Iridoid Compound 10-*O-trans-p*-Coumaroylcatalpol Extends Longevity and Reduces Alpha Synuclein Aggregation in *Caenorhabditis elegans*

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Abstract: Aging, the major cause of several ailments has led to intense exploration of potential drugs that delay aging and its associated effects. We mined the information on traditional Indian medicines and identified an iridoid, 10-*O-trans-p*-Coumaroylcatalpol (OCC), a major ingredient of *Premna integrifolia* Linn. (syn: *Premna serratifolia*). OCC forms an important constituent of famous herbal formulation ''Dashmula'', a ten herb formulation, commonly used for its various medicinal properties. Employing model system *C. elegans,* the effect of OCC on life span, stress resistance, chemotaxis, the content of reactive oxygen species (ROS) and on the aggregation of alpha synuclein was studied. OCC extended the mean life span of nematodes, increased their tolerance against chemical induced stress, improved the chemotaxis index and reduced the ROS content. Further, the aggregation of Parkinson's disease (PD) associated protein, alpha synuclein (a-syn), was decreased when transgenic a-syn expressing worms were raised on OCC mixed diet. We extended the studies further to explore the possible genetic mechanism that mediates the observed effects of OCC. Employing the genetic knockout mutants TK22 [*mev-1(kn1)III]*; GR1307 *[daf-16(mgDf50)I]*; VC199 [*sir-2.1(ok434)IV]* and transgenic GFP expressing strain TJ356 [*zls356; DAF-16::GFP],* our studies revealed that the effects were mediated by *daf-16* and not by *sir-2.1* or *mev-1*. Our results indicate that OCC has the ability to ameliorate a-syn aggregation, reduce oxidative stress and promote longevity in *C. elegans via* activation of longevity promoting transcription factor DAF-16. Thus, OCC may serve as a lead compound of plant origin for important nutraceutical intervention against aging and age associated PD.

Keywords: 10-*O-trans-p*-Coumaroylcatalpol, aging, *Caenorhabditis elegans,* lifespan, Parkinson's disease, ROS.

INTRODUCTION

Aging and age-related cardiovascular and neurodegenerative diseases viz. Alzheimer's disease, Parkinson's disease have a world-wide increasing economic impact demanding a sincere effort for the development of novel remedies to delay or prevent the decline in tissue-function with age. Reactive oxygen species (ROS) are chemically active molecules able to damage physiologically vital macromolecules like DNA, lipids, and proteins causing several age related diseases like cancer, arthritis, osteoporosis, type 2 diabetes, hypertension and neurodegenerative diseases [1, 2]. ROS can be generated as an outcome of cellular metabolism and various stresses like UV- radiation, chemicals and heat [3]. Although several chemical options are available but owing to their hefty costs and numerous side-effects, the world's major population still relies on the natural sources for the cure of diseases [4]. Thus, the central focus towards the exploration of novel drug sources from plant origin has become a critical evaluation aspect for antiaging (age related diseases).

Premna integrifolia Linn. (syn: *Premna serratifolia*) commonly known as ''Agnimantha'' is reportedly important for its biological activities such as antirheumatic, carminative, galactogenic, bechic, febrifuge, stomachic and anti-inflammatory [5]. Besides several phytochemicals viz. beta-sitosterol, polyisoprenoid, spermine alkaloids, aphilandrine, and premnin, 10-*O-trans-p*-Coumaroylcatalpol (OCC) forms a major ingredient of *P. integrifolia* [6, 7]. OCC, an Iridoid compound (monoterpene) in nature forms an important constituent of the famous herbal formulation ''Dashmula'', commonly used for its various medicinal properties. Iridoids possess defensive roles in certain plant species [8], and are also reported for a wide range of pharmacological actions such as treatment of hepatic dysfunction, stimulation of bile acid excretion [9], antitumor [10], and anti-inflammatory activities [11]. However, the antiaging activity of iridoids is yet not explored and serious efforts are required to detect the possible roles and mode of action of these compounds.

In order to accomplish this aspect, the present study was designed to isolate and characterize an iridiod compound, OCC from *P. integrifolia.* The potential of isolated

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1871-5273/12 $58.00+.00

compound were evaluated for age related ailments using animal model *C. elegans*. *C. elegans,* a free living soil nematode is an emerging model for aging research as most of the *C. elegans* genes have human orthologues [12] suggesting the conserved mechanisms of longevity regulation in humans. Besides the short lifespan, ease of genetic and dietary manipulations also adds to the forefront choice of *C. elegans* in aging studies. The present study will help to explore and authenticate OCC, a plant-derived molecule in the development of profitable pro-longevity pharmaceuticals and as an economic benefit to the mankind.

MATERIALS AND METHODS

Plant material Stem bark of *P. integrifolia* was collected from Tambarum, Chennai, India. Plant identification was done by Prof. P. Jayaraman, Director, National Institute of Herbal Science, Plant Anatomy Research Centre, West Tambaram, Chennai, India. A voucher specimen (no. 22706) has been deposited in the herbarium of the above institute.

Apparatus for Isolation and Identification

The 300 MHz NMR spectra were recorded with tetramethyl silane (TMS) as internal standard on Bruker Avance instrument. ^{13}C NMR and DEPT spectra were recorded at 75 MHz. The DEPT experiments were used to resolve multiplicities of carbon atoms. Chemical shifts are given in parts per million. COSY, HSQC and HMBC were performed using standard Bruker pulse programs. IR spectras were obtained on a Nicolet 380 FTIR spectrophotometer. Mass spectras were measured on API 3000 triple quadrapole mass spectrometer (Applied Biosystems/MDS SCIEX). Optical rotations were measured on a HORIBA polarimeter (SEPA-300). Column chromatography was executed using Silica gel (60-120 mesh, Merck, India). Preparative HPLC (Shimadzu, Japan) consisting column (Supelcosil LC-18, 21.2mm×250mm, 12µm), pumps LC-8A and PDA detector was used for isolation and purification of reference compounds.

Extraction and Isolation of OCC from *P. integrifolia*

Air dried and finely powdered stem bark of the plant (900 g) was extracted with methanol (2 L X 24 h) at room temperature and the process was repeated five times. The combined extracts were evaporated under reduced pressure to obtain 53 g residue. Methanolic extract was suspended in water and then partitioned with hexane, chloroform and butanol, successively. The *n*-hexane partitioned fractions were combined and concentrated in vacuo to give a residue (2.73 g). Similarly, chloroform and butanol fractions were also concentrated to give residues 1.5g and 23g, respectively. The butanol extract was chromatographed over a silica gel column (60-120 mesh, 100x5 cm,) eluted with $CHCl_3$ and increasing polarity with MeOH and was fractionated into 10 frs: fr.B1-B10 (each 4.5 L, *n*-hexane-EtOAc 1:0, 19:1, 23:2, 9:1, 17:3, 4:1, 3:1, 7:3, 1:1, EtOAc). Fr.B4 (1.8 g) was chromatographed to reverse phase preparative HPLC column (RP-C18, MeOH: H_2O; 70:30, 15 mL/min) which yielded compound OCC (75 mg) (tR 7.1). The structure elucidation of compound (Fig. **1**) was performed with the help of UV, ^1H NMR, ^{13}C NMR, 2D experiments (HSQC, HMBC, COSY, and NOESY), mass analysis (ESI-MS))and confirmed finally by comparison with reported data [13]. The ^1H- and ^{13}C-NMR data of marker compounds is presented in Table **1**.

