

# Crocin, a Carotenoid Mitigates Acrylamide Induced Phenotypic Alterations, Oxidative Stress and Dopaminergic Neurodegeneration in *Caenorhabditis Elegans*: Implications for Neuropathy

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**Research Article**

Volume 1 Issue 1

**Received Date:** July 24, 2018

**Published Date:** August 03, 2018

## Abstract

Earlier we demonstrated the potential of Crocin, (CR) a well known carotenoid to attenuate experimentally induced neurotoxicity employing a *Drosophila* model. In the present study we sought to obtain insights on the neuroprotective propensity of CR using acrylamide (ACR) model of neurotoxicity in the nematode, *Caenorhabditis elegans*. We exposed age-synchronized L4 worms (both wild-type N2 and *BZ555* strain to ACR (250mg/L) for 48 h in the presence or absence of CR (10 $\mu$ M). The protective efficacy of CR was assessed in N2 worms by analyzing phenotypic alterations (locomotory behaviour), reproductive functions (brood size and egg laying), biochemical parameters and longevity. The neuroprotective propensity was assessed by direct visualization and quantification of dopaminergic (DA) neurons in the transgenic strain (*BZ555*). CR treatment not only normalized reproductive functions, but significantly alleviated ACR-induced oxidative stress and mitochondrial dysfunction. Further, CR restored the activity levels of acetylcholinesterase, dopamine levels and markedly reduced the dopaminergic degeneration. CR also extended the lifespan in N2 worms and markedly enhanced the expectancy of short-lived mutant worms (*MEV-1*). Collectively, these experimental findings reveal that CR significantly attenuates the dopaminergic neurodegeneration and associated physiological/ biochemical dysfunctions induced by ACR in *C. elegans*. Based on these data we propose that Crocin may be an effective therapeutic adjuvant in the management of oxidative stress mediated neurodegenerative conditions.

**Keywords:** Crocin; Acrylamide; Oxidative Stress; *Caenorhabditis Elegans*; Neuroprotection

**Abbreviations:** CR: Crocins; ACR: Acrylamide; PD: Parkinson's disease; GFP: Green Fluorescent Protein; DA: Dopamine; NGM: Nematode Growth Medium; NCRR: National Centre for Research Resources; ROS: Reactive oxygen species; HP: Hydro Peroxides; SOD: Superoxide Dismutase; HPLC: High-Performance Liquid Chromatography; ECD: Electro Chemical Detector; GST: Glutathione-S-Transferase.

## Introduction

*Crocus sativus* L. commonly known as saffron is cultivated in many countries such as Iran, India, Italy, Spain and Greece. Commercially available saffron is the dried red stigmas of the flower. The main bioactive constituents of saffron are crocins, picrocrocin, and safranal [1]. Saffron extract or its active constituents have been demonstrated to have diverse biological properties such as antioxidant, anti-inflammatory and neuroprotective effects [2-4]. Crocins (CR) are the glucosyl esters of crocetin, which are water-soluble carotenoids. CR is responsible for its characteristic colour of saffron and numerous studies have attributed its antioxidant property to be responsible for the protective efficacy in a wide range of diseases [5]. Both *in vitro* and *in vivo* studies have demonstrated the cytoprotective, anti-inflammatory and anti-oxidant effects of CR on nerve cells [6,7]. Several recent reports suggest the potential of CR to improve cognitive power and to reduce anger and depression [8,9].

Acrylamide (ACR), a well-documented human neurotoxin is a commonly used chemical for various industrial applications and gel electrophoresis in laboratories [10]. ACR is a water soluble monomer, formed in carbohydrate-rich foods when they are prepared in high temperature. In recent times, ACR has gained considerable attention owing to its formation during heat processing of carbohydrate rich foods (eg., French fries, chips). Compelling evidence from different experimental models indicate its toxic effects leading to neuropathic signs. ACR monomer is known to affect both central and peripheral nervous system and impacts both sensory, motor and cognitive functions [11-14]. Various undesirable effects of ACR including reproductive toxicity, carcinogenicity and neurotoxicity have been described [15-17]. ACR also induces metabolic alterations, genotoxicity and mutagenicity [18,19]. Industrial exposure of humans to ACR results in neurological symptoms, including ataxia, skeletal muscle flaw, numbness of hands/ feet, neuropathy and Parkinsonism [20]. *In vivo* and *in vitro* studies have shown that ACR

alters dopamine levels by affecting the dopaminergic system [21,22].

Dopaminergic neuronal loss, motor impairment, formation of lewy bodies, increased oxidative stress and mitochondrial dysfunction are the characteristic features of Parkinson's disease (PD) [23]. The advantages of employing the nematode *Caenorhabditis elegans* (*C. elegans*) to investigate the association between oxidative stress and DAergic neurodegeneration are well known [24]. The simplicity of *C. elegans* to high-throughput genomic, proteomic and drug screening approaches make this organism a striking option for recognizing new curative compounds for PD and understanding their mechanism of action. The simple nervous system of *C. elegans* contain only 302 neurons (out of 959 somatic cells) in adult hermaphrodites and various neurons are categorized as sensory, interneuron, or motor neurons. It has 8 DA neurons, with completely mapped neuronal networks [25]. *C. elegans* is extensively used for studying varying cellular pathways because of the availability of genetic, behavioural, biochemical and physiological endpoints associated with normal aging and neurodegeneration including PD [26]. Since DA neurons can be directly visualized through the expression of a fluorescent protein (Green fluorescent protein (GFP), this model has also been employed to induce DA neuron degeneration utilizing chemical neurotoxins to understand the mode of action of therapeutic compounds [27,28].

Recently we demonstrated the neuroprotective effects of Saffron extract and CR in a *Drosophila* model of Parkinsonism [29]. In this study, utilizing *C. elegans* as a model system, we investigated the neuroprotective efficacy of CR focusing on the aspects related to its potential to abrogate ACR mediated neurotoxic outcome. Initially, we determined the potential effects of CR in worms and recapitulated the neurotoxic effect of ACR. Further, employing a co-exposure paradigm, the efficacy of CR to alleviate ACR-induced locomotor and physiological effects, oxidative impairments, cholinergic function and dopamine levels were determined. The neuroprotective potential of CR was assessed by quantifying the extent of neuronal degeneration in the transgenic strain (*BZ555*). Furthermore, we also investigated its ability to extend the longevity employing both wild and short lived mutants (*MEV-1*).

## Materials and Methods

Crocins (CR, Figure 1), Acrylamide (ACR), Dopamine (DA), and other fine chemicals were procured from Sigma

Chemical Co. (St. Louis, MO, USA). All other chemicals used were of analytical grade. *C. elegans*, wild-type strain (N2), BZ555 strain (GFP tagged in dopamine neuronal stroma and processes), a mutant strain of *C. elegans*

(MEV-1) and *Escherichia coli* (OP50-uracil auxotroph) were obtained from the *Caenorhabditis* Genetics Center (CGC, Minneapolis, MN, USA) which is funded by the National Centre for Research Resources (NCRR).

