Pharmacogn. Mag.
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ORIGINAL ARTICLE

5,7-Dihydroxy-4-Methoxyflavone a Bioactive Flavonoid Delays Amyloid Beta-induced Paralysis and Attenuates Oxidative Stress in Transgenic *Caenorhabditis elegans*

Jyotsna Asthana[1,2], B. N. Mishra[2], Rakesh Pandey[1]

[1]Department of Microbial Technology and Nematology, CSIR-Central Institute of Medicinal and Aromatic Plants, [2]Department of Biotechnology, Dr. A. P. J. Abdul Kalam Technical University, Lucknow, Uttar Pradesh, India

Submitted: 04-07-2017 Revised: 09-08-2017 Published: 28-06-2018

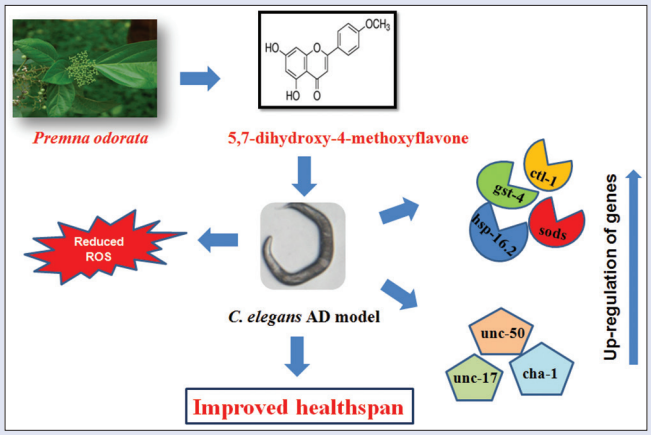
ABSTRACT

Background: The various herbal remedies have been used in Ayurveda which symbolizes traditional medicine system since ancient times. The increasing popularity of herbal medicines has prompted us toward the development of natural therapeutics for preventing neurodegenerative disease such as Alzheimer's disease (AD) in living organisms. This study focused on a flavonoid compound 5,7-dihydroxy-4-methoxyflavone (DMF) also known as acacetin which is a major constituent of *Premna odorata* (L.) plant. This bioactive flavonoid exhibits several medicinal properties such as antimicrobial, anti-inflammatory, antioxidant, and anti-carcinogenic. **Objective:** The aim is to determine the protective effects of DMF against amyloid beta (Aβ)-induced toxicity and oxidative stress in transgenic *Caenorhabditis elegans* model of AD. **Materials and Methods:** The therapeutic potential of DMF treatments (5, 25, and 50 μM) was investigated to counteract Aβ paralysis and oxidative stress through paralysis assay, reactive oxygen species (ROS) detection, protein carbonylation, aldicarb assay, and mRNA quantification using transgenic *C. elegans* model of AD. **Results:** The present study reports that DMF effectively delayed Aβ-induced paralysis, attenuated ROS level reduced protein carbonylation and conferred aldicarb resistance. In addition, DMF was also found to up-regulate the expression of stress modulating (*sod-1, sod-2, sod-3, ctl-1, hsp-16.2,* and *gst-4*), acetylcholine transporter(*unc-17*), regulator of nicotinic acetylcholine receptor (*unc-50*), and choline acetyltransferase (*cha-1*) related genes. **Conclusion:** These findings suggest DMF may provide protection against Aβ toxicity and oxidative stress due to its antioxidant activity. Therefore, the bioactive flavonoid DMF may provide invaluable medicinal and health benefits which can delay the onset of age-related diseases.