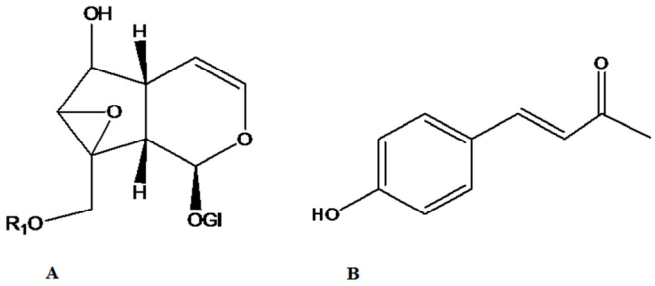

Fig. (1). Structure of 10-*O-trans-p*-Coumaroylcatalpol (10-OCC) (**A**) and R_1 (**B**).

Table 1. **^1H and ^{13}C NMR Shifts in δ for Compound OCC (in $CDCl_3$)**

Position	δ H (*m*, *J* in Hz)	δ C
1	5.06(1H, *d*, 10)	94.6
2	-	-
3	6.29(1H, *dd*, 6.0, 2.1)	140.8
4	5.09 (1H, *m*)	102.7
5	2.05(1H, *m*)	38.0
6	3.96(1H, *m*)	78.5
7	3.38(1H, *d*, 1)	61.8
8	-	62.6
9	2.66 (1H, *dd*, 10, 8)	43.5
10A	4.27 (1H, *d*, 13)	62.0
10B	4.98(1H, *d*, 13)	
1'	4.64(1H, *d*, 8.4)	99.3
2'	3.20(1H, *dd*, 9.0, 8.0)	73.8
3'	3.42(1H, *m*)	77.5
4'	3.18 (1H, *m*)	70.4
5'	3.32 (1H, *m*)	76.8
6a'	3.82 (1H, *dd*, 12.0, 2.0)	63.2
6b'	3.93(1H, *dd*, 12, 2)	
1″	-	126.0
2″ 6″	7.37(2H, *d*, 6.6)	130.3
3″ 5″	6.70(2H, *d*, 6.6)	115.8
4″	-	161.0
7″	7.55 (1H, *d*, 15.6)	145.9
8″	6.27 (1H, *d*, 15.6)	113.9
9″	-	168.0

Isolated compound was then dissolved in double distilled water to prepare stock solutions of 20mM, 2mM and 0.2mM for further experimentation.

C. elegans Strains and Worm Culture

C. elegans strains N2 Bristol (Wild type); TK22 *[mev-1(kn1)]*; GR1307 *[daf-16(mgDf50])*; VC-199 *[sir-2.1(ok434)],* TJ356 [*zls356; DAF-16::GFP]* and NL5901 (P_{unc-54}::alphasynuclein::YFP+unc-119) used in this study, were procured from *Caenorhabditis* Genetics Center, University of Minnesota, MN, USA. These worms were grown on Nematode growth medium (NGM) which was

prepared by adding 50mM Sodium chloride, 2.5gL^{-1} Peptone, 17 gL^{-1} Agar in 975ml double distilled water, q.s. and autoclaved for 40 minutes at 15lb/inch^2. After the medium was cooled to 50°C - 60°C, 5µgml^{-1} cholesterol solution (dissolved in ethanol), 1mM Calcium chloride, 1mM Magnesium sulphate and 25mM Potassium dihydrogen phosphate were added. All experiments were carried out at 20°C (except where otherwise mentioned) and worms were propagated following standard conditions as described earlier [14-16]. For isolation of age synchronized worms, gravid nematode populations were subjected to axenization on the day of initiation of treatment [17].

Treatment of Worms with OCC

OCC was mixed with bacteria OP50 to achieve a final concentration of 2µM, 20µM and 200µM and then seeded onto the NGM plates followed by an overnight incubation at room temperature. Embryos retrieved by hypochlorite treatment for obtaining synchronous worm populations, were transferred onto these 'OCC treated plates', for control groups embryos were transferred onto NGM plates seeded with OP50 only. Worms were raised on these plates until early adulthood and then processed for further analysis as described for various endpoints.

Lifespan Assay

Age synchronized N2 worms were used for lifespan assay. Isolated eggs were allowed to hatch on NGM plates previously spotted with or without different concentrations of OCC viz. 200µM, 20µM and 2µM till L4 stage. Approximately 100-120 L4 moults were then transferred to NGM plates previously spotted with corresponding test concentration and 50µM FUdR (Sigma, St. Louis, MO, USA) to block progeny development. 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 experiment [18].

Stress Resistance Assay

Effect of OCC on resistance of worms against oxidative stress, Paraquat (Sigma, St. Louis, MO, USA) which is an intracellular ROS generator was used. Wild type N2 worms were synchronized on NGM plates as described for lifespan assay. Adult day 2 worms were transferred to 10mM paraquat and assayed for survival after 72 hrs of continuous exposure [19].

Pharyngeal Pumping Assay

To investigate the effect of OCC on pharyngeal pumping of the worms, movement of the pharynx terminal bulb was recorded. Worms were grown on NGM plates, treated with or without different concentrations of OCC from L1 stage. Pumping records were made for 20 sec interval in adult day 0 and day 5 OCC treated worms against a untreated control. The experiment was performed at room temperature and worms were scored on bacterial lawn [20].

Chemotaxis Assay

Chemotaxis assay was performed according to Bargmann *et al.*, 1993 [21] using adult day 5 worms with slight modifications. Briefly treated and non-treated worms were washed in M9 buffer, and worms were placed in the centre of agar plates containing 10µl of attractant on one side (1M sodium acetate) and 10µl of water on the other side. 10µl of 1M sodium azide was added on both sides to paralyze the worms if they happen to venture in the opposite direction of the attractant. Worms were counted after 90 min. and chemotaxis index was calculated as:

Chemotaxis Index = A-B/A+B

where A= number of worms at the attractant location, B = number of worms at the control location, A+B = Total number of worms in the plate. The experiments were repeated independently thrice.