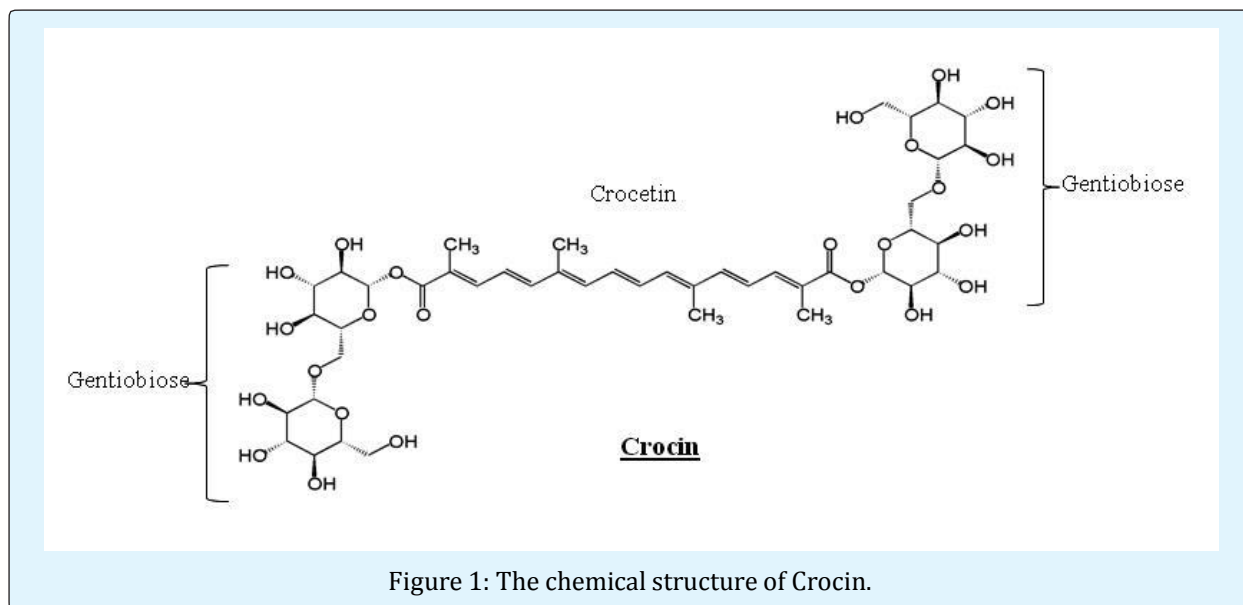


Figure 1: The chemical structure of Crocin.

### C. elegans Maintenance and Treatment

*C. elegans* were maintained on Nematode Growth Medium (NGM) seeded with *E. coli* OP50 and sustained at 20°C. Age-synchronized worms were obtained by the alkali-bleaching method [30].

### Crocin (CR) Concentrations: Effect on Endogenous Levels of Oxidative Markers

For this study, L4 stage worms were exposed to varying concentrations of CR (5, 10 and 20 μM) for 48h and the effect on locomotor function was evaluated. Further, the effect of CR on endogenous levels of biochemical markers of oxidative stress was determined.

### Selection of ACR Concentration and Exposure of Worms

In a preliminary study, worms were exposed to ACR at varying concentrations (75-250 mg/L) in K-medium for 48h and induction of locomotor deficits were assessed. Further we determined the impact of ACR exposure on selected biochemical markers of oxidative stress. Based on these results, a concentration of 250 mg/L was selected for further experimentation in the co-exposure paradigm.

### ACR Exposure - Recapitulation of ACR Induced Effects in Worms

The basic experimental design comprised of exposing L4 stage worms to ACR for a period of 48 h. Subsequently, worms were washed thrice with K-medium and subjected to various studies. Assessment of behavioural phenotypes consisted of thrashing behaviour, pharyngeal pumping and locomotor movements. Biochemical analysis comprised of quantification of oxidative stress markers, activity levels of antioxidant enzymes, mitochondrial enzymes, cholinergic function and dopamine content.

### Protective Effect of CR Treatment Against ACR-Co Exposure Paradigm

**Modulation of phenotypic and biochemical alterations in N2 worms:** In order to assess the modulatory effect of CR on ACR-induced phenotypic alterations, a co-exposure paradigm was employed. L4 worms were exposed to sublethal concentration of ACR (250 mg/L) and CR (10 μM) for 48 h in 6-well tissue culture plate. Following the exposure period, worms were washed thrice with K-medium and subjected to select physiological measurements and processed further for biochemical assays.

**Neuroprotective effect of CR: Analysis of neuronal degeneration in BZ555:** Strain Synchronized L4 stage of *BZ555* (egls1 [dat-1p: GFP] i.e., bright GFP observable in Dopamine neuronal soma and processes) were exposed to ACR and CR at the concentrations stated above for 48 h. After the exposure period, the worms were washed thrice with K-medium to dispose of bacterial debris. Microscopy of living young adults was executed by introducing the worms on glass slides with a small aliquot of 100 mM sodium azide (utilized as an anesthetic) and GFP was visualized with a fluorescence microscope. All images were developed with Image J software for quantifying the green pixel number. Two independent experiments were conducted for each dose with at least 10 worms/per exposure group.

**Potential of CR to extend Lifespan of worms exposed to ACR:** In this study, we examined the effect of CR on the lifespan of worms exposed to ACR employing both wild type (N2) and short lived mutant (*MEV-1*) strains employing a co-exposure paradigm. Worms ( $n=20 \pm 1$ ) from both control and ACR exposed groups after 48h were transferred to a 24-well plate containing K-medium with OP50 and 5-fluoro-2-deoxyuridine (FUdR: 50  $\mu$ M). The worms were maintained at 20° C and checked daily for survivability until all the worms died [31].

### Methods of Analysis

**Analysis of behavioural response:** Following exposure to ACR, worms were washed in K-medium and subjected to the behaviour analysis as briefly described below.

**Thrashing:** This study was conducted by placing worms in K-medium and counting the number of tangential swimming movements (thrashes  $\text{min}^{-1}$ ), thereby measuring a significant feature of locomotion. The effects of toxin on the locomotion of worms were quantified as described previously [32].

**Pharyngeal pumping:** Pharyngeal pumping is defined as the backward grinding movements in the terminal bulb of the worm in the presence of *Escherichia coli* OP50. To assay pumping on bacteria, pumps were counted for 30 s. At least 5 worms per group were analyzed for pumping behaviour and the results pooled [33].

**Locomotion:** We analyzed rates of locomotion behaviour by measuring the incidence at which young adult animals initiated body bends. NGM plates with thin lawns of OP50 bacteria were used as the stage for the worms to perform locomotory behaviour. We counted the number of body bends by direct observation for a total of 30 s. A body

bend was counted each time a bend reached a maximum just posterior to the pharynx, either on the dorsal or ventral side of the animal [34].