Key words: 5,7-dihydroxy-4-methoxyflavone, Alzheimer's disease, *Caenorhabditis elegans*, flavonoid, neurodegenerative disease

SUMMARY

- DMF effectively delayed amyloid beta induced paralysis in transgenic *C. elegans* model of AD
- DMF reduced intracellular ROS level, protein carbonylation and conferred aldicarb resistance in transgenic worms
- DMF up-regulated the expression of stress modulating (*sod-1*, *sod-2*, *sod-3*, *ctl-1*, *hsp-16.2* and *gst-4*), acetylcholine transporter (*unc-17*), regulator of nicotinic acetylcholine receptor (*unc-50*) and choline acetyltransferase (*cha-1*) associated genes
- DMF exhibited protection against amyloid beta toxicity and oxidative stress due to its antioxidant activity.



Abbreviations used: DMF: 5, 7-dihydroxy-4-methoxyflavone; ROS: Reactive oxygen species; *C. elegans*: *Caenorhabditis elegans*; AD: Alzheimer's disease; Aβ: Amyloid beta; DMSO: Dimethyl sulphoxide; NGM: Nematode growth media; ACh: Acetylcholine; Ald: Aldicarb; APP: Amyloid precursor protein.

Correspondence:

Dr. Rakesh Pandey,
Department of Microbial Technology and Nematology Department, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow - 226 015, Uttar Pradesh, India.
E-mail: r.pandeycimap@gmail.com

DOI: 10.4103/pm.pm_290_17

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INTRODUCTION

Alzheimer's disease (AD) is a growing menace for modern societies which increases with age and occurs with frequency of 5% in people above the age of 60 years. According to AD international report, approximately 36 million people were affected from this disease.[1] Although, the effective treatment has not been found yet for this disease. Therefore, it is necessary to focus our research to develop new possible disease-modulating natural bioactive compounds. The medicinal plants and their secondary metabolites are rich sources of biologically active compounds. The naturally occurring dietary flavonoids have been demonstrated to possess a wide range of biological activities such as antioxidative, detoxification, anti-inflammatory, and regulation of signalling pathway which have been considered to be promising

Cite this article as: Asthana J, Mishra BN, Pandey R. 5, 7-dihydroxy-4-methoxyflavone a bioactive flavonoid delays amyloid beta-induced paralysis and attenuates oxidative stress in transgenic *caenorhabditis elegans*. Phcog Mag 2018;14:S57-64.

approach for preventing age-related neurodegenerative diseases.[2-4] *Premna odorata* L.(*Lamiaceae*) plant is a medicinal plant conventionally used in the treatment of tuberculosis, vaginal irrigation, and respiratory diseases.[5,6] 5,7-dihydroxy-4-methoxyflavone (DMF) is most abundant flavonoid found in this plant which exhibits several pharmacological and medicinal properties.[7-9] Besides different properties of DMF, in our previous study lifespan extension and stress resistance properties of DMF were reported in wildtype *Caenorhabditis elegans*.[10] Therefore, it has been previously reported that the anti-aging compounds also possess potential to protect against several age-related neurodegenerative diseases.[11-13] AD is characterized by the aggregation of amyloid beta peptide ($A\beta_{1-42}$) and cell death in the brain. The amyloid beta (Aβ)-induced toxicity and oxidative stress both are thought to be genetic and lifestyle-related major risk factors for AD which may cause neuronal cell injury in the brain.[14,15] Aβ is one of the important proteins in the AD which can take distinct conformation, form aggregates, and act together with different cellular processes. Furthermore, the aggregation of oligomeric Aβ form senile plaques which are abundant in the brain of AD patient. The oxidative damage elevates the formation of Aβ resulted from cleavage product of amyloid precursor protein and aggregation in AD.[16-18] The model organism *C. elegans* has gained increasing importance for studying biological processes on the molecular and cellular level because they are inexpensive and easy to experimentally manipulate.[19,20] The genetics of soil nematode *C. elegans* is being deployed in opposition to few of the most intractable and economically significant neurodegenerative diseases in modern medicine. To investigate the neuroprotective effect of DMF on the whole organism, we utilized the unique transgenic *C. elegans* model (CL4176) expressing the human $A\beta_{1-42}$ which exhibits paralysis on the induction of human $A\beta_{1-42}$. To understand the therapeutic potential of flavonoid DMF against AD, we performed several experiments with different doses of DMF (5, 25, and 50 μM) using a transgenic *C. elegans* models CL4176 and CL2006.