Assay of Alpha Synuclein Protein Aggregation

The aggregation of PD associated protein alpha synuclein was studied in transgenic strain NL5901 (P_{unc-54}::alphasynuclein::YFP+unc-119) that expresses human a-syn tagged with Yellow Fluorescence Protein (YFP). Worms of NL5901 strain were raised on normal OP50 (control group) or OCC treated plates (OCC group) at 22°C. After 48 hrs of treatment, worms were washed thrice with M9 buffer to remove any adhering bacteria and then transferred to slides with agar pads (2% agarose). Worms were mounted using 100mM sodium azide (Sigma, cat no. 71289) for immobilization, followed by sealing with coverslip. Imaging of live (immobilised) worms using upright fluorescence microscope (Nikon) was carried out to monitor the aggregation of protein alpha synuclein *via* observing fluorescence pattern of YFP. The fluorescence intensity was quantified using Image J analysis software (Image J, National Institutes of Health, Bethesda, MD) and represented in terms of relative fluorescence units (RFU) from n = 3 subjects from each group. The software quantifies the fluorescence intensity across the selected region of interest (kept same for each subject) and then provides numbers in terms of relative fluorescence units. Comparison of control *vs* treated subjects, gives a measure of the fluorescence intensity, hence alpha synuclein aggregation in the subjects. Statistical analysis was performed using SPSS software package and Student's t test was employed to calculate the statistical significance between groups [22, 23].

GFP Quantification and Visualization

The intracellular localization of DAF-16 was observed by using transgenic strain TJ356 (DAF-16::GFP), in which green fluorescent protein is fused with *daf-16* gene. Age synchronized L4 worms were transferred to NGM plates treated or non-treated with OCC and kept at 20°C for 48 hours. Worms were then mounted on 2% agarose pad and visualized under microscope (Carl Zeiss Axio Scope A1) for GFP localization. Non-treated worms with short thermal stress at 37°C for 20min were used as positive control [24].

ROS Detection Assay

Intracellular ROS level was determined using a non-fluorescent dye dichlorodihydrofluorescin diacetate (H_2DCF-DA, Sigma, St. Louis, MO, USA). Worms were synchronized on NGM plates as described earlier. At day 2 of adulthood 30 age synchronized worms were collected in 300µl of 0.1%PBST. Worms were washed three times with PBST and the lysate was prepared by equally timed homogenization and sonication (Branson Sonifier 250, VWR Scientific, Suwanee, GA). The homogenized samples were transferred to 96 well plate and prior to reading, 15µl of 10mM H_2DCF-DA was added to each well. Fluorescent readings were measured in every 20 minute interval for 2hrs 30 min at 37^0C by using a fluorescent microplate reader (Spectramax M_2, Molecular devices). Excitation was read at 485nm and emission was read at 535nm [2].

Statistical Analysis

Significant difference between the lifespan of treated and control worms under both normal and stress conditions was determined using Kaplan-Meier survival assay in MedCalc software. Data other than lifespan were statistically analyzed using ANOVA in ASSISTAT statistical assistance software. Difference between the data was considered as significant at $p \leq 0.05$.

RESULTS

OCC Extends the Mean Lifespan of Wild Type N2 Worms

To investigate the effects of OCC supplementation on longevity, the lifespan of OCC treated worms was compared with untreated worms. Worms were observed daily for survival by touch provoked method [25]. It was observed that 20µM concentration of OCC increased the mean lifespan of wild type of worms by 3.31 days which is 17.96% greater as compared to the non-treated control worms (p=0.0001) (Table **2**; Fig. **2A**). Although the 200µM and 2µM tested concentrations failed to significantly enhance the mean lifespan of wild type worms at 20^oC, the treatment with 20µM concentration depicted 13.31% increase in the maximum lifespan of N2 worms (Table **2**).

OCC Enhances Stress Resistance of *C. elegans*

Increased longevity has been reportedly associated with improved survival under stressed conditions [26]. To investigate the longevity promoting ability of OCC under oxidative stress wild-type worms were exposed to 10mM paraquat. In the present experiment it was observed that OCC pretreated worms were more resistant to parquat in comparison to non-treated control worms (Fig. **3**). The results revealed that all the observed concentrations of OCC significantly enhanced the percent survival of the parquat exposed worms. The worms pretreated with 20µM OCC showed the highest significant ($p < 0.007$) survival.

OCC Decreases the Intracellular ROS Levels in *C. elegans*

To evaluate the potential effects of OCC for ROS scavenging, the intracellular free radical levels of wild type N2 worms was determined. It was observed that all the tested concentrations of OCC significantly reduced ROS generation in wild type worms (Fig. **4**). The results clearly indicate that OCC could reduce ROS production as the 20µM OCC treated worms reduced 40.1% ROS levels as compared to untreated control ($p < 0.001$).

OCC Enhanced the Pharyngeal Pumping of *C. elegans*

Speed of pharynx contraction or pumping is an important parameter of aging in *C. elegans* and reduction in pumping can induce dietary restriction (DR) like effects in worms [27]. The results showed that OCC concentrations were able to enhance the rate of pharyngeal pumping in adult day 0 and adult day 5 treated worms (Fig. **5A**). The 2µM OCC treatment was unable to increase the pumping rates in adult day 5 worms while in 200µM and 20µM treated adult day 5

Table 2. Effect of OCC on the Lifespan of *C. elegans*

Strains	Treatment (mg/ml)	Mean Lifespan ± SE	No. of Worms (N)	% Change	P Value	Max. Lifespan ± SE	Min. Lifespan ± SE
N2	Control	18.42 ± 0.94	140			25 ± 0.71	11.5±0.29
	OCC (200)	19.42 ± 0.67	96	5.14	= 0.67	24.66 ± 0.76	15±0.50
	OCC (20)	21.73 ± 0.65	114	17.96	=0.0001***	28.33± 1.25	17±0.87
	OCC (2)	19.94± 1.13	134	8.25	=0.019	28± 1.08	13±1.00
mev-1 (kn-1)	Control	15.7± 0.77	100			23.66±0.58	10.33±0.58
	OCC (20)	15.42±0.65	136	1.7	= 0.36	21.67±1.60	11±0.0
daf-16 (mgDf50)	Control	16.13± 0.43	166			22.33±1.04	12.33±0.76
	OCC (20)	15.74±0.45	166	-2.4	= 0.32	21.33±0.28	11.33±0.57
sir-2.1 (ok434)	Control	17.13 ± 0.45	180			23.25 ± 0.47	10.5 ± 0.50
	OCC (20)	19.71 ± 0.23	138	15.06	<0.0001***	25.67 ± 0.88	12 ± 1.15

The lifespan assay was done with wild type N2, *mev-1*(*kn-1*), *daf-16*(*mgDf50*) and *sir-2.1 (ok434)* at 20^oC. Worms were treated with different test concentrations of OCC from L1 stage till the completion of the assay. The mean lifespan was calculated as the average number of days worms survived in each test concentration (***$p < 0.001$).