### Assessment of physiological responses

**Lifespan:** L4 worms ( $20 \pm 1$ ) were transferred to a 24-well plate containing K-medium with OP50 and 5-fluoro-2-deoxyuridine (FUdR: 50  $\mu$ M). The worms were maintained at 20° C and checked daily for survivability until all the worms died [31].

**Egg laying:** About ten worms picked from each treatment group were shifted to NGM plates. The number of eggs laid by every worm during 1 h after exposure period was counted [35]. **Brood size:** A single worm was picked and relocated to 12-well tissue culture plate containing 1 ml K-medium, with OP50. The plates were incubated at 20° C for 72 h. After the incubation period, the worms were washed, pelleted and the progeny was counted under the dissecting microscope [36]. Assays were repeated three times in triplicate.

### Biochemical estimations

**Measurement of ROS generation:** Reactive oxygen species (ROS) produced was evaluated using dihydro dichlorofluorescein diacetate, a non-polar compound that after conversion to a polar derivative by intracellular esterases, can swiftly react with ROS to form the highly fluorescent compound, dichlorofluorescein in [37]. In brief, an aliquot equivalent to 0.1 mg protein was incubated in Locke's solution (pH 7.4; NaCl – 154 mM, KCl – 5.6 mM, NaHCO<sub>3</sub> – 3.6 mM, HEPES – 5 mM, CaCl<sub>2</sub> – 2 mM and glucose – 10 mM) containing 5  $\mu$ M DCFDA for 30min at room temperature and the fluorescence was considered at excitation and emission wavelengths of 480 and 530 nm, respectively. The ROS generation was projected from a DCF standard curve and expressed as pmol DCF/min/mg protein.

**Measurement of HP:** The levels of hydroperoxides (HP) were determined based on oxidation of xylenol orange reconciled by ferrous ions [38]. An aliquot of homogenate was added to 1 ml of FOX reagent (250  $\mu$ M Ferrous ammonium sulphate; 100  $\mu$ M sorbitol; 25 mM H<sub>2</sub>SO<sub>4</sub>; 100  $\mu$ M xylenol orange), and incubated for 30 min at room temperature. The absorbance was read at 560 nm and expressed as nmoles hydroperoxides (HP) /mg protein.

**Estimation of Reduced glutathione (GSH) and total thiols:** The levels of reduced glutathione were established by following o-phthalaldehyde fluorescence [39]. An aliquot of the sample (0.1 mg protein) was deproteinized by adding formic acid (0.1 M) and subsequent centrifugation (10,000 rpm, 10 min). The supernatant was mixed with alkaline formaldehyde (8% in 20 mM



Na<sub>2</sub>HPO<sub>4</sub>) and incubated (45 min) with sodium phosphate buffer (0.1 M, pH 8.0, containing 5 mM EDTA, 100 mg/ml o-phthalaldehyde). Reduced glutathione levels were read (excitation – 345; emission – 425) against a standard curve and expressed as  $\mu\text{mol GSH/mg protein}$ . The total thiols levels were determined following a previously described method with slight modification [40].

**Activities of antioxidant enzymes:** Superoxide dismutase (SOD) activity was calculated indirectly by observing the inhibition of quercetin auto-oxidation [41]. In brief, to a reaction mixture of phosphate buffer (0.016 M, pH 7.8, containing TEMED-4 mM and EDTA- 0.04 mM) quercetin (50  $\mu\text{M}$ ) was added and the rate of its auto oxidation was monitored at 406 nm for 3 min. The ability of the test sample to inhibit quercetin oxidation by 50% is defined as one unit of the enzyme and activity expressed as units/mg protein. Catalase activity was measured by adding an aliquot of cytosol (0.25 mg protein) to phosphate buffer (0.1 M, pH 7.4, containing H<sub>2</sub>O<sub>2</sub> – 10 mM) [42]. The collapse of H<sub>2</sub>O<sub>2</sub> was monitored at 240 nm and activity expressed as nmol substrate/ min/mg protein ( $\epsilon$ – 44.2/mM/cm). Thioredoxin reductase (TR) activity in the test sample was estimated by monitoring the reduction of DTNB at 412 nm, in a potassium phosphate buffer (0.1 M, pH 7.0, containing in mM: EDTA – 10, NADPH – 0.2) [43]. The activity was expressed as nmol substrate reduced/min/mg protein. Glutathione-S-transferase (GST) activity was quantified by scrutinizing the conjugation of glutathione to CDNB at 340 nm [44]. The reaction was started by adding a cytosolic aliquot (0.01 mg protein) to the phosphate buffer (0.1 M, pH 6.5 containing in mM: EDTA – 0.5, CDNB – 0.075, GSH – 0.05). The increase in the optical density at 340 nm was recorded for 3 min and the activity expressed as nmol conjugate formed/min/mg protein.

**Determination of acetylcholinesterase (AChE) activity:** AChE activity was quantified according to a standard method [45]. The reaction was initiated by the addition of acetylthiocholine iodide (ATCI, 1.95 mM) to the phosphate buffer (0.1 M, pH 8.0) containing DTNB (2.5 mM) and cytosolic sample (0.01 mg protein). The change in absorbance was monitored at 412 nm for 3 min. The enzyme activity was expressed as nmol substrate hydrolysed/min/ mg protein.

**Analysis of dopamine concentrations by HPLC:** After the exposure period, the worms were washed thrice with K-medium, pelleted and then homogenized in buffer A (50 mM Tris-HCl buffer pH 7.4 + 0.1% Tween 20) followed by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatant was used for dopamine quantification after filtration through 0.25m nylon filters. The quantification was done by high-performance liquid chromatography

(Shimadzu HPLC) with an electrochemical detector (ECD) consisting of a high-pressure isocratic pump, a 20  $\mu\text{l}$  sample injector valve, C18 reverse phase column and electro chemical detector. Retention time (RT) for dopamine peak was between 11.9 and 13.3 min of the run time. Dopamine (DA) standard was used to obtain a standard curve and the DA content in the control and experimental worms were calculated and DA was expressed as pg/mg protein [46].

**Determination of mitochondrial enzyme activity:** NADH-cytochrome C reductase activity was quantified following the reduction of cytochrome C by the sample (0.1 mg protein) in phosphate buffer (0.1 M, pH 7.4, having NADH– 0.2 mM, KCN – 1 mM) [47]. The decrease in absorbance was monitored at 550 nm for 3 min and activity was expressed as nmol cytochrome-c reduced/min/mg protein. The MTT reduction assay was performed by incubating the mitochondrial aliquot (0.015 mg protein) in the mitochondrial re-suspension buffer (containing sodium succinate – 20 mM, MTT- 75 mg/ml) for 60 min at 37°C. The formazan formed was suspended in SDS reagent (pH 7.4, 10%-SDS, 45%-DMF), measured at 570 nm and expressed as OD/mg protein [48].

