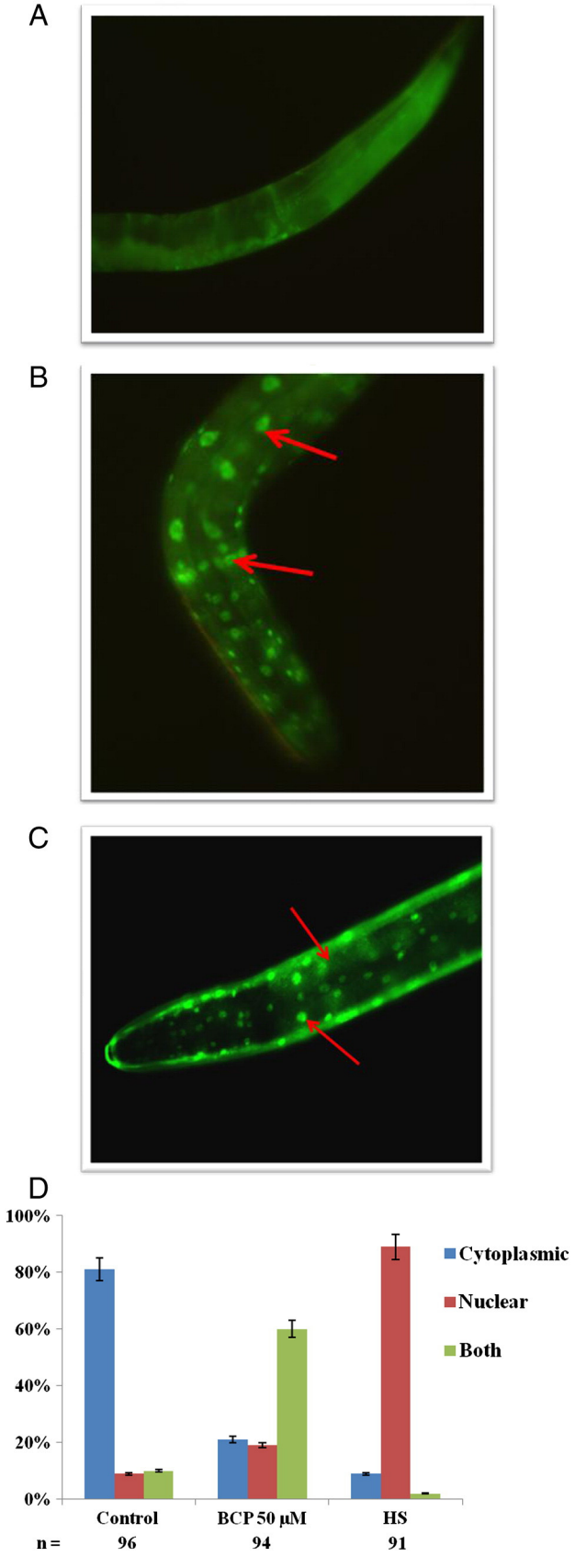


**Fig. 8.** Effect of BCP 50 µM on DAF-16 nuclear localization. Age-synchronized L1 TJ356 worms were continuously exposed to BCP 50 µM for 72 h. Worms that underwent a small heat shock at 37 °C for 20 min were used as positive controls. Supplementation of BCP 50 µM resulted in nuclear translocation of DAF-16. Worms were mounted on a 3% agarose pad on a glass slide and the subcellular (nuclear, cytoplasmic, and both (nuclear–cytoplasmic) localization of DAF-16 was analyzed under a fluorescence microscope. A) Control *daf-16*::gfp transgenic strain of *C. elegans*. (B) Heat shock (HS) mediated nuclear translocation of DAF-16. C) BCP 50 µM treated worm. D) Quantification of the DAF-16::GFP localization. The results are presented as mean ± S.E.M. of the percentages of DAF-16 localization. The experiment was repeated three times and images were captured using a 20× objective. n, number of worms imaged. The experiment was repeated three times. The data is statistically analyzed using ANOVA (Duncan test) in ASSISTAT 7.6 beta statistical assistance software. Differences between the data were considered significant at $P \leq 0.05$. Error bars represent mean ± S.E.M.