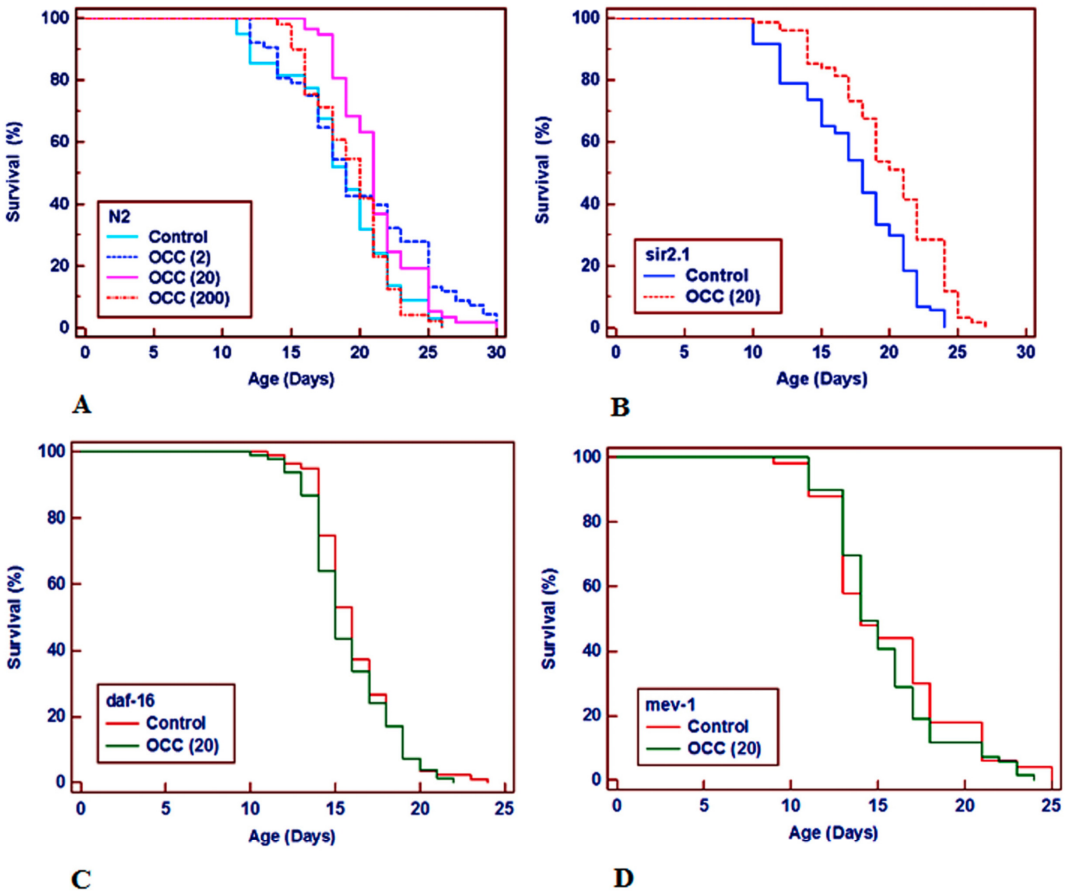

Fig. (2). Effect of OCC on lifespan of *C. elegans*. Worms were synchronized on NGM plates spotted with different test concentrations of OCC (2, 20 and 200µM) on OP50 lawn and allowed to grow till L4 stage. L4 moults were then transferred to fresh NGM plates of corresponding test concentration with 50µM FUdR. Worms were scored daily for survival. 20µM of the test concentration showed a significant increase in lifespan of wild type N2 worms (p=0.0001) (**A**) and *sir-2.1* mutant (p< 0.001) (**B**), while no increase in mean lifespan was observed in *daf-16* (**C**) and *mev-1* mutant (**D**). Survival plot was drawn by Kaplan-Meier survival assay.

worms, a significantly ($p < 0.004$, $p < 0.001$ respectively) higher pumping rate was recorded.

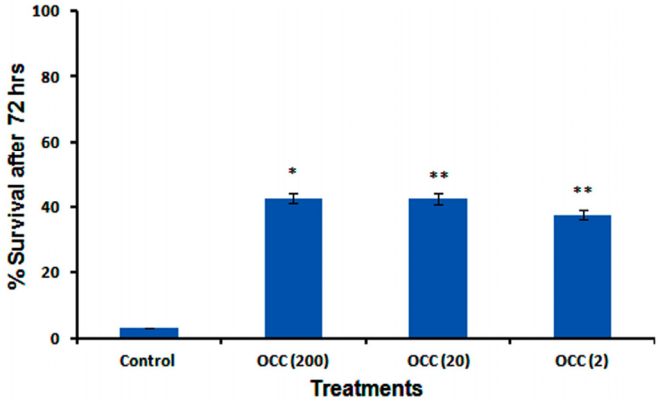

Fig. (3). Effect of OCC on paraquat induces oxidative stress on wild type N2 worms. Worms were synchronized on NGM plates supplemented with or without OCC. On adult day 2 worms were transferred to paraquat (10mM) treated plates and survival of the worms was measured after 72 hrs. All test concentration significantly reduced the paraquat sensitivity in pretreated worms than the non treated control worms. Error bars represent the standard error of mean (SEM). Statistically significant at *p<0.05, **p<0.01.

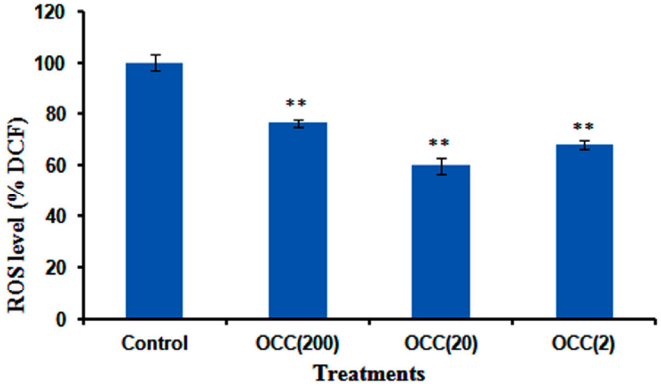

Fig. (4). Effect of OCC on ROS in *C. elegans.* To measure intracellular ROS level age synchronized wild type N2 worms were treated with different concentrations of OCC since L1 stage. On adult day 2 worms were homogenized and ROS level was detected using 96 well micro plate reader at 37°C. Graph was plotted as relative change in ROS compared to control as 100%. All the test concentration significantly reduced the ROS level, 20µM concentration showing the best results. Error bars represent the standard error of mean (SEM). Statistically significant at *p<0.05, **p<0.01.