**Measurement of ADP/ATP ratio:** ADP/ATP ratio was quantified by ApoSENSOR™ ADP/ATP Ratio Bioluminescent Assay Kit (from Biovision Catalog #K255-200) according to the manufacturer's instruction. This kit utilized bioluminescent detection of the ADP and ATP levels for a rapid screening of apoptosis, necrosis, growth arrest, and cell proliferation simultaneously in cells. The assay utilized the enzyme luciferase to catalyze the formation of light from ATP and luciferin, and the light was measured using a luminometer or Beta Counter. ADP level was measured by its conversion to ATP that is subsequently detected using the same reaction in a 96 well plate.

**Protein estimation:** Protein concentrations were determined by incubating an aliquot of the sample with Folin-Ciocalteu's phenol in an alkaline medium and measuring the optical density at 750 nm using a UV-VIS spectrophotometer [49]. The amount of protein was enumerated using bovine serum albumin as the standard.

### Statistical Analysis

Every experiment was repeated at least thrice with three replicates each. Mean and standard error (SE) were evaluated for all parameters, and the results were expressed as mean SE. The data were analyzed employing one-way ANOVA followed by the Tukey's multiple comparison tests. The longevity data were subjected to Kaplan-Meier survival analysis to prepare survival curves and the data were compared with untreated control

worms. Values  $\leq 0.05$  were considered as significant. All the calculations were carried out by Graph pad prism software.

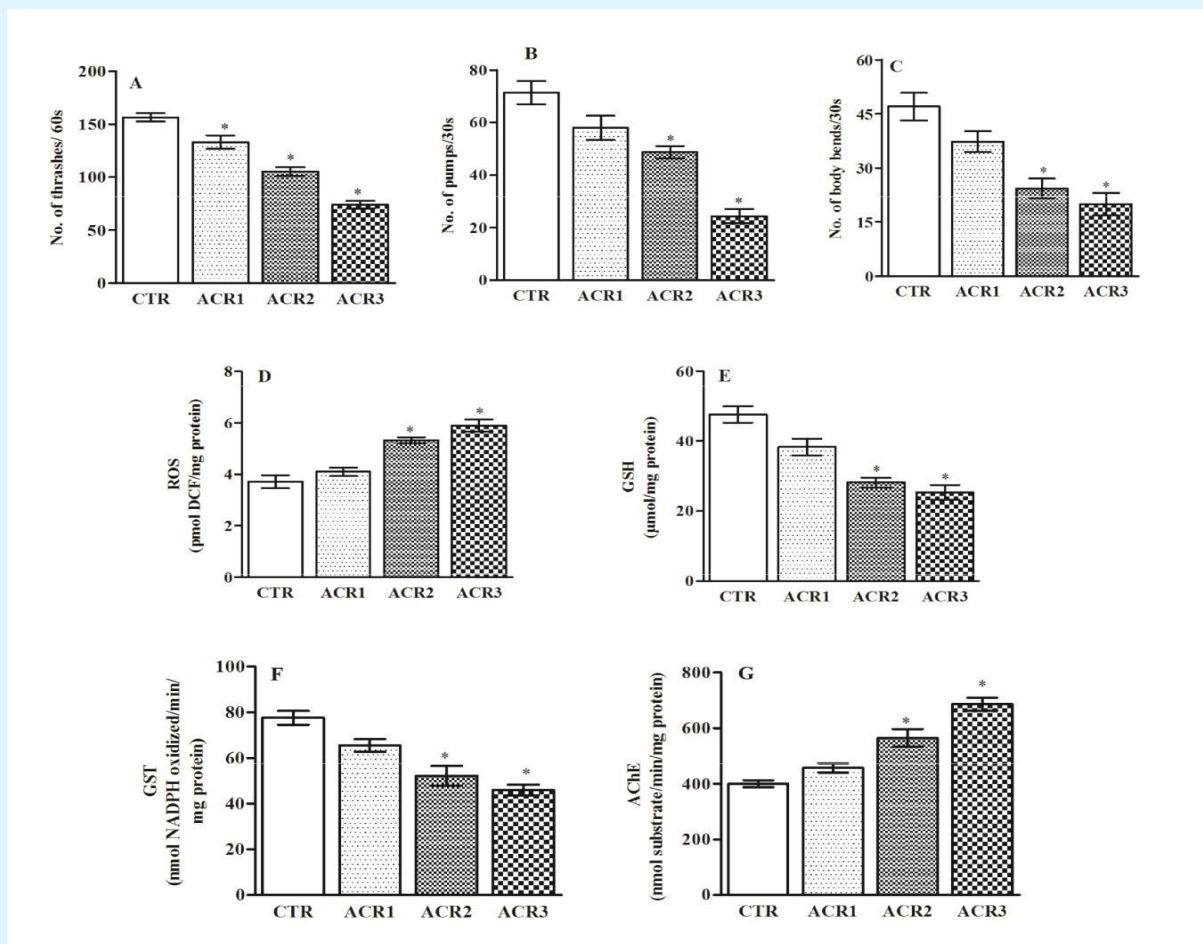
## Results

### Effect of Crocin (CR) Treatment

Worms maintained in K-media enriched with CR (5-20 $\mu$ M) for 48h exhibited no locomotor dysfunction or lethality (data not shown). However, CR treatment (48h) significantly influenced the basal levels of some important biochemical parameters. CR decreased the levels of ROS, increased GSH levels, enhanced GST activity and reduced the AChE activity levels in worms. All further experiments were conducted employing CR at concentrations of 5-10 $\mu$ M.

### Recapitulation of ACR Effects in Worms

Worms exposed to different sublethal concentrations of ACR (75-250mg/L) demonstrated a concentration-dependent alteration on locomotor behaviour. While the lowest concentration of ACR had no significant effect on worms, at higher concentrations (150 and 250mg/L) it caused a significant reduction in thrashing behavior, pharyngeal pumping and locomotion Figures 2A-C. Further, ACR induced significant oxidative stress as evidenced by elevated ROS levels, decreasing GSH levels, diminished GST activity levels and enhanced AChE activity Figures 2D-G. Based on these, a concentration of 250mg/L of ACR was used for further studies to assess the protective efficacy of CR.



**Figure 2(A-G):** Effect of ACR (75-250mg/L) *per se* on the behaviour parameters {thrashing (A), pharyngeal pumping (B) and locomotion (C)} and biochemical response (ROS (D), GSH (E), GST (F) and AChE (G) levels) in worms. Values are mean  $\pm$  SE (n=10, 3 replicates). ACR1= 75mg/L, ACR2=150mg/L and ACR3=250mg/L. Data analyzed by One-way ANOVA ( $P \leq 0.0010$ ) followed by Dunnett Test. \*- Significantly different compared to control.