The present study suggests that flavonoid DMF delays Aβ-induced paralysis, alleviates oxidative stress, reduces protein carbonyl contents and provides resistance against aldicarb in transgenic *C. elegans* model of AD. Therefore, the study findings may provide a better understanding of neuroprotective and antioxidant properties of DMF to develop new strategies for natural therapy against age-related neurodegenerative diseases, which, however, requires further investigation in other model organisms.

MATERIALS AND METHODS

Test compound

DMF [Figure 1] was obtained from Sigma-Aldrich (St. Louis, MO, USA), stock solution (10 mM) of DMF was prepared in 10% of dimethyl sulfoxide (DMSO, Sigma-Aldrich). The final concentration of DMSO did not exceed 0.05% in the food (*Escherichia coli* strain OP50). The treatments for experimental strains were added directly to the food source, and the test concentrations of DMF were used as 5, 25, and 50 μM with respect to 0.05% DMSO control for experiments.

Caenorhabditis elegans strains, culture and maintenance

C. elegans strains, namely, wild-type strain N2 (Bristol), the transgenic nematode strains CL4176 (*smg*-1^{ts} [*myo-3*/$A\beta_{1-42}$ long 3'-untranslated region]), CL2006 (*unc-54*/$A\beta_{1-42}$), and *E. coli* OP50 were obtained from *Caenorhabditis* Genetics Centre (University of Minnesota, Minneapolis, MN, USA). The expression of muscle-specific $A\beta_{1-42}$ in CL4176 depends on temperature up-shift from 16°C to 25°C, whereas CL2006 strain constitutively expresses a body wall muscle-specific $A\beta_{1-42.}$ The wild-type N2 and transgenic CL2006 were cultured at 20°C, whereas CL4176 at 16°C on solid nematode growth medium (NGM) seeded with live *E. coli* OP50 food lawn. The age synchronized worms were obtained after sodium hypochlorite treatment.[21] The worms were fed with compound starting from the egg.

Paralysis assay

The accumulation of protein aggregates and oxidatively damaged proteins play a significant role in neurological decline which contributes aging and age-related disease.[22,23] Thus, the paralysis assay was performed to investigate the potential efficacy of DMF treatments by using a transgenic strain CL4176. The age synchronized CL4176L1 worms (100 worms/plate) were grown on NGM plates containing live OP50 food with or without DMF treatments (5, 25, and 50 μM) at 16°C. After reaching the L3 stage, the temperature was increased from 16°C to 25°C to induce the expression of $A\beta_{1-42}$.[24] The paralysis in the worms was scored 18–20 h after temperature upshift at 2 h interval until all the worms were paralyzed. The worms were counted as paralyzed if they failed to respond after prodding. The assay was performed three times independently. Kaplan–Meier method was used to compare the survival curves. The time in hours posttemperature upshift at which 50% of worms were completely paralyzed/dead (PT_{50}) was calculated from the paralysis curve. The experiment was performed in three independent trials.

Reactive oxygen species detection in transgenic *Caenorhabditis elegans*

The aggregation of Aβ peptide causes an elevation in free radical formation and intracellular ROS which create an imbalance in cellular redox system. The intracellular ROS levels were measured in DMF treated (5, 25, and 50 μM) or untreated day 4 transgenic *C. elegans* (CL2006) using 2,7-dichlorofluorescein diacetate (DCF-DA) dye. The age synchronized worms were washed with M9 buffer and collected into 300 μL of phosphate buffered saline with 0.1% Tween 20 in the Eppendorf tubes. The worms were transferred into the 96-well plate and 1.5 μL of 10 mM DCF-DA dye were added to each well at 37°C.[25] The quantification of fluorescence was recorded for 120 min at every 20 min intervals using SpectraMax M2 multimode microplate reader (molecular devices) at 485 nm excitation and 530 nm emission. The experiment was performed three times independently.