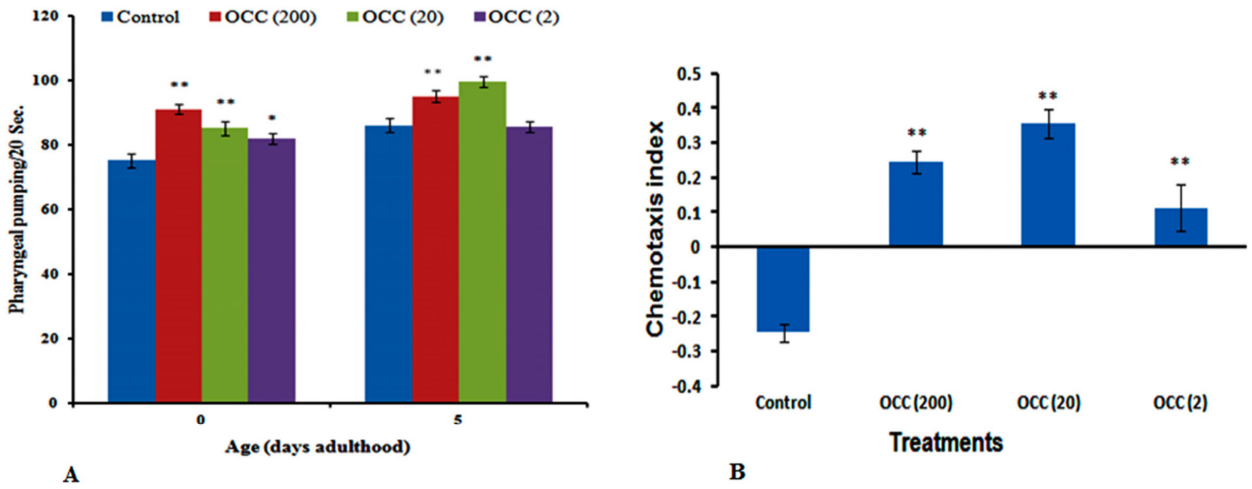

Fig. (5). Effect of OCC on the pharyngeal pumping and CI of wild type worms. (**A**) Pharyngeal pumping of worms treated with different concentrations of OCC was recorded for 20sec and compared with non-treated control worms. On adult day 0 and adult day 5 200µM & 20µM concentrations enhanced the movement of pharynx bulb significantly. 20 worms per experimental condition were scored and experiment was repeated thrice. (**B**) For CI wild type *C. elegans* were synchronized on NGM plates with or without OCC. CI were determined using adult day 5 worms. Sodium acetate was used as attractant and sodium azide was applied to paralyze the worm. After 90 min worms were scored and CI was calculated. 20µM concentrations enhance the CI index significantly. Error bars represent the standard error of mean (SEM). Statistically significant at ** $p < 0.01$.

OCC Increased the Chemotaxis Index of the Worms

To investigate the effect of OCC on age dependant physiological behavior Chemotaxis index (CI) was determined, which is a measure of learning behavior and motor responses to sensory stimulus in *C. elegans* [21]. In the present experiment all the OCC concentrations increased the CI index but a highly significant increase in CI was observed in 20µM treated worms ($p < 0.001$). The CI index of 200µM and 2µM OCC treated worms was also found to be enhanced in comparison to untreated control worms (Fig. **5B**).

OCC Treatment Reduced Aggregation of Alpha Synuclein

After observing significant anti-aging effects of OCC, we endeavored to study its effect on age-associated PD. Employing alpha synuclein expressing transgenic *C. elegans* strain tagged with YFP, we observed a significant reduction in YFP fluorescence when worms were treated with OCC as compared to the worms of control group (Fig. **6A**, **B**). When quantified using Image J analysis, the worms of control group were observed to have a fluorescence intensity of 26.32 ± 1.97 RFU whereas worms raised on OCC had a fluorescence intensity of 19.92 ± 1.01 RFU, thus exhibiting a 1.32 fold reduction (Fig. **6C**).

Lifespan Extension Due to OCC Requires Insulin Signaling and ROS Detoxification Pathway

The identification of genes extending lifespan in *C. elegans* empowers its mutants to be utilized for genes/ pathway identification modulated by various drugs. Thus the opportunity to identify the genetic mechanism of OCC treatment was investigated using different *C. elegans* mutants (*sir 2.1, daf-16* and *mev-1*). In *C. elegans sir-2.1* encodes a histone deacetylase-like protein that brings metabolic status together with lifespan [28, 29]. OCC supplementation increased the lifespan of *sir-2.1* mutant significantly by 15.06% ($p < 0.0001$) (Fig. **2B**; Table **2**). *C. elegans* mutant *daf-16(mgDf50)* has a null mutation in its gene encoding for DAF-16, a major transcription factor of IIS pathway conferring stress tolerance and longevity [12]. It was recorded that OCC supplementation was unable to increase the lifespan of this mutant (Fig. **2C**; Table **2**). The lifespan of *daf-16* control worm was 16.13 days which is almost equal to OCC treated *daf-16* worms 15.74 days. Similarly no significant increase in lifespan was observed in *mev-1(kn-1)* after OCC supplementation (Fig. **2D**; Table **2**). Hence after OCC supplementation, there was negligible change in lifespan of *daf-16* and *mev-1* mutants.

Further, the genetic requirement of insulin signaling for the effects mediated by OCC was also examined by TJ356 mutant carrying *daf-16::gfp* transgene. To begin with, the nuclear localization of DAF-16 was studied under control conditions, where a diffused homogenous distribution was observed. After this worms were treated with 20µM and 200µM OCC for 48 hrs and then visualized under fluorescent microscope. It was observed that OCC supplementation was able to translocate DAF-16 into nucleus (Fig. **7**). Similar observation was found in worms exposed to a short heat shock at 37°C for 20 min, used as a positive control.