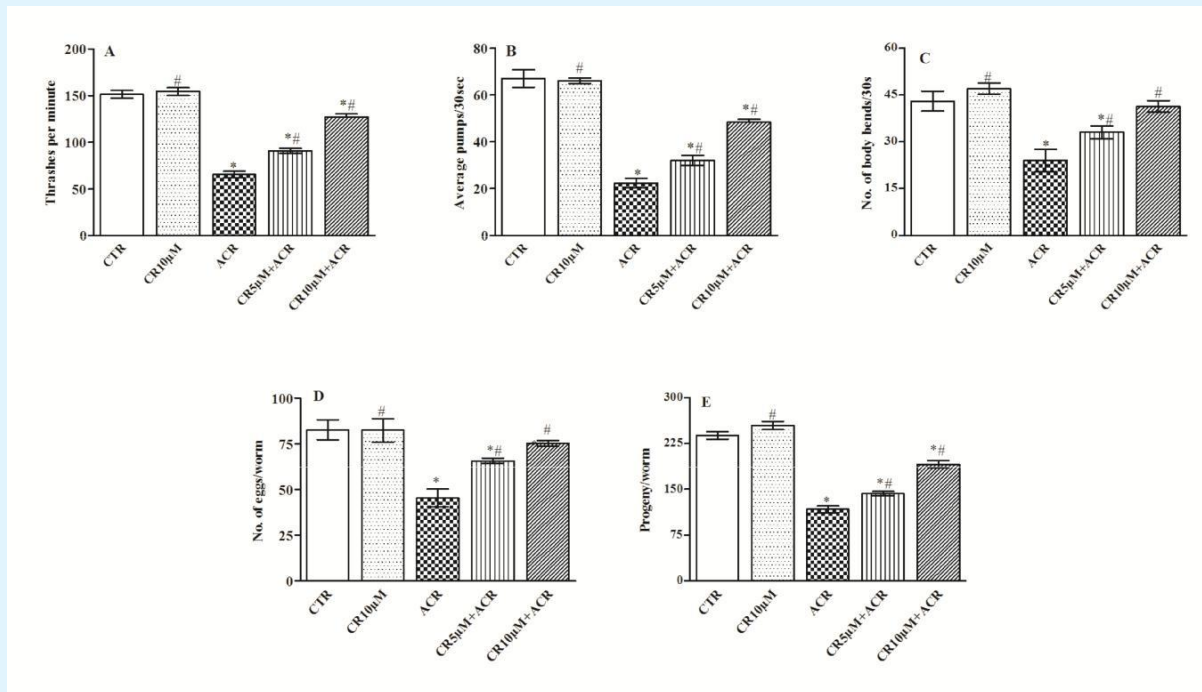
### Modulatory Effect of CR on Behaviour Parameters of Worms Exposed To ACR

ACR exposure caused a significant decrease in the locomotor activity of worms. While ACR reduced the thrashing movements (by 57%), body bending (by 44%), and pharyngeal pumping was markedly (by 67%) diminished. Interestingly, in the co-exposure paradigm, CR treatment significantly improved all the three behaviour parameters. While the thrashing behaviour and locomotion was significantly restored (by 28-48%) Figure

3A, higher degree of restoration (30-54%) was evident with pharyngeal pumping Figures 3B & C.

### Effect of CR on Egg Laying and Brood Size of Worms

Worms exposed to ACR showed a significant decrease in egg laying (45%) and brood size (51%), which were normalized by CR treatment in a concentration-dependent manner Figures 3D & E.



**Figures 3A-E:** Protective effect of Crocin (CR) (10µM), on ACR (250mg/L) induced alterations in behaviour parameters like thrashing (A), pharyngeal pumping (B) and locomotion (C), egg laying (D) and brood size (E) in worms. Values are mean ± SE (n=10, 3 replicates). Data analyzed by One-way ANOVA (P≤0.0010) followed by Tukey's Multiple Comparison Test. \*- Significantly different compared to control; #- Significantly different compared to ACR.

### Protective Efficacy of CR on Redox Status and Antioxidant Enzyme Levels

The effect of ACR exposure on markers of oxidative stress and antioxidant enzyme levels were quantified in worms. ACR caused a significant increase in ROS (56%) and HP (63%) levels. While a lower concentration of CR offered only a marginal protective effect, CR at higher concentrations significantly reduced both ROS (40%) and HP (46%) levels in worms Table 1. Further, the levels of reduced GSH and total thiols which were significantly (40-

45%) depleted among worms exposed to ACR, were restored markedly with CR treatment Table 1. Furthermore, ACR exposure caused significant reduction in the activity levels of enzymatic antioxidants viz., SOD, catalase, TR and GST and CR treatment attenuated this effect as evident with enhancement in the levels of the enzymic antioxidants Table 2.

Effect of Crocin supplementation on oxidative markers, GSH and total thiols against acrylamide toxicity in *C. elegans*.

Group	ROS <sup>a</sup>	HP <sup>b</sup>	GSH <sup>c</sup>	TSH <sup>d</sup>
CTR	2.64±0.28	0.96±0.01	45.09±3.17	22.35±1.07
CR (10µM)	2.21±0.4 <sup>#</sup>	1.03±0.03 <sup>#</sup>	46.33±2.04 <sup>#</sup>	29.49±0.57 <sup>#</sup>
ACR (250mg/L)	5.99±0.18 <sup>*</sup>	2.57±0.01 <sup>*</sup>	24.87±2.03 <sup>*</sup>	13.26±0.59 <sup>*</sup>
CR(5 µM)+ACR	5.16±0.07 <sup>*#</sup>	2.18±0.12 <sup>*#</sup>	26.99±0.88 <sup>*</sup>	15.73±0.47 <sup>*#</sup>
CR(10 µM)+ACR	3.15±0.22 <sup>*#</sup>	1.38±0.03 <sup>*#</sup>	33.73±1.13 <sup>*#</sup>	18.58±0.50 <sup>*#</sup>

**Table 1:** Values are mean ± SE. Data analyzed by one-way ANOVA followed by Tukey's test ( $p < 0.05$ ); <sup>\*</sup>= significantly different compared to control; <sup>#</sup>= significantly different compared to acrylamide ROS-Reactive oxygen species, HP- Hydroperoxides, GSH- Reduced glutathione, TSH= Total thiols

a- pmolDCF/min/mg protein

b- µmol hydroperoxides/ min/mg protein

c- µmol GSH/mg protein

b- nmol thiols/mg protein

Modulatory effect of Crocin supplements on the activity of antioxidant enzymes against acrylamide toxicity in *C. elegans*.