Protein carbonylation assay

The effect of DMF (5, 25 and 50 μM) treatments on protein carbonyl content was demonstrated in wild-type N2 and CL2006 transgenic

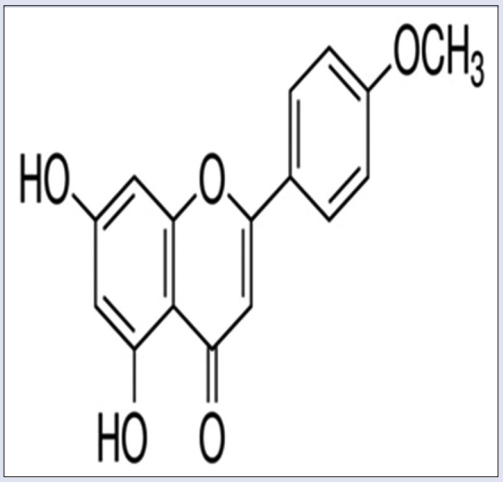

Figure 1: Structure of 5,7-dihydroxy-4-methoxyflavone compound

worms according to the protocol described by Hawkins *et al.*[26] The adult day 5 worms were homogenized to obtain diluted protein concentration approximately 1 mg/ml. About 250 µL of diluted homogenate mixture was taken into separate centrifuge tubes. The 250 µL volume of 10 mM 2,4-dinitrophenylhydrazine (dissolved in 2 N HCl) was added to tubes, vortexed and kept in the dark for 20 min. The 125 µl volume of 10% (w/v) trichloroacetic acid was added, mixed thoroughly and kept at −20°C for 20 min. The tubes were then centrifuged, the obtained pellet was washed off thrice with organic solvent ice-cold ethanol:ethylacetate (1:1 v/v). The pellet was redissolved in 1 ml of 6 M guanidine hydrochloride and the absorbance was taken at 370 nm. The experiment was performed in three independent manners.

Aldicarb assay

This assay was carried out to investigate the effect of DMF (5, 25 and 50 µM) on day 2 wild-type N2 and CL2006 worms in the presence of acute and chronic aldicarb. For acute aldicarb assay, the aldicarb (1 mM) plates were prepared before 1 h of experiment and age synchronized embryos were grown on treatment NGM plates containing live OP-50 till their adulthood. The adult wildtype and CL2006 worms were washed off with M9 and placed on aldicarb plate and scored paralysis for 6 h at the interval of every 30 min.[27,28] The worms which did not response to repeated prodding considered as paralyzed.

In the chronic aldicarb assay, the plates were prepared by adding aldicarb (final concentration 0.1 mM) to the agar before pouring. For the chronic aldicarb assay, DMF treatment/aldicarb was given from embryo stage till death in wild-type and CL2006 worms. The lifespan of wildtype and CL2006 worms were recorded till their death. The assay was repeated three times independently.

Quantitative real-time polymerase chain reaction

The age synchronized CL2006 worms were treated with DMSO (0.05%) or 25 µM DMF for 72 h and grown under standard laboratory conditions at 20°C. Total RNA was isolated from day 2 adult worms using RNAzol (MRC, USA). The first strand cDNA was synthesized using the cDNA synthesis kit (Invitrogen) according to manual's instruction. The mRNA expression level of genes *sod-1*, *sod-2, sod-3, hsp-16.2, ctl-1, gst-4, unc-17, unc-50,* and *cha-1* was quantified with respect to housekeeping gene *β-actin* (actin-1) used as an endogenous control. Furthermore, this experiment was performed using the SYBR green detection method on an Applied Bio-systems 7900HT fast real-time polymerase chain reaction (PCR) system. Relative-fold changes of the expressed gene were calculated using the comparative C_t ($\Delta\Delta C_t$) method. The experiment was repeated in three independent trials.