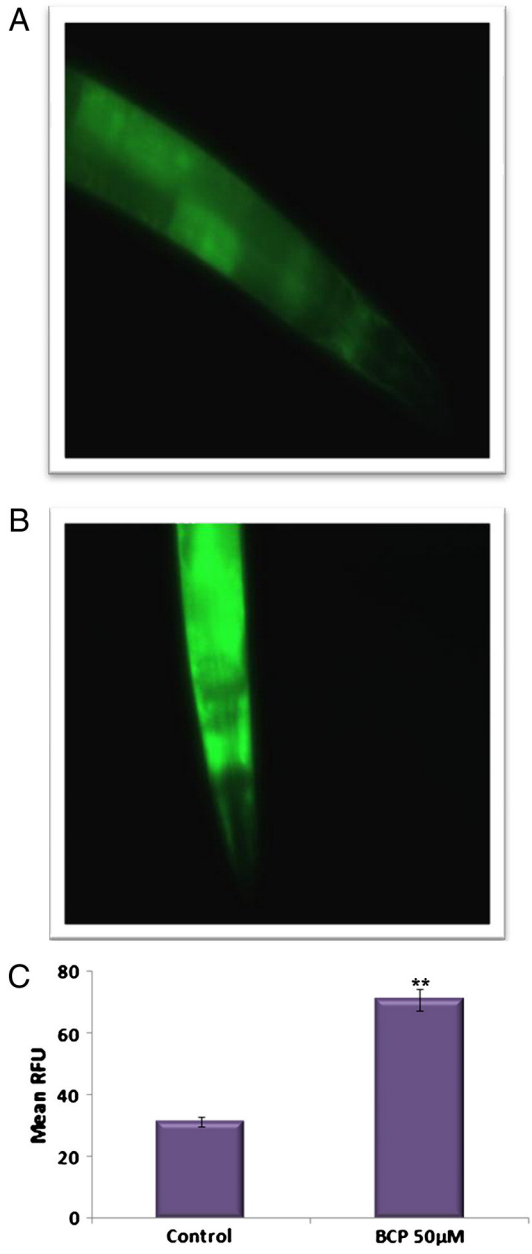


**Fig. 9.** Effect of BCP 50 μM on GST-4 expression. The age-synchronized L1 CL2166 *gst-4*::gfp (*dvls19*) transgenic strains were continuously exposed to BCP 50 μM for 72 h. Supplementation of BCP 50 μM resulted in enhanced expression of GST-4. Worms that were mounted on a 3% agarose pad on a glass slide were analyzed under a fluorescence microscope. BCP supplementation enhanced the expression of GST-4. A) Control *gst-4*::gfp (n = 99). B) BCP 50 μM treated *gst-4*::gfp (n = 96). C) Quantification of the *gst-4*::gfp expression. The results are presented as mean ± 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. The data is statistically analyzed using ANOVA (Duncan test) in ASSISTAT 7.6 beta statistical assistance software. Differences between the data were considered significant at $P \leq 0.05$. Error bars represent mean ± S.E.M. $^{**}P \leq 0.001$.

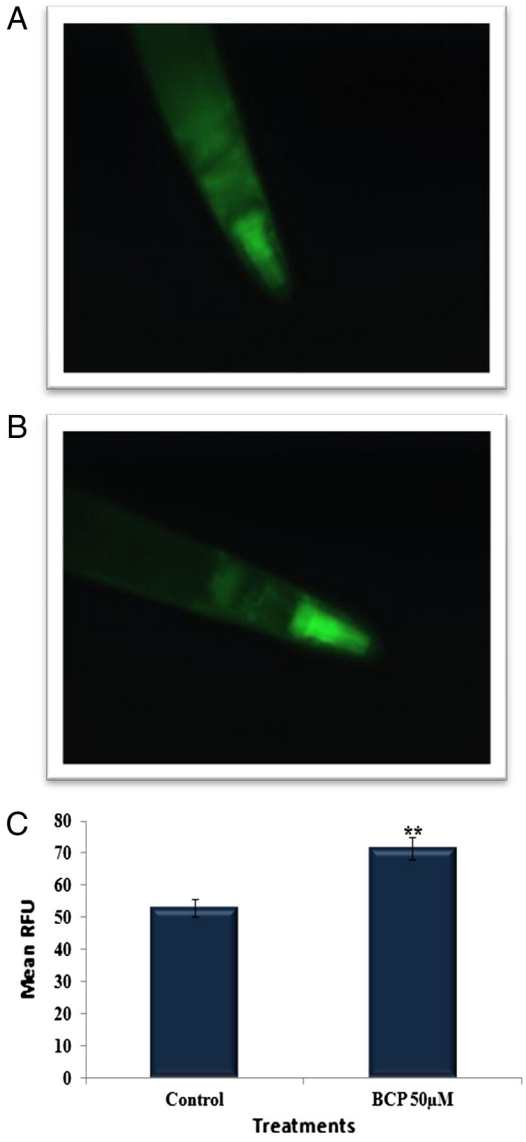


**Fig. 10.** Effect of BCP 50 μM on SOD-3 expression. The age-synchronized L1 CF1553, *muls84* (pAD76 (*sod-3*::gfp)) transgenic strains were continuously exposed to BCP 50 μM for 72 h. Supplementation of BCP 50 μM resulted in enhanced expression of SOD-3. Worms that were mounted on a 3% agarose pad on a glass slide were analyzed under a fluorescence microscope. BCP supplementation enhanced expression of SOD-3. A) Control *sod3*::gfp (n = 96) transgenic strain of *C. elegans*. B) BCP 50 μM treated *sod3*::gfp transgenic strain (n = 90). C) Quantification of the *sod3*::gfp expression. The results are presented as mean ± S.E.M. of the relative fluorescence intensity. The experiment was repeated three times and images were captured using a 20× objective. n, number of worms imaged. The data is statistically analyzed using ANOVA (Duncan test) in ASSISTAT 7.6 beta statistical assistance software. Differences between the data were considered significant at $P \leq 0.05$. Error bars represent mean ± S.E.M. $^{**}P \leq 0.001$.

signaling pathway there are various parallel pathways that control longevity in *C. elegans*. Caloric restriction (Lakowski and Hekimi, 1998), anti-oxidative and oxidative stress pathways are well known to alter lifespan in *C. elegans* (Baumeister et al., 2006; Honda and Honda, 1999). The gene mutants for these pathways were used for identifying the mechanism of longevity action of BCP in *C. elegans*, as a specific pathway is required for lifespan extension if the treatment is unable to prolong lifespan in that mutant.

To elucidate the mode of action of BCP, we examined the lifespan of different mutants viz. *daf-16* (*mgDf50*), *eat-2* (*ad1116*), *sir-2.1* (*ok434*), *skn-1* (*zu67*) and *mev-1* (*kn1*). DAF-16 is a major transcription factor that modulates stress resistance and longevity in *C. elegans* (Murphy et al., 2003). BCP treatment extended lifespan by 6.3% ($P = 0.006$) in *daf-16* mutants as compared to untreated worms (Table 2, Fig. 8A, B & C). A marginal percentage increase was observed indicating partial involvement of insulin signaling pathway in lifespan extension. In addition to *daf-16* mutants, we evaluated the effect of BCP on mutant for anti-oxidative enzyme MEV-1. *mev-1* mutants have a mutation in the cytochrome b subunit of complex II and have a short lifespan (Ishii et al., 1998). Overproduction of ROS enhances oxidative stress limiting lifespan. We observed significant increase in mean lifespan in BCP treated worms by 11.31% ($P \leq 0.0001$) indicating reduction in oxidative stress in treated worms as compared to untreated worms and involvement of