Group	SOD <sup>a</sup>	CAT <sup>b</sup>	TR <sup>c</sup>	GST <sup>d</sup>
CTR	49.52±1.67	23.47±1.04	27.86±1.32	78.4±2.57
CR (10µM)	66.86±2.03 <sup>*#</sup>	23.36±0.66 <sup>#</sup>	30.81±0.80 <sup>#</sup>	87.93±0.87 <sup>#</sup>
ACR (250mg/L)	29.01±0.74 <sup>*</sup>	10.36±0.65 <sup>*</sup>	16.26±0.62 <sup>*</sup>	47.43±0.91 <sup>*</sup>
CR(5 µM)+ACR	31.45±0.35 <sup>*#</sup>	11.40±0.35 <sup>*</sup>	18.75±0.4 <sup>*#</sup>	52.88±0.86 <sup>*#</sup>
CR(10 µM)+ACR	39.60±1.45 <sup>*#</sup>	17.23±0.55 <sup>*#</sup>	23.34±0.45 <sup>*#</sup>	69.93±2.16 <sup>*#</sup>

**Table 2:** Values are mean ± SE. Data analyzed by one-way ANOVA followed by Tukey's test ( $p < 0.05$ ); <sup>\*</sup>= significantly different compared to control; <sup>#</sup>= significantly different compared to acrylamide

a= nmol cytochrome-C reduced/min/mg protein

b=OD/mg protein

c=ADP/ATP value

### Effect on Mitochondrial Enzyme Markers

ACR exposure resulted in a significant diminution in the activity levels of mitochondrial enzymes. While ACR markedly reduced the activity levels of Complex I-III (NADH-cyt C reductase) (52%), CR treatment significantly improved the activity. A significant (38%) increase in the activity levels of complex I-III was evident at a higher concentration of CR Table 3. Likewise, ACR reduced (by

49%) the MTT reduction property of mitochondria which was restored to normalcy by CR. While ACR caused a marked (87%) increase in ADP/ATP ratio, CR treatment restored the ratio to near normalcy Table 3.

Protective Effect of Crocin supplements on the activity levels of mitochondrial enzyme and ADP/ATP ratio against acrylamide toxicity in *C. elegans*.

Groups	NADH-Cytochrome-C reductase <sup>a</sup>	MTT reducion <sup>b</sup>	ADP/ATP ratio <sup>c</sup>
CTR	37.62±1.92	4.46±0.19	0.46±0.07
CR (10µM)	39.92±2.33 <sup>#</sup>	5.13±0.17 <sup>*</sup>	0.37±0.04 <sup>*</sup>
ACR (250mg/L)	18.08±1.55 <sup>*</sup>	2.3±0.07 <sup>*</sup>	3.99±0.24 <sup>*</sup>
CR(5 µM)+ACR	20.86±0.81 <sup>*#</sup>	2.83±0.09 <sup>*#</sup>	2.67±0.15 <sup>*#</sup>
CR(10 µM)+ACR	29.09±0.34 <sup>*#</sup>	3.03±0.06 <sup>*#</sup>	2.07±0.06 <sup>*#</sup>

**Table 3:** Values are mean ± SE. Data analyzed by one-way ANOVA followed by Tukey's test ( $p < 0.05$ ); <sup>\*</sup>= significantly different compared to control; <sup>#</sup>= significantly different compared to acrylamide.

a= nmol cytochrome-C reduced/min/mg protein

b=OD/mg protein

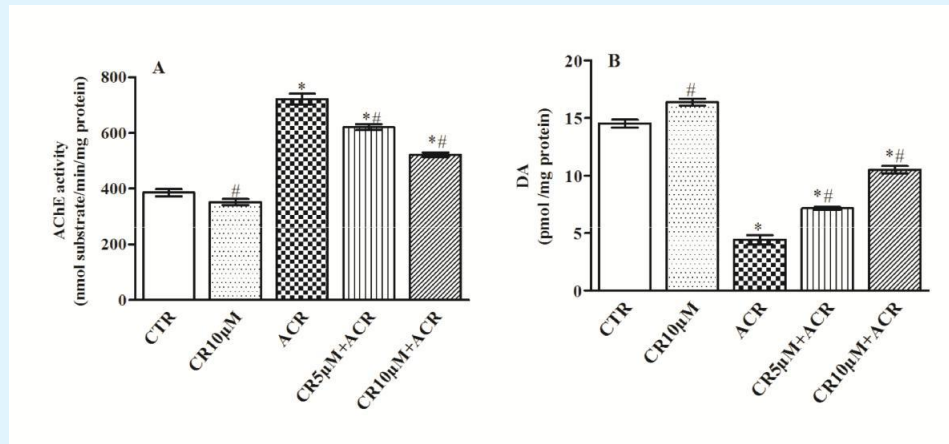
c=ADP/ATP value



### Protective Efficacy of CR on AChE and DA Activity

Worms exposed to ACR exhibited a significant (47%) increase in AChE activity compared to controls, while CR

treatment diminished (14-28%) the activity levels. Further, the DA content was robustly depleted (69%) among worms exposed to ACR, and CR treatment markedly normalized the levels Figures 4A & B.

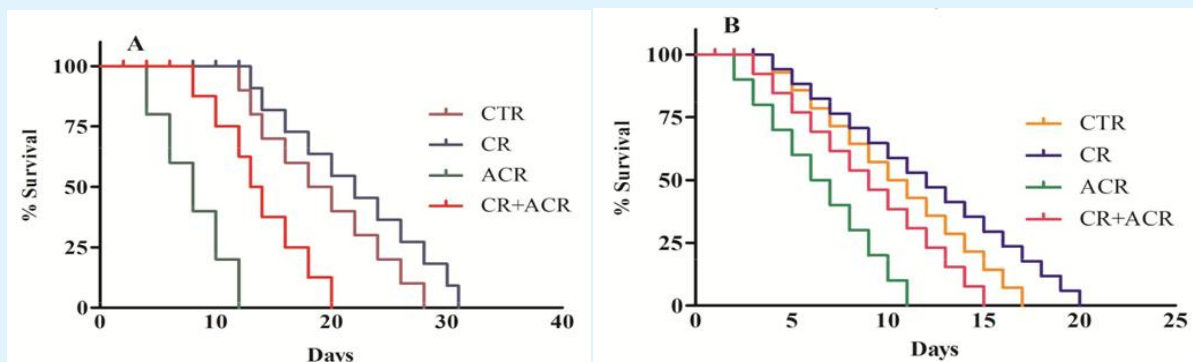


**Figures 4A & B:** Protective effect of CR (10 $\mu$ M) on ACR (250mg/L) induced changes in the activity levels of AChE (A) and DA (B). Values are mean  $\pm$  SE (3 replicates). Data analyzed by One-way ANOVA ( $P \leq 0.05$ ) followed by Tukey's Multiple Comparison Test. \*- Significantly different compared to control; #- Significantly different compared to ACR.