DISCUSSION

The present study demonstrates the isolation and longevity promoting activity of iridoid OCC from an important Indian medicinal plant *P. integrifolia*. The study first reports the antiaging and anti-neurodegenerative activity of one of the iridoids using *C. elegans, a* promising animal model for aging research. Numerous natural compounds such as blueberry polyphenols [19], EGCG [20], DATS [27], resveratrol [30], reserpine [31], quercetine [32] and curcumin

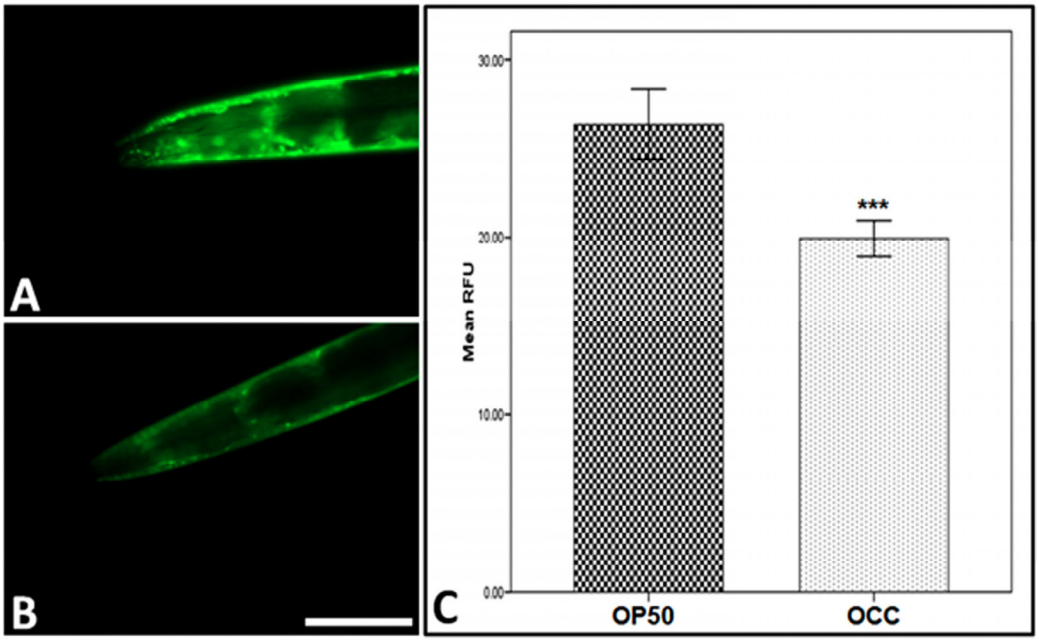

Fig. (6). Fluorescent photomicrographs depicting alpha synuclein aggregation in NL5901 strain of C. elegans: (**A**) Control, (**B**) OCC treated. (**C**) is graphical representation of fluorescence intensity as quantified by Image J analysis.***p≤0.001, Scale bar, 50µm.

[33] have been studied using this model. These reports resulted in seeking the longevity promoting activity of OCC, contributing to the ongoing search for a novel lifespan extension compound.

The present experiment demonstrated that OCC at its 20µM concentration significantly increased the mean lifespan of wild type N2 worms under normal culture conditions. Similarly, a number of experiments have been carried out with different phytomolecules which also increased the life span of *C. elegans* at variant concentrations [19, 20, 31, 33]. In the present experiment OCC supplemented worms showed resistance to paraquat thereby increasing the survival of worms after 72 hours as compared to untreated control (Fig. **3**). Maximum survival was recorded in 200 µM and 20 µM OCC supplemented worms after 72 hours. Free radicals contribute to the formation of ROS, damaging the biomolecules leading to several biological pathologies. These results demonstrated that intracellular ROS level was lower in OCC treated worms (Fig. **4A**). Similar with previous findings [2, 33] the low level of ROS can be assumed as an important factor for increased resistance against oxidative stress and the enhancement of lifespan under normal culture conditions. Alpha synuclein is a Parkinson's disease (PD) associated protein which aggregates more in diseased and aged person. The reduced aggregation of alpha synuclein (a-syn) in OCC treated a-syn transgenic worms signifies the potentials of OCC in reducing PD incidence, extending the further genetic possibility to be explored. However, it was also observed that OCC supplementation did not prolong lifespan of pro-aging mitochondrial mutant *mev-1(kn-1)* (Fig. **2D**; Table **2**), which has a short lifespan due to accumulation of ROS as a result of mutation in its cytochrome *b* subunit of the succinate dehydrogenase (complex II) of the electron transport chain [34]. This suggested the involvement of an endogenous ROS detoxification pathway conferring oxidative stress tolerance and reduced ROS level. In 2010, Yu *et al.* [35] also demonstrated the similar findings in *C. elegans* treated with *Cinnamomum cassia* bark. Based on our results, it is concluded that OCC reduces the ROS levels in wild type worms with a requirement of an endogenous pathway for such effects.

In the present experiment it was observed that OCC supplementation enhanced other age dependant physiological parameters like pharyngeal pumping and CI. At adult day 0, OCC supplementation at all the concentration slightly increased the pumping rates, whereas in adult day 5 worms the pharyngeal pumping was significantly more at 20µM concentration (Fig. **5A**). The increase in pumping rates suggested the proper food intake and diminished the probability of DR like effects. It has been already reported that *C. elegans* can modify its behavior to avoid various adverse conditions and this change in learning behavior and motor activity is associated with aging [36, 37]. Consistent with previous findings [20], improved CI in adult day 5 wild type worms was also observed (Fig. **5B**), suggesting the ability of lifespan extending compounds to attenuate age dependent decline in physiological behaviours.

In the later part of the study, the genetic requirements for the longevity promoting effect of OCC in *C. elegans* were investigated. To find the possible mechanism of action of OCC, the lifespan of *sir-2.1* mutant was examined. The increased lifespan of worms lacking SIR-2.1 activity showed that SIR-2.1 is not involved in effect mediated by OCC supplementation [38]. Further, the lifespan of *daf-16* mutant, after OCC supplementation was examined and it was observed that OCC supplementation failed to extend the lifespan of *daf-16* mutant (Fig. **2C**; Table **2**). This showed the possibility of requirement of major longevity regulating transcription factor DAF-16. Later, in an experiment with TJ356 mutant carrying *daf-16::gfp* transgene, it was observed that OCC treatment was able to translocate DAF-