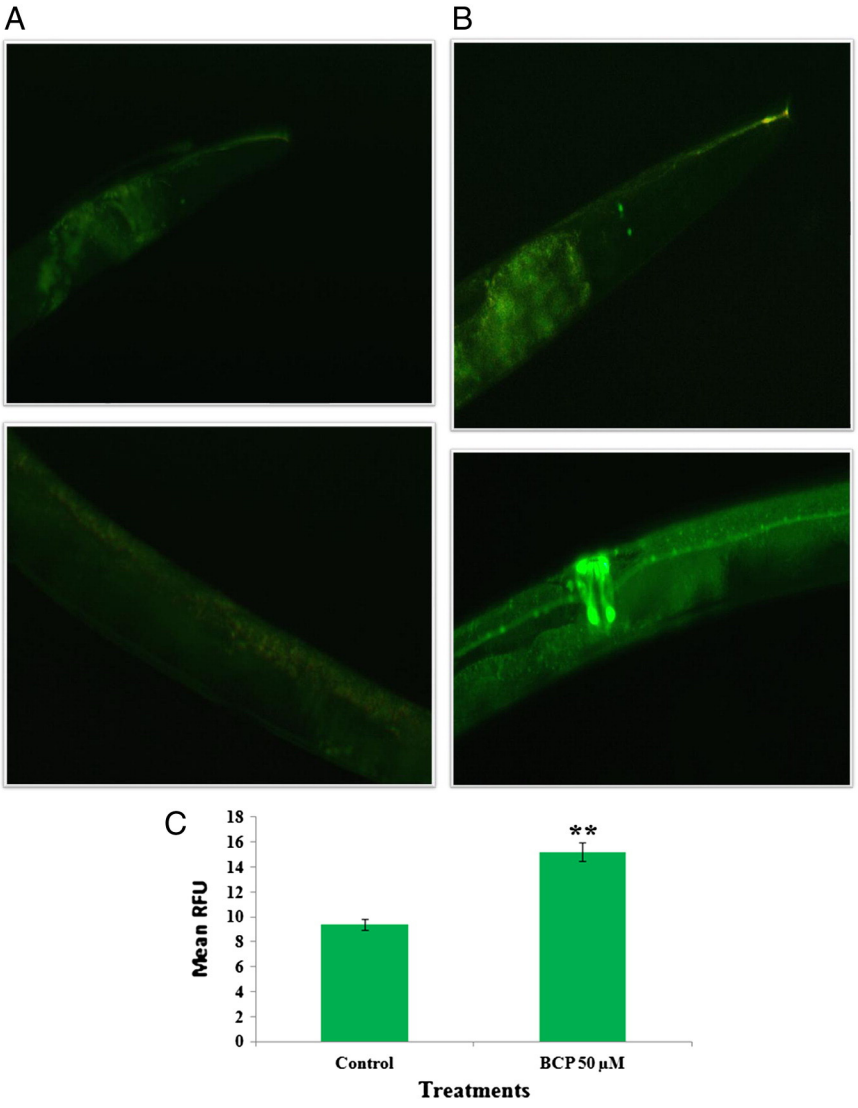


**Fig. 11.** Effect of BCP 50 µM on SKN-1 expression. The age-synchronized L1 ldIs7 [skn-1B/C::GFP+pRF4(rol-6(su1006))] transgenic strains were continuously exposed to BCP 50 µM for 72 h. Supplementation of BCP 50 µM resulted in enhanced expression of SKN-1 in both tissues i.e. neurons and intestine. Worms were mounted on a 3% agarose pad on a glass slide was analyzed under a fluorescence microscope. BCP supplementation enhanced expression of SKN-1. A) Control *skn-1*::gfp (n = 96) transgenic strain of *C. elegans* (photomicrograph of head and intestinal region). B) BCP 50 µM treated *skn-1*::gfp transgenic strain (n = 90) (photomicrograph of head and intestinal region). C) Quantification of the *skn-1*::gfp expression. The results are presented as mean ± S.E.M. of the relative fluorescence intensity. The experiment was repeated three times and images were captured using a 20× objective. n, number of worms imaged. The data is statistically analyzed using ANOVA (Duncan test) in ASSISTAT 7.6 beta statistical assistance software. Differences between the data were considered significant at $P \leq 0.05$. Error bars represent mean ± S.E.M. $^{**}P \leq 0.001$.

endogenous detoxification pathway in lifespan extension (Table 2, Fig. 2C). Non-significant lifespan extension was observed in *sir-2.1* ($P = 0.58$) and *skn-1* ($P = 0.50$) and a slight increase in mean lifespan is observed in *eat-2* ($P = 0.65$) mutant (Fig. 2E, F, G) mutants indicating involvement of DR and oxidative stress resistance pathway (Table 2).

BCP treatment does not augment lifespan in *sir-2.1* and *skn-1* mutants and extends lifespan in *eat-2* marginally; suggesting the involvement of these genes in BCP mediated lifespan extension.

Further, involvement of insulin signaling in lifespan extension mediated by BCP was also investigated by using the TJ356 transgenic strain carrying a DAF-16::GFP reporter gene (Lee et al., 2001). The nuclear localization of DAF-16 is essential for its transcriptional activity which is induced by thermal and oxidative stress. A short heat shock at 37 °C resulted in approximately 100% nuclear localization of DAF-16. We evaluated the nuclear localization of DAF-16 on treatment with BCP without heat shock which resulted in partial nuclear localization (20%) indicating only a minor involvement of the insulin signaling pathway. The results are also in accordance with the mean lifespan of the *daf-16* mutant (Fig. 8A, B & C).