### CR Treatment Extends Lifespan of N2 and Mutant *MEV* Strains

Worms exposed to only ACR showed a significant (54%) reduction in survival rate, while worms treated with ACR along with CR showed a marked extension of survival period i.e., 8 days life extension (40%). CR alone treatment could also improve the life span of worms by expanding the lifespan 2 d additional compared to control

worms Figure 5A. In another study, well-known short-lived mutant worms (*MEV-1*) exposed to ACR lived only for 11 d compared to control worms which lived for 17 d. However, CR treatment resulted in marked (36%) extension of life expectancy among these worms Figure 5B. Interestingly, CR alone extended the life of mutant worms significantly (3d more) in comparison to control worms Figure 5B.

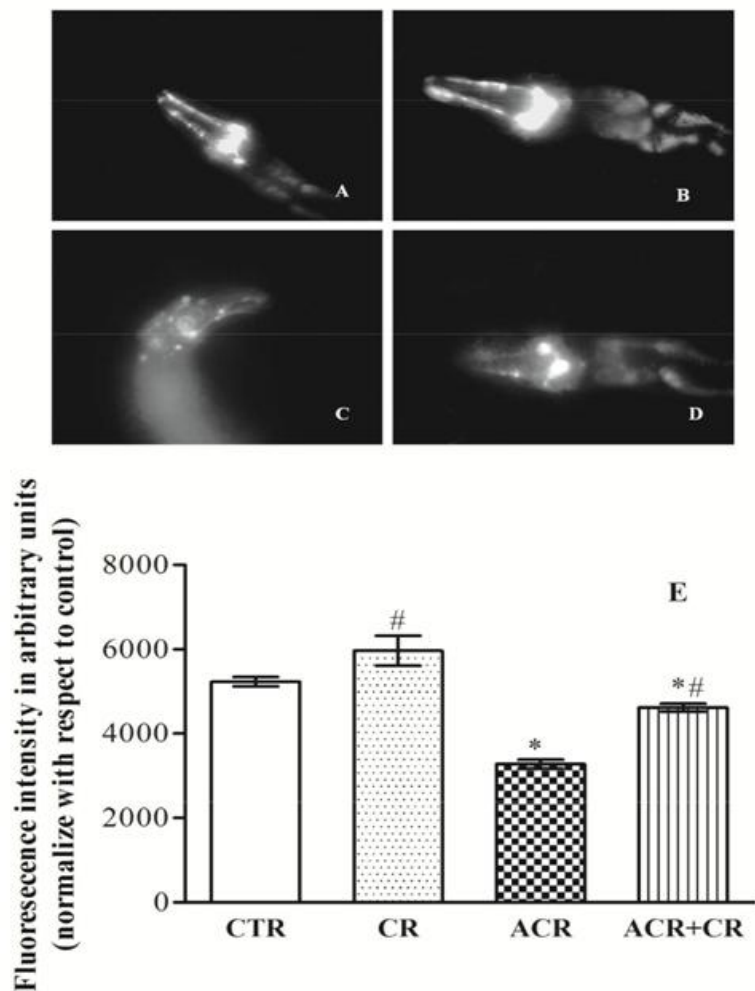


**Figures 5A & B:** Survival curves of worms in different treatment groups: Lifespan of wild type N2 worms (A) and mutant *MEV-1* worms (B). Values are percent (%) survival (3 replicates). Data analyzed by Kaplan-Meier ( $P \leq 0.0070$ ) followed by Log-rank (Mantel-Cox) Test.

### Protective Efficacy of CR To Abrogate ACR Toxicity in *BZ555* Strain

When compared to control worms, those treated with ACR exhibited a statistically significant (38%) reduction

in the green pixel number. Worms exposed to ACR along with CR exhibited a marked increase in GFP expression (29%) compared to ACR-exposed worms Figure 6.



**Figures 6A-E:** GFP expression in dopaminergic neurons of *Caenorhabditis elegans* (*BZ555* strain). A = Control, B = ACR (250mg/L), C = CR (10 $\mu$ M) + ACR (250mg/L), D = CR (10 $\mu$ M) and E = graphical depiction of fluorescence intensity of GFP in dopaminergic neurons in different treatment groups as quantified using Image J software. Values represented are mean  $\pm$  SE (n = 6). Data analyzed by One-way ANOVA ( $P \leq 0.05$ ) followed by Tukey's Multiple Comparison Test. \*- Significantly different compared to control; #- Significantly different compared to ACR.

### Discussion

Chronic exposure of children and adult humans to ACR entails understanding the underlying mechanism/s and developing newer strategies to alleviate the adverse effects [50]. While experimental evidences have

implicated reproductive toxicity and carcinogenicity owing to ACR exposure, neurotoxicity is the only outcome identified by epidemiological studies among occupationally exposed human populations [17]. Numerous evidences have accumulated over the years which show that ACR induces ataxia, distal muscle

weakness and neuronal damage [51,52]. In view of this, several newer strategies have been attempted to mitigate ACR-mediated neurotoxicity employing experimental models such as *Drosophila* and rodents [12,53,54]. In the present study, we assessed the propensity of CR, a well known carotenoid to modulate ACR neurotoxicity in *C. elegans*. Saffron and its active constituent CR have been reported to possess various beneficial effects on the central nervous system such as antidepressant, antioxidant, anti-inflammatory, neuromodulant, anticonvulsant, memory enhancing in various studies [29,55-60]. Recently we demonstrated the protective effect of CR against rotenone induced neurotoxicity in *Drosophila* model [29].

Previously few researchers have reported the adverse effects of ACR in *C. elegans* system. ACR exposure is known to produce adverse effects on metabolic processes, growth, life span and locomotion in worms [21,61]. Consistent with this, in the present study, worms exposed to ACR showed a significant decrease in egg laying and brood size while CR treatment significantly restored these effects. ACR is reported to be a reproductive toxin affecting male and female reproductive system in rodents [15,62]. In the present model, ACR exposure significantly decreased the reproductive capability of worms. Interestingly, CR treated worms showed improved reproductive performance clearly suggesting its ability to alleviate the same. This finding corroborates earlier data which have demonstrated that CR exerts protective effects on the development of reproductive organs, fertilization and development of the embryo [63,64].

Several studies have shown that ACR exposure causes specific phenotypic alterations in experimental models. In the present model, we observed a significant decrease in the locomotor behaviour, thrashing and pharyngeal pumping among worms exposed to ACR which were normalized by CR treatment suggesting its potential protective efficacy. Decreased locomotor activity and DA depletion with ACR exposure have been previously reported in both *Drosophila* and *C. elegans* [21,54].