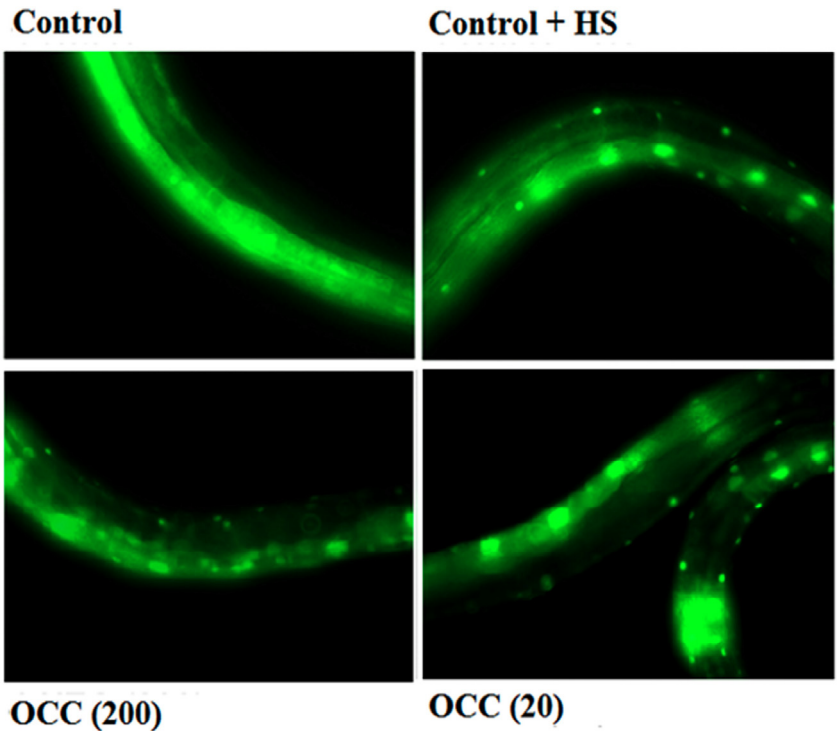

Fig. (7). Effect of OCC on nuclear localization of DAF-16. Age synchronized L4 TJ356 worms were continuously exposed to 200µM and 20µM concentration of OCC for 48 hours. Worms with small heat shock at 37°C for 20min. were used as positive control. Supplementation of OCC resulted in nuclear translocation of DAF-16. 100 worms per experimental condition were mounted on 2% agarose pad on a glass slide and subcellular localization of DAF-16 was analyzed under fluorescent microscope. Experiment was repeated thrice and images were captured using 40X objective.

16 into nucleus (Fig. **7**). In *C. elegans* DAF-16 is considered as a major transcription factor regulating a number of longevity and stress tolerance associated genes [12, 39]. It has also been suggested that compounds restraining IIS pathway and DAF-16 localization are provocative contenders for pharmacological manipulation of lifespan [40]. Previously other studies have also shown increased longevity by naturally occurring molecules required DAF-16 activation [24, 32]. The present findings were consistent with previous studies indicating that OCC supplementation resulted in translocation of DAF-16 leading to an increased lifespan and improved physiological processes.

CONCLUSION

This study reports for the first time the longevity promoting activity of OCC from *P. integrifolia* using *C. elegans* model system. The results demonstrated OCC potentials for the lifespan extension and significant increased resistance against oxidative stresses. Besides the reduction in ROS levels and alpha synuclein aggregation capabilities indicate towards the possibility of OCC to be used as therapeutic remedy for age related ailments. Importantly, these results can be transferred and explored in mammalian systems due to the high homology between *C. elegans* and the human beings.

ABBREVIATIONS

CI = Chemotaxis index

GFP = Green fluorescent protein

OCC = 10-*O-trans-p*-Coumaroylcatalpol

PD = Parkinson's Disease;

ROS = Reactive oxygen species

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ACKNOWLEDGEMENTS

Authors are sincerely grateful to CGC Center, Minneapolis, MN, USA, which is funded by NIH National Center for Research Resources, USA for providing the worm culture. The senior author would like to thank to Council of Scientific and Industrial Research for providing grant in-aid. Authors are also thankful to Prof. Ram Rajasekharan Director, CSIR-Central Institute of Medicinal and Aromatic Plants for his valuable support and suggestions. Authors are grateful to Mrs. Shilpi K. Saikia for her editorial help.

REFERENCES

[1] Finkel, T.; Holbrook, N.J. Oxidants, oxidative stress and the biology of ageing. *Nature*, **2000**, *408*, 239-247.

[2] Smith, J.V.; Luo, Y. Elevation of oxidative free radicals in Alzheimer's disease models can be attenuated by Ginkgo biloba extract EGb 761. *J. Alzheimer's Dis.*, **2003**, *5*(4), 287-300.

[3] Gutteridge, J.M.; Halliwell, B. Free radicals and antioxidants in the year 2000. A historical look to the future. *Ann. NY Acad. Sci.*, **2000**, *899*, 136-147.

[4] Zang X. *Traditional medicine: its importance and protection*. In: Twarog S, Kapoor P, editors. Protecting and promoting traditional knowledge: systems, national experiences and international

dimensions. Part I. The role of traditional knowledge in health care and agriculture. **2004**; pp. 3-6.

[5] Husain, A.; Virmani, O.P.; Popli, S.P.; Misra, L.N.; Gupta, M.M.; Srivastava, G.N.; Abraham, Z.; Singh, A.K. *Dictionary of Indian Medicinal Plants.* Central Institute of Medicinal and Aromatic Plants, New Delhi. **1992**; p. 375.

[6] Rao, B.; Susheela, K.; Rao, P.V.S.; Krishna, P.G.; Raju, G.V.S. Chemical examination of some Indian medicinal plants. *Ind. J. Chem.*, **1984**, *23B*, 787- 788.

[7] Dasgupta, B.; Sinha, N.; Pandey, V.; Ray, A. Major alkaloid and flavonoid of Premna integrifolia. *Planta Med.*, **1984**, *50*(3), 281-283.

[8] Konno, K.; Hirayama, C.; Yasui, H.; Nakamura, M. Enzymatic activation of oleuropein: A protein crosslinker used as a chemical defense in the privet tree. *Proc. Natl. Acad. Sci. USA*, **1999**, *96*(16), 9159-9164.

[9] Chang, I-M. Liver-protective activities of aucubin derived from traditional original medicine. *Res. Commun. Mol. Pathol. Pharmacol.*, **1998**, *102*(2), 189-204.

[10] Konoshima, T.; Takasaki, M.; Tokuda, H.; Nishino, H. Cancer chemopreventive activity of an iridoid glycoside, 8-acetylharpagide, from Ajuga decumbens. *Cancer Lett.*, **2000**, *157*(1), 87-92.

[11] Park, K.S.; Kim, B.H.; Chang, II-M. Inhibitory Potencies of Several Iridoids on Cyclooxygenase-*1*, Cyclooxygnase-2 Enzymes Activities, Tumor Necrosis factor-α and Nitric Oxide Production *In Vitro*. *Evid. Based Comp. Alternat. Med.*, **2010**, *7*(1), 41-45.

[12] Kenyon, C.J. The genetics of ageing. *Nature*, **2010**, *464*, 504-512.

[13] Sudo, H.; Ide, T.; Otsuka, H.; Hirata, E.; Takushi, A.; Takeda, Y. Iridoid glucosides with different acyl moieties from globularinin and globularimin from leaves of *Premna subscandens*. *Phytochemistry*, **1998**, *49*(3), 783-786.

[14] Brenner, S. The genetics of *Caenorhabditis elegans*. *Genetics*, **1974**, *77*(1), 71-94.

[15] Jadiya, P.; Chatterjee, M.; Sammi, S.R.; Kaur, S.; Palit, G.; Nazir; A. Sir-2.1 modulates 'calorie-restriction-mediated' prevention of neurodegeneration in *Caenorhabditis elegans*: implications for Parkinson's disease. *Biochem. Biophys. Res. Commun.*, **2011**, *413*(2), 306-310.