### 3.9. BCP enhanced expression of SOD-3, SKN-1 and GST-4 in transgenic strain of C. elegans

As aforementioned, the increased ROS scavenging and lifespan extension activity in BCP treated *mev-1* mutant attributes to its high antioxidative property. For further validation, we evaluated the effect of BCP on transgenic strains, CF1553 (*sod-3*::gfp), ldIs7 [skn-1B/C::GFP+pRF4(rol-6(su1006))] and CL2166 (*gst-4*::gfp). SOD-3 is an antioxidative enzyme which is induced in response to oxidative stress and its expression is enhanced in variety of long-lived mutants of *C. elegans* (Honda and Honda, 1999). It was observed that BCP (50 µM) treatment enhanced SOD-3 expression ($P \leq 0.001$) in transgenic strain CF1553 (*sod-3*::gfp) as compared to untreated control worms (Fig. 10A, B & C). The skn-1/Nrf-2 transcription factor is expressed in both the worm intestine and the ASI neurons mediating longevity in worms (An and Blackwell, 2003; Bishop and Guarente, 2007). The localization in ASI neuron is essential for DR mediated longevity and expression in intestine is essential for the induction of the phase II detoxification pathway. We evaluated the effect of BCP (50 µM) on SKN-1 localization and we observed induction of GFP

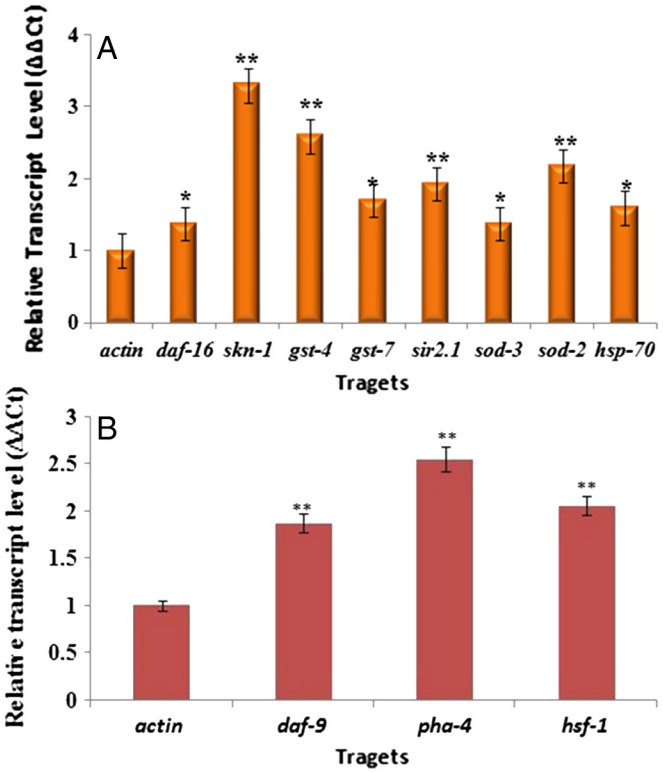


**Fig. 12.** A) Influence of BCP 50 μM treatment on expression level of stress and longevity promoting gene *daf-16*, *skn-1*, *sir2.1*, *sod-3*, *sod-2*, *hsp-70*, *gst-4* and *gst-7*. B) Influence of BCP 50 μM treatment on expression level of stress and longevity promoting gene *daf-9*, *pha-4* and *hsf-1*. The housekeeping gene *β-actin* (*act-1*) was used as endogenous control and the relative expression level of gene observed through real-time PCR using comparative $C_t$ ($\Delta\Delta C_t$) method. Actin-1 was taken as an internal control. The experiment was repeated three times. The data is statistically analyzed using ANOVA using Duncan test in ASSISTAT 7.6 beta statistical assistance software. Differences between the data were considered significant at $P \leq 0.05$. Error bars represent mean ± S.E.M. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

expression in ASI neuron as well as intestinal nuclei in response to BCP (50 μM) treatment (Fig. 11A, B & C). Our results suggest that BCP treatment induces SKN-1 mediated DR response as well as induction of phase II detoxification. Furthermore, to validate the role of BCP in mediating oxidative stress response, we investigated the effect of BCP on GST-4 expression because it is a major phase II detoxification enzyme regulated by SKN-1 in response to oxidative stress. BCP (50 μM) was found to enhance the expression of phase-II detoxification enzyme GST-4 ($P \leq 0.001$), which is a known molecular marker for intracellular oxidative stress affecting cellular signaling (Kampkötter et al., 2008). The expression of GST was enhanced in BCP (50 μM) treated worms as compared to untreated control worms (Fig. 9A, B & C).

### 3.10. BCP augments mRNA expression of anti-aging genes

Previous studies suggest that there are number of longevity pathways that modulate lifespan in *C. elegans* viz. insulin/IGF-1 signaling (IIS), caloric restriction (Lee et al., 2002), and mitochondrial respiration (Chang and Min, 2002). *daf-16*, *sod-3*, *sod-2*, *sir-2.1*, *hsp-70*, *gst-4*, *gst-7*, *skn-1*, *hsf-1*, *pha-4* and *daf-9* are the major genes that regulate multiple cellular pathways associated with longevity. *sod-3* and *sod-2* are mitochondrial anti-oxidant genes that regulate expression of enzyme superoxide dismutase which scavenge ROS and reduce oxidative stress, thereby promoting lifespan extension. In *C. elegans*, *sir-2.1* encodes a histone deacetylase-like protein that brings metabolic status together with lifespan (Chang and Min, 2002). To evaluate the association of selected stress responses and genes relevant with BCP mediated lifespan extension, real time quantification for longevity promoting genes viz. *daf-16*, *sod-3*, *sod-2*, *hsp-70*, *sir-2.1*, *skn-1*, *gst-4*, *gst-7*, *hsf-1*, *pha-4* and *daf-9* was performed (Fig. 12A & B). Our results suggest that BCP treatment significantly upregulated mRNA expression of *daf-16* (1.37-fold, $P = 0.021$), *sod-2* (2.18-fold, $P \leq 0.01$), *sod-3* (1.37-fold, $P = 0.033$), *hsp-70* (1.60-fold, $P = 0.02$), *sir-2.1* (1.93-fold, $P \leq 0.001$), *skn-1* (3.3-fold, $P \leq 0.001$), *gst-4* (2.5 fold, $P \leq 0.001$), *gst-7* (1.7-fold, $P = 0.042$). Whereas, DAF-9, a cytochrome P450 which belongs to the CYP2 class, resembles steroidogenic and fatty acid hydroxylases, as well as xenobiotic detoxifying enzymes (Gerisch et al., 2001; Jia et al., 2002) is upregulated by BCP treatment by (1.87 fold, $P \leq 0.001$) suggesting induction of phase I detoxification in response to hormetic stress mediated by BCP. BCP significantly enhanced expression of PHA-4, a FOX a transcription factor (2.55 fold, $P \leq 0.001$) and *hsf-1* (2.06 fold, $P \leq 0.001$) essentially required for DR mediated lifespan extension and for longevity phenotype exhibited by *eat-2* mutants. The upregulation of *sod-3*, *daf-16*, *skn-1* and *gst-4* is consistent with DAF16: :GFP, SOD-3: :GFP, SKN-1: :GFP and GST4: :GFP reporters. The increased expression of *hsp-70* and *hsf-1* is in accordance with thermotolerance shown by BCP treated N2 worm. Additionally, *sir-2.1* and *skn-1* upregulation validates the finding of no observable change in the mean lifespan of *sir-2.1* and *skn-1* mutants.