Growing data in experimental models suggest that increased oxidative stress occurs in almost all neurodegenerative diseases. Oxidative stress refers to disparity between the production of free radicals and the ability of the cell to defend against them. Antioxidants are now being looked upon as convincing curatives against neuronal loss, owing to their potency to neutralize free radicals [65]. ACR is known to cause deleterious effects on antioxidant enzyme activities and elevate oxidative stress in animal models [12,54,66]. In the worm model, ACR

exposure caused a rigorous oxidative insult as evidenced by significantly elevated levels of ROS and HP and CR co-treatment resulted in a marked reduction. ROS and HP are not just endogenous oxidants, but serve as secondary messengers activating different enzyme cascades activating specific biochemical pathways [67]. Hence the considerable protection rendered by CR treatment against ACR-induced locomotor deficits, as well as the oxidative markers, suggests its potential to modulate ACR mediated oxidative damage.

In the present study, ACR exposure caused a significant depletion in the levels of reduced glutathione and total thiols, whereas, CR treatment significantly restored the levels. Glutathione-allied metabolism is key machinery for cellular protection against conditions of oxidative stress. Glutathione participates in detoxification and may scavenge free radicals, diminish peroxides or be conjugated with electrophilic compounds. Thus, glutathione offers the cell with multiple defenses against a variety of toxins [68]. In the present model, CR caused a significant restoration of ACR-induced activity levels of antioxidant enzymes viz., CAT, GST, TR, and SOD, signifying its efficacy to restore the enzymic antioxidant defense system in worms. Growing number of reports in *C. elegans* have shown the vital role of antioxidants on enzymic antioxidant defense systems, detoxification of various exogenous peroxides and oxidative stress resistance and their efficacy in life extension [69-71]. Hence we speculate that the enhanced activity levels of antioxidant enzymes (TR and GST) may be partly responsible for the neuroprotective effect of CR in the current model. Interestingly, we have previously observed a similar effect in *Drosophila* model with CR supplementation [29]. It would be interesting to examine if CR causes a similar up regulation of antioxidant defences in brain regions under neurotoxicant exposure in rodent models.

Disturbances in neurotransmitter turnover and cholinergic/dopaminergic dysfunctions have been reported amongst the notable features of PD. AChE plays a vital role in the functioning of the neuromuscular system, stopping continuous muscular contraction. Alterations in AChE affect ACh release leading to altered motor coordination. Analysis of the cholinergic function is likely to contribute to further understanding dementia and neurodegenerative diseases [72]. In the present model, ACR exposure resulted in enhanced activity levels of AChE which was brought to near normal range by CR treatment. This finding corroborates earlier reports of AChE inhibitory potency of plant compounds [13,73]. Dopamine (DA) is a crucial neuromodulator, endowed

with diverse roles in the regulation of neural networks controlling sensory modalities, locomotor activity and stereotypical behaviours [74]. In the present study, ACR exposure resulted in significant depletion of DA level consistent with a recent report [21]. Conversely, among ACR flies co-exposed with CR, DA was maintained at basal levels, which is indicative of their prospective specific effect on the dopaminergic neurons. The better performance of worms in the locomotory behaviour may be attributable to the restoration of DA levels among worms co-exposed with CR. Interestingly CR has shown a protective effect in a *Drosophila* model of Parkinsonism [29].

Besides oxidative stress, mitochondrial dysfunction and decreased ATP production are the key features of aging and neurodegeneration [75]. Recent studies have speculated that disruption or alterations in the activation of pathways including the mitochondrial unfolded protein response (UPR<sup>MT</sup>) and mitophagy, which regulate mitochondrial function are linked to neurodegenerative diseases due in part to the central role of mitochondria in metabolism, ROS regulation, and proteostasis [76]. In the present model, with ACR exposure we observed significant decrease in the activity levels of complex I-III in worms, which were restored to normalcy with CR treatment. This restorative effect clearly suggests the specific protective effect of CR in modulating the ACR-induced mitochondrial dysfunction. Further, MTT assay was performed to confirm the fate of NADPH-dependant oxidoreductases. Our data revealed the ability of CR to significantly restore the reduced MTT level induced by ACR exposure. The ADP/ATP assay is a quantitative assay revealing the energy status of the cells. ADP/ATP ratio provides a rapid method to measure ADP and ATP levels for the screening of apoptosis, necrosis and proliferation in cells. In the present study, we observed a significant increase in ADP/ATP among ACR exposed worms. Previously, ACR has been shown to affect energy production by inhibiting glycolytic pathway in both in vitro and in vivo models [77,78]. Reduction in ATPase activity was reported previously illustrating the relationship of ACR-induced neurotoxic targets among rodent and fly models. Alterations in the ADP/ATP ratio have been used to differentiate modes of cell death and viability [12,13]. While, increased levels of ATP and decreased levels of ADP indicate proliferating cells, conversely, decreased levels of ATP and increased levels of ADP represent apoptotic or necrotic cells [79]. Interestingly, with ACR exposure we evidenced increased levels of ADP/ATP ratio which was effectively attenuated by CR co-treatment.

In the transgenic strain, following ACR exposure we measured neuronal viability by measuring the loss of expression of a GFP in DA neurons. We found a significant loss of GFP expression in CEP dopaminergic neurons after ACR treatment which was visualised by diminished fluorescence in photomicrographs. Interestingly, CR treatment showed a marked increase in GFP expression which is evident by the enhanced increase in green pixel number, a finding which clearly suggests the neuroprotective efficacy of CR in *C. elegans*. We have previously employed this transgenic strain and demonstrated the potential of Monocrotophos, an organophosphorous insecticide to induce DAergic degeneration and also the modulatory effect of several compounds [80].

Lifespan is a biological process controlled by several genetic and biological factors. Extensive studies on longevity trials in various animal models suggest that the components responsible for lifespan extension affect gene expression, signaling pathways and reduce oxidative stress [81]. Oxidative stress shortens the lifespan of worms. We employed *MEV-1* mutant strains which are short lived and more importantly sensitive to oxidative stress and aging. Data obtained in our present lifespan experiment suggests that CR enrichment resulted in a significant extension of lifespan of both wild type N2 worms and also short-lived *MEV-1* mutant. *MEV-1* mutants which accumulate markers of aging i.e., fluorescent material and protein carbonyls [82,83]. Further studies are essential to understand the underlying mechanisms by which CR treatment caused increase in life extension.

## Conclusion

In summary, our results suggest the propensity of Crocin to alleviate acrylamide-induced phenotype, physiological changes and dopaminergic neurodegeneration in *C. elegans*. Crocin markedly attenuated ACR induced oxidative stress, mitochondrial dysfunction and significantly enhanced the life span among acrylamide-exposed worms. Taken together, our findings clearly reveal the neurotherapeutic benefits of Crocin and suggest its possible usage as an adjuvant in the management against oxidative –mediated neurodegenerative disorders.

## Conflict of Interest

The authors declare that there are no conflicts of interest.



## Acknowledgments

The authors thank the Director, CFTRI for his encouragement. The first author thanks the Indian Council of Medical Research (ICMR), New Delhi, for the award of Senior Research Fellowship, while the second author thanks UGC for the award of junior/ Senior Research Fellowships.

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