[16] Sulston, J.E.; Brenner, S. The DNA of Caenorhabditis elegans. *Genetics*, **1974**, *77*(1) 95-105.

[17] Stiernagle, T. Maintenance of C. elegans. *WormBook*, **2006**, 1-11.

[18] Zhang, L.; Jie, G.; Zhang, J.; Zhao, B. Significant longevity-extending effects of EGCG on *Caenorhabditis elegans* under stress. *Free Rad. Biol. Med.*, **2009**, *46*(3), 414-421.

[19] Wilson, M.A.; Hale, B.S.; Kalt, W.; Ingram, D.K.; Joseph, J.A.; Wolkow, C.A. Blueberry polyphenols increase lifespan and thermotolerance in *C. elegans*. *Aging Cell*, **2006**, *5*(1), 59-68.

[20] Brown, M.K.; Evans, J.L.; Luo, Y. Beneficial effects of natural antioxidants EGCG and α-lipoic acid on lifespan and age-dependent behavioral declines in *Caenorhabditis elegans*. *Pharmacol. Biochem. Behav.*, **2006**, *85*(3), 620-628.

[21] Bargmann, C.I.; Hartwieg, E.; Horvitz, H.R. Odorant-selective genes and neurons mediate olfaction in *C. elegans*. *Cell*, **1993**, *74*(3), 515-527.

[22] Jadiya, P.;Khan, A.; Sammi, S.R.; Kaur, S.; Mir, S.S.; Nazir, A. Anti-Parkinsonian effects of *Bacopa monnieri*: insights from transgenic and pharmacological *Caenorhabditis elegans* models of Parkinson's disease. *Biochem. Biophys. Res. Commun.*, **2011**, *413*(4), 605-610.

[23] Kaur, S.; Sammi, S.R.; Jadiya, P.; Nazir, A. RNAi of *cat-2*, a Putative Tyrosine Hydroxylase, Increases Alpha Synuclein Aggregation and Associated Effects in Transgenic *C. elegans*. *CNS Neurol. Disord. Drug Targets*, **2012**, *11*, 387-394.

[24] Wiegant, F.A.C.; Surinova, S.; Ytsma, E.; Langelaar-Makkinje, M.; Wikman, G.; Post, J.A. Plant adaptogens increase lifespan and stress resistance in *Caenorhabditis elegans*. *Biogerontology,* **2008**, *10*(1), 27-42.

[25] Lithgow, G.J.; White, T.M.; Melov, S.; Johnson, T.E. Thermotolerance and extended lifespan conferred by single-gene mutations and induced by thermal stress. *Proc. Natl. Acad. Sci. USA*, **1995**, *92*(16)7540-7544.

[26] Ristow, M.; Schmeisser, S. Extending life span by increasing oxidative stress. *Free Rad. Biol. Med.*, **2011**, *51*(2), 327-336.

[27] Powolny, A.A.; Singh, S.V.; Melov, S.; Hubbard, A.; Fisher, A.L. The garlic constituent diallyl trisulfide increases the lifespan of *C. elegans via* skn-1 activation. *Exp. Gerontol.*, **2011**, *46*(6), 441-452.

[28] Tissenbaum, H.; Guarente, L. Increased dosage of a *sir-2* gene extends lifespan in *Caenorhabditis elegans*. *Nature*, **2001**, *410*(6825), 227-230.

[29] Wood, J.; Rogina, B.; Lavu, S.; Howitz, K.; Helfand, S.; Tatar, M.; Sinclair, D. Sirtuin activators mimic caloric restriction and delay aging in metazoans. *Nature*, **2004**, *430*(5), 686-689.

[30] Basss, T.M.; Weinkovem, D.; Houthoofd, K.; Gems, D.; Partridge, L. Effects of resveratrol on lifespan in *Drosophilla melanogeter* and *Caenorhabditis elegans*. *Mech. Ageing Dev.*, **2007**, *128*(10), 546-552.

[31] Srivastava, D.; Arya, U.; SoundaraRajan, T.; Dwivedi, D.; Kumar, S.; Subramaniam, J.R. Reserpine can confer stress tolerance and lifespan extension in the nematode C. elegans. *Biogerontology*, **2008**, *9*(5), 309-316.

[32] Kampkötter, A.; Timpel, C.; Zurawski, R.F.; Ruhl, S.; Chovolou, Y.; Proksch, P.; Wätjen, W. Increase of stress resistance and lifespan of Caenorhabditis elegans by quercetin. *Comp. Biochem. Physiol. Part B*., **2008**, *149*(2), 314-323.

[33] Liao, V.H.C.; Yu, C.W.; Chu, Y.J.; Li, W.H.; Hsieg, Y.C.; Wang, T.T. Curcumin- mediated lifespan extension in *Caenorhabditis elegans*. *Mech. Aging Devlop.*, **2011**, *132*(10), 480-487.

[34] Ishii, N.; Fujii, M.; Hartman, P.S.; Tsuda, M.; Yasuda, K.; Senoo-Matsuda, N.; Yanase, S.; Ayusawa, D.; Suzuki, K. A mutation in succinate dehydrongenase cytochrome b causes oxidative stress and aging in nematodes. *Nature*, **1998**, *394*(6694), 694-697.

[35] Yu, Y.B.; Dosanjh, L.; Lao, L.; Tan, M.; Shim, B.S.; Luo, Y. Cinnamomum cassia bark in two herbal formulas increases life span in Caenorhabditis elegans *via* insulin signaling and stress response pathways. *PLoS One*, **2010**, *5*(2), e9339.

[36] Zhang, Y.; Lu, H.; Bargmann, C.I. Pathogenic bacteria induce aversive olfactory learning in Caenorhabditis elegans. *Nature*, **2005**, *438*,179-84.

[37] Murakami, S.; Murakami, H. The effects of aging and oxidative stress on learning behavior in C. elegans. Neurobiol. *Aging*, **2005**, *26*(6), 899-905.

[38] Houthoofd, K.; Vanfleteren J.R. The longevity effect of dietary restriction in *Caenorhabditis elegans*. *Exp. Gerontol.*, **2006**, *41*(10), 1026-1031.

[39] Murphy, G.T.; McCarroll, S.A.; Bargmann, C.I.; Fraser, A.; Kamath, R.S.; Ahringer, J.; Li, H.; Kenyon, C. Genes that act downstream of DAF-16 to influence the lifespan of Caenorhabditis elegans. *Nature*, **2003**, *424*(6946), 277-284.

[40] Gami, M.S.; Wolkow, C.A. Studies of Caenorhabditis elegans DAF-2/insulin signaling reveal targets for pharmacological manipulation of lifespan. *Aging Cell*, **2006**, *5*(1), 31-37.

Received: June 14, 2012 **Revised: July 20, 2012** **Accepted: August 14, 2012**