## 4. Discussion

The present study elucidates the lifespan modulating potential of a bicyclic sesquiterpene BCP, found as an active ingredient of edible essential oils and spices. The present study for the first time reports the stress modulating and anti-aging effects of BCP. Previously, antioxidant and anti-cancerous activities have been studied (Calleja et al., 2013; Legault and Pichette, 2007). Most of the knowledge about interaction and effect of this sesquiterpene from different *in vitro* and *in vivo* studies, provide a restricted insight for the interaction of BCP with living systems. Therefore, we studied the interaction of BCP with the *C. elegans* model system and its effect on longevity. Earlier this model system has been used for studying anti-aging and stress modulating effects of natural compounds. A variety of natural bio-active phytomolecules (quercetin, epigallocatechin gallate, reservatol) are well reported for enhancing stress resistance and longevity in *C. elegans* (Abbas and Wink, 2009; Kampkötter et al., 2008; Valenzano et al., 2006). Majorly these compounds are anti-oxidative in nature and are able to scavenge intracellular ROS, maintaining cellular redox homeostasis. These studies motivated us to study the stress modulating and anti-aging effects of BCP in *C. elegans*. In our experiment, we demonstrated that the 50 μM concentration of BCP significantly increased mean survival ($P \leq 0.0001$) in N2 wild type worms under normal culture conditions at 20 °C as well as in the case of BCP (50 μM) treatment given during early adulthood (Table 1, Fig. 2B). The high intracellular ROS levels are the outcome of various metabolic activities. It has also been reported that longevity and stress resistance are interrelated phenomenon (Lithgow and Walker, 2002). In the present study, the lifespan modulation was found to be associated with stress resistance as BCP treated worms were also resistant to juglone ($P = 0.0051$) and heat ($P \leq 0.0001$) as compared to untreated worms (Fig. 5A, B &C). Similar results have been observed by previous workers for different phytomolecules (Shukla et al., 2012; Surco-Laos et al., 2012).

Age-related disorders are followed by enhanced oxidative stress and according to free radical theory, the increase in oxidative stress decreases lifespan or vice versa. The antioxidant enzymes like superoxide dismutase and glutathione peroxidase scavenge intracellular ROS and balance oxidative stress in living systems (Honda and Honda, 1999; Lee et al., 2001). Aging marks an increase in ROS levels and oxidative stress which leads to several biological disorders. Similar to the previous findings, we also observed a significant decrease in intracellular ROS levels in BCP treated worms (Brown et al., 2006). The reduction in ROS levels can be assumed to be the chief reason behind oxidative stress resistance and lifespan extension. In addition to the ROS scavenging effect of BCP, we observed that BCP extended percentage mean survival

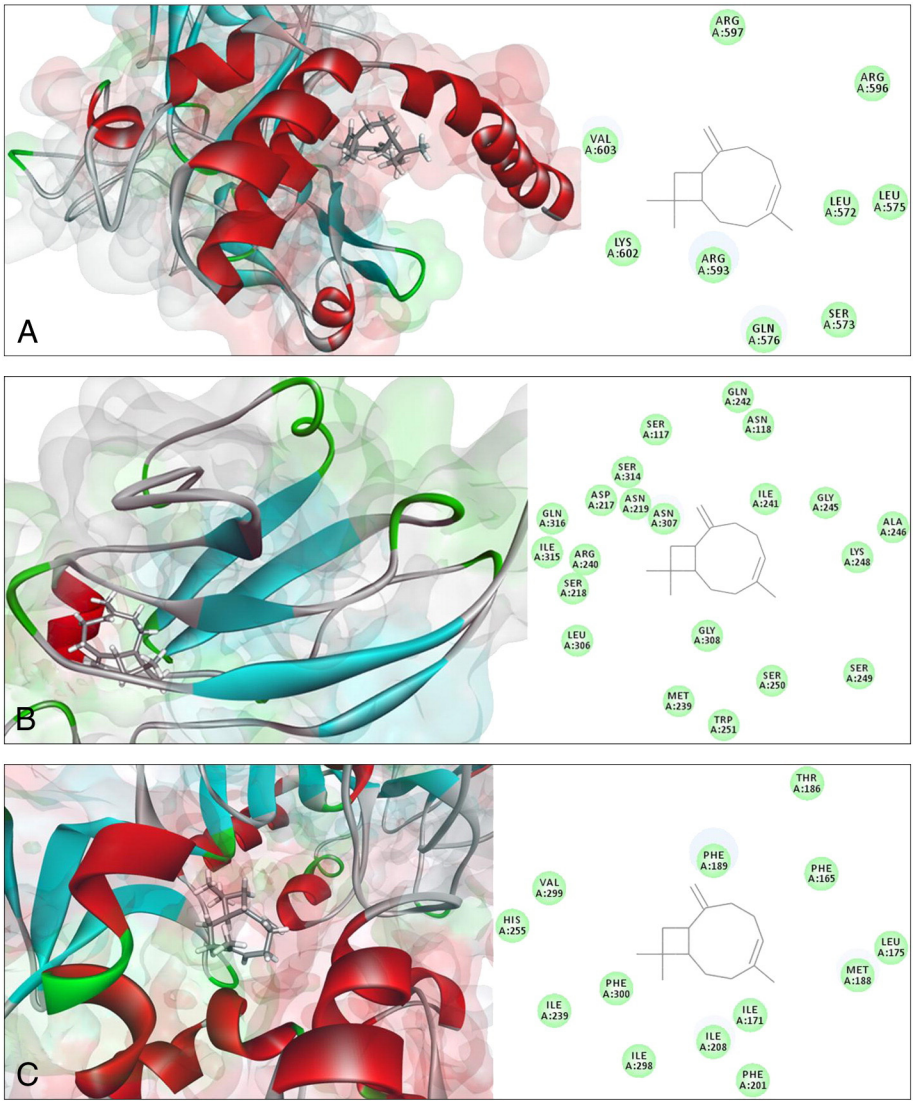


**Fig. 13.** Docked conformation of top ranked poses of BCP with (A) SKN-1, (B) DAF-16, and (C) SIR-2.1. The interaction of the important residues of the selected proteins with the BCP is also shown.

by 11.31% ($P \leq 0.0001$) in oxidative stress prone mutant *mev-1* (Table 2, Fig. 2C). The *mev-1* deletion mutant is hypersensitive to oxidative stress as there is 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). The study suggests that the antioxidative property of BCP is attributed to enhanced survival in the *mev-1* mutant. Furthermore, effect of BCP treatment on lipofuscin level in worms was observed. Lipofuscin is a product of lipid peroxidation, which constitutes protein residues cross linked due to an iron catalyzed oxidative process (Moldovan and Moldovan, 2004) and is fluorescent in nature. The lipofuscin level begins to build up at initial stages in worms but it is accelerated with age and serves as a marker of aging. We observed a reduction in lipofuscin level in BCP treated worms as compared to control (Fig. 7) indicating its contribution to longevity in BCP treated worms.

For predicting possible biological molecular targets for BCP molecular docking approaches was used. The interaction of BCP with SIR-2.1, SKN-1 and DAF-16 was studied *in silico*. Usually, ligand efficacy is ranked according to the estimated free energy of binding with the particular receptor, and molecular docking is commonly used for this purpose. Therefore to calculate the binding affinity of BCP with the SKN-1, SIR-2.1, and DAF-16 proteins, we conducted docking studies using the LibDock program and the PLP1 score was used as a scoring function to assess the BCP binding. Using the BEST Conformation Method, the program generated 11 lowest energy conformers of BCP and the docking of BCP into the binding sites of SKN-1, SIR-2.1, and DAF-16 resulted in 20, 7 and 5 BCP poses respectively. The PLP1 scores of the observed poses are presented in Table 3. It is clear from the data summarized in Table 3 that SKN-1 has better docking score (−PLP1 Score: 48.29) than SIR-2.1 (−PLP1 Score: 46.3), followed by DAF-16 (−PLP1 Score: 45.18). The best binding pose of the BCP (highest PLP1 scores) for the selected proteins is depicted in Fig. 13 and the interacting residues of the SKN-1, SIR-2.1, and DAF-16 receptors are also shown.

To further gain insights into the genetic mechanism underlying BCP mediated lifespan extension *daf-16*, *eat-2*, *sir-2.1* and *skn-1* mutants were screened. DAF-16 is a major transcription factor homologous to the mammalian FOXO family and has been characterized as a major regulator of longevity, oxidative and thermal stress. Previous studies suggest that compounds restricting the insulin signaling pathway and DAF-16 transcriptional activity are provocative contenders for pharmacological manipulation of lifespan (Gami and Wolkow, 2006). The subcellular distribution of this transcription factor and its activity is controlled by the insulin signaling pathway. Thus the effect of BCP on *daf-16* mutant was observed. BCP treatment enhanced mean lifespan

**Table 2**
Lifespan assay analysis: effect of BCP on mean lifespan of different mutants.

| Genotype | Treatment (μM) | Mean | ±S.D. | ±S.E. | Sample size | N (trial no.) | % change | *P*-value |
|---|---|---|---|---|---|---|---|---|
| *mev-1* (*kn1*) | Control | 11.58 | 2.46 | 0.191 | 165 | 1 | | |
| | BCP (50) | 12.89 | 5.20 | 0.39 | 177 | 1 | 11.31 | <0.0001** |
| | Control | 10.35 | 2.97 | 0.262 | 128 | 2 | | |
| | BCP (50) | 11.73 | 3.19 | 0.279 | 130 | 2 | 13.33 | <0.0001** |
| | Control | 11.82 | 3.19 | 0.25 | 162 | 3 | | |
| | BCP (50) | 13.54 | 3.34 | 0.264 | 160 | 3 | 14.55 | <0.0001** |
| *daf-16* (*mgDf50*) | Control | 15.1 | 3.83 | 0.325 | 138 | 1 | | |
| | BCP (50) | 16.16 | 4.15 | 0.364 | 129 | 1 | 7.01 | 0.053* |
| | Control | 15.12 | 3.94 | 0.211 | 348 | 2 | | |
| | BCP (50) | 16.08 | 3.91 | 0.217 | 321 | 2 | 6.3 | 0.006** |
| | Control | 14.78 | 2.14 | 0.167 | 164 | 3 | | |
| | BCP (50) | 15.69 | 3.30 | 0.264 | 156 | 3 | 6.15 | 0.0002** |
| *skn-1* (*zu 67*) | Control | 15.77 | 4.39 | 0.244 | 322 | 1 | | |
| | BCP (50) | 15.65 | 5.05 | 0.257 | 387 | 1 | 1 | 0.5001ns |
| | Control | 15.79 | 3.76 | 0.317 | 140 | 2 | | |
| | BCP (50) | 15.39 | 4.43 | 0.367 | 145 | 2 | 2.53 | 0.84ns |
| | Control | 15.38 | 1.88 | 0.182 | 106 | 3 | | |
| | BCP (50) | 15.29 | 3.34 | 0.304 | 120 | 3 | 0.5 | 0.26ns |
| *eat-2* (*ad1116*) | Control | 24.23 | 6.54 | 0.503 | 168 | 1 | | |
| | BCP (50) | 24.74 | 7.01 | 0.543 | 166 | 1 | 2.1 | 0.02* |
| | Control | 24.6 | 8.72 | 0.745 | 136 | 2 | | |
| | BCP (50) | 25.3 | 8.41 | 0.709 | 140 | 2 | 2.84 | 0.65ns |
| *sir-2.1* (*ok434*) | Control | 16.78 | 4.38 | 0.383 | 130 | 1 | | |
| | BCP (50) | 16.7 | 4.45 | 0.351 | 160 | 1 | 0.47 | 0.679ns |
| | Control | 16.5 | 4.30 | 0.374 | 132 | 2 | | |
| | BCP (50) | 16.61 | 4.51 | 0.36 | 156 | 2 | 0.66 | 0.581ns |

The mutant worms were treated with BCP (50 μM) from L1 stage until the completion of lifespan. The mean lifespan was calculated as the average number of days that the worms survived. The data were processed using the Kaplan–Meir survival analysis in Medcalc 12.7.7.0 software.
Mean ± S.E.M.
ns non-significant.
* $P < 0.05$.
** $P < 0.001$.

in *daf-16* mutant by 6.3% ($P = 0.006$) suggesting that the insulin signaling pathway might not be involved in lifespan augmentation by BCP. BCP treatment also resulted in nuclear translocation of DAF-16 but in comparison to stress mediated conditions the percentage localization was less, supporting partial involvement of DAF-16 in BCP mediated lifespan extension (Fig. 8A, B, C & D). The nuclear localization of DAF-16 is essential for expression of stress reducing proteins. *sod-3* is a well known target of DAF-16 and the expression of SOD-3 is upregulated in response to stress (McElwee et al., 2003). We observed upregulated SOD-3 protein expression in BCP treated *sod-3*::gfp transgenic lines as compared to untreated control ($P \leq 0.001$) which also suggests for the anti-oxidative effects of BCP (Fig. 10A,B & C).

**Table 3**
List of docked poses of BCP along with their PLP1 scoring function.

| S. No. | SKN-1 | | SIR2.1 | | DAF-16 | |
|---|---|---|---|---|---|---|
| | Pose number | −PLP1 Score | Pose number | −PLP1 score | Pose number | −PLP1 score |
| 1 | 3 | 48.29 | 1 | 46.3 | 1 | 45.18 |
| 2 | 4 | 46.23 | 4 | 40.6 | 2 | 40.56 |
| 3 | 2 | 45.56 | 3 | 40.37 | 3 | 38.98 |
| 4 | 1 | 45.07 | 2 | 40.32 | 4 | 34.68 |
| 5 | 7 | 44.56 | 5 | 38.67 | 5 | 31.92 |
| 6 | 8 | 43.55 | 6 | 38.13 | – | – |
| 7 | 9 | 42.32 | 7 | 35.96 | – | – |
| 8 | 6 | 42.27 | – | – | – | – |
| 9 | 10 | 41.91 | – | – | – | – |
| 10 | 5 | 40.14 | – | – | – | – |
| 11 | 12 | 38.77 | – | – | – | – |
| 12 | 11 | 37.34 | – | – | – | – |
| 13 | 15 | 34.26 | – | – | – | – |
| 14 | 14 | 32.57 | – | – | – | – |
| 15 | 19 | 28.29 | – | – | – | – |
| 16 | 13 | 27.69 | – | – | – | – |
| 17 | 17 | 27.14 | – | – | – | – |
| 18 | 16 | 26.92 | – | – | – | – |
| 19 | 20 | 23.34 | – | – | – | – |
| 20 | 18 | 23.25 | – | – | – | – |

Previous studies suggest that DR modulates lifespan in variety of model organisms including *C. elegans* (Walker and Gems, 2004). Nutrient restriction without causing malnutrition alleviates age related decline and promotes longevity in all species (Masoro, 2005). The decline in pharyngeal pumping rate causes reduction in food intake which leads to lifespan extension. BCP treated worms exhibited decline in pharyngeal pumping rate with progression in lifespan. Our results suggest that BCP treated worms induced DR like characteristics in *C. elegans* viz. extension in mean lifespan and decline in pharyngeal pumping rate (Fig. 4A). In addition to this BCP enhanced the mean lifespan marginally by approximately 2% in *eat-2* mutant ($P = 0.65$) which serves as a model for DR. The genetic mutation (*eat-2*) exhibits reduction in pharyngeal pumping rate of worms changing its feeding behavior (Lakowski and Hekimi, 1998). Fox a/PHA-4 is essential for longevity induced by *eat-2* mutation due to DR (Panowski et al., 2007). PHA-4, a homologue of mammalian Fox a protein is a member of FOXO family of transcription factor. PHA-4 is known to play important role in pharynx development and glucose metabolism regulation in worm. The upregulation of PHA-4 is specific for DR mediated lifespan extension. BCP treatment upregulated the expression of *pha-4* gene validating DR response mediated by BCP (Fig. 12B). This suggests that signaling pathways linked with DR could overlap or interact with pathways activated by BCP. Furthermore, BCP failed to alter mean lifespan in *sir-2.1* ($P = 0.58$) (Table 2). SIR-2.1, the histone deacetylase is widely known to modulate lifespan during DR and enhance stress resistance (Kenyon, 2010). The stimulation of heat shock response is necessary for maintaining cellular stress and SIR-2.1 is known to play key role in regulating expression of *hsp-70* (Raynes et al., 2012). Another transcription factor HSF-1 known to play key role in longevity mediated by DR is upregulated by BCP treatment. Thermotolerance is key feature of

DR response and *hsf-1* is essential for DR induced lifespan extension. Additionally, *hsf-1* and *hsp-70* upregulation supports our results where we demonstrated the thermotolerance by BCP treatment (Fig. 12A & B). Additionally, BCP failed to augment mean lifespan of *skn-1*($P = 0.50$) mutants suggesting its role in BCP mediated lifespan extension and oxidative stress resistance. Previous studies suggest that SKN-1 is the *C. elegans* homologue of the mammalian Nuclear Factor Erythroid-derived 2-Related Factor (Nrf2) which coordinates DR and oxidative stress response in worms (An and Blackwell, 2003; Oliveira et al., 2009; Park et al., 2009). SKN-1 has also been shown to play an important role in promoting longevity in worms. SKN-is known to enhance longevity under DR when it is functional in two ASI neurons (Bishop and Guarente, 2007) and phase II detoxification response when localizes in intestine (An and Blackwell, 2003). To evaluate whether SKN-1 acts in either the ASI neurons or the intestine to mediate BCP-induced lifespan extension, we subjected *skn*-1 (b/c) gfp tagged transgenic strain ldIs7 to BCP treatment and localization was found in both ASI neuron and intestine respectively (Fig. 11A, B & C). We observed expression of SKN-1 in both tissues. Our results are supported by similar findings observed as in case of worms treated with metformin where lifespan extension was found to be associated with *skn*-1 actions in both tissues (Onken and Driscoll, 2010). Altogether our data strongly suggests that BCP promotes longevity in *C. elegans* in a DR like manner, where PHA-4, HSF-1 SIR-2.1 and SKN-1 are playing key role.

In addition to the induction of DR response, BCP induces xenobiotic response thereby promoting lifespan extension in *C. elegans*. The phenomenon of hormesis is known to extend lifespan by upregulating distinct stress response genes. Eukaryotic system exhibit three phase detoxification defense system against toxic or reactive compounds (Sarkadi et al., 2006). This process also constitutes the major response to protective antioxidants including many natural compounds which hereby stimulate xenobiotic detoxification. The phase I comprises of enzymes such as Cytochrome P450s (CYPs) which solubilizes and modifies lipophilic endobiotics or xenobiotics allowing these compounds to be excreted. The Phase I detoxification may also produce damaging reactive compounds therefore, Phase II detoxification enzymes like GSTs defend cell against such compounds, as well as ROS. They encompass a diverse group of enzymes that metabolize free radicals and in phase III conjugated toxins are pumped out of cell by ATP-binding cassette (ABC) or other transporters (Sarkadi et al., 2006). To study the xenobiotic response induced by BCP, we evaluated the expression of *daf-9* gene encoding Cytochrome P450s (CYPs) Phase I detoxification enzyme in *C. elegans*. BCP treatment upregulated the expression of *daf-9* by 1.87 fold ($P \leq 0.001$) suggesting hormetic stress response is induced by BCP. The phenomenon of hormesis is known to extend lifespan by upregulating distinct stress response genes (Gems and Partridge, 2008; Jia et al., 2002). In addition to induction of phase I detoxification BCP may modulate the conserved oxidative stress response transcription factor SKN-1 as discussed earlier, which upon activation in intestine induces the phase II detoxification response and plays important roles in regulating multiple signaling pathways controlling lifespan in *C. elegans* (Oliveira et al., 2009). GST-4 is a phase II detoxification enzyme which encodes a glutathione –requiring prostaglandin D synthase, which is a well known target gene of SKN-1 transcription factor which mediates oxidative stress response in *C. elegans*. Consistent with the present findings, the *gst-4*::gfp transgene treated with BCP exhibited enhanced expression of GST-4 in comparison to untreated *gst-4*::gfp transgenic strain (Fig. 9A & B). The upregulation of GST-4 reduces oxidative stress in *C. elegans* and alters lifespan. Thus, modulation in lifespan and oxidative stress by BCP can be attributed to SKN-1 transcription factor. Simultaneously, real-time quantification of *daf-16*, *sod-3*, *sod-2*, *hsp-70*, *sir-2.1*, *skn-1*, *gst-4*, *gst-7*, *hsf-1*, *pha-4* and *daf-9* was also determined. The gene expression was found to be upregulated in all the genes, supporting the above findings (Fig. 12A & B). The upregulation of these genes significantly promotes stress resistance and longevity. BCP treatment enhanced the mean lifespan of *daf-16* mutant marginally and partial nuclear localization of *daf16*::gfp supported the partial involvement of *daf-16*. There is upregulation of *sod-3* suggesting that nuclear localization of DAF-16 alone is not sufficient for its transcriptional activity but intensive interaction of 14-3-3, the histone deacetylases SIR-2.1 proteins with DAF-16 is necessary for upregulation of SOD-3 and stress resistance as suggested in previous studies (Berdichevsky et al., 2006). The upregulation of *sod-2* and *sod-3* supports the antioxidant activity of BCP, reducing mitochondrial stress modulating lifespan. The induction in expression of *gst-4* can be attributed to upregulated expression of *skn-1* as suggested by previous studies (Park et al., 2009). The outcome of the present study is supported by previous findings where lifespan is modulated by upregulation of DR and stress responsive genes like *pha-4*, *skn-1*, *sir-2.1*, *hsf-1*, *daf-9* and *gst-4*. BCP mediates DR response and xenobiotic stress response thereby promoting lifespan extension. BCP modulates the function of SIR-2.1 and SKN-1 and regulates many downstream gene/s and pathways which can be subjected for future investigations.

## 5. Conclusion

The present experiment demonstrated the potential of BCP (50 μM) in modulating lifespan in the *C. elegans* model system under standard laboratory conditions. The study for the first time reports the ROS scavenging potential of BCP *in vivo* using *C. elegans*. BCP was able to modulate oxidative and thermal stress, balancing intracellular ROS thereby maintaining cellular redox homeostasis. The study demonstrates the involvement of various cellular signaling pathways including the major transcription factors viz. SKN-1, SIR-2.1, DAF-16, HSF-1, PHA-4 and upregulation of stress regulating target genes *sod-3*, *sod-2*, *gst-4*, *gst-7*, *hsp-70* and *daf-9*. This study for the first time reports the lifespan extending and stress modulating potential of BCP in the *C. elegans* model system.

## Conflict of interest

The authors have no conflicts of interests.

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