Statistical analysis

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

RESULTS

5,7-dihydroxy-4-methoxyflavone alleviates amyloid beta-induced paralysis in transgenic *Caenorhabditis elegans*

To determine the effects of DMF treatments on Aβ-induced toxicity, we conducted paralysis assay using temperature inducible transgenic strain CL4176 which expresses human $A\beta_{1-42}$ peptide in muscle cells. The age synchronized worms were fed with different concentrations of DMF (5 µM, 25 µM, and 50 µM) or control (0.05% DMSO) for 48 h before temperature upshift and then scored for paralysis. The result exhibited time course study of Aβ expression induced paralysis by comparing survival curves between nontreated control and DMF treated CL4176 worms. Furthermore, we observed that control worms were started to become paralyzed and die due to the expression of $A\beta_{1-42}$ after 24 h of the temperature upshift. Therefore, Figure 2a represents that Aβ-induced paralysis was delayed in DMF treated worms as compared to nontreated control. In addition, we determined PT_{50} value, the time at which 50% of worms were paralyzed or dead after temperature upshift.[29,30] In contrast, the 50% worms were paralyzed or dead in control (0.05% DMSO) worm at 29 h (PT_{50}) after temperature upshift. The PT_{50} in 25 µM was significantly increased by 34 h ($P = 0.01$) as compared to other tested concentrations 5 µM ($PT_{50} = 32$ h, $P = 0.03$), 50 µM ($PT_{50} = 30$ h, $P = 0.23$) and control [Figure 2b and Table 1]. The Aβ-induced paralysis was delayed in the presence of all three concentrations of DMF (5 µM, 25 µM and 50 µM). The maximum percentage of transgenic worms were found nonparalyzed in the presence of DMF 25 µM as compared to other tested doses and control. Thus, DMF possesses potential to alleviate Aβ-induced toxicity/paralysis in transgenic AD model.

5,7-dihydroxy-4-methoxyflavone attenuates reactive oxygen species level in amyloid beta expressing transgenic *Caenorhabditis elegans*

Previous studies have established that oxidative stress plays a pivotal role in neurodegenerative diseases like AD.[15,22] This study investigated that whether antioxidant properties of DMF might provide protection against Aβ-induced toxicity in transgenic AD model. To determine the protective effect of DMF against intracellular reactive oxygen species (ROS), transgenic CL2006 worms were grown in presence or absence of DMF treatments (5, 25 and 50 µM) as compared to nontreated control (0.05% DMSO). The DMF supplementation exhibited low intracellular accumulation of ROS in 25 µM (39%, $P = 0.0005$) followed by 5 µM (60.7%, $P = 0.0013$) and 50 µM (62.5%, $P = 0.0006$) in treated CL2006 worms as compared to untreated control [Figure 3]. These findings indicate that alleviation in Aβ induced toxicity might be due to antioxidative properties of DMF which provides protection against intracellular ROS in the transgenic worms.

5,7-dihydroxy-4-methoxyflavone reduces the protein carbonyl content in wild-type and transgenic *Caenorhabditis elegans*

The most proximal cause of age-related neurodegenerative diseases is an accumulation of cellular and molecular damage over time driven by the production of ROS in the normal mitochondrial functions. The increments in oxidative damage cause disruption in protein structure and function by altering the intracellular protein homeostasis and affecting the stability of oxidative protein modification.[31] For this reason, the present study demonstrated the effect of DMF treatments (5, 25, and 50 µM) on protein carbonyl content in wild-type N2 worms and transgenic *C. elegans* (CL2006). The result exhibited protein carbonyl content level in 25 µM (43.9%, $P < .0001$), followed by 5 µM (68.6%, $P < .0001$) and 50 µM (61.7%, $P < .0001$) in DMF exposed wild-type worms as compared to 100% control (0.05% DMSO) [Figure 4a]. Similarly, DMF treated CL2006 worms exhibited protein carbonyl contents level in 25 µM (59.5%, $P < 0.0001$) followed by 5 µM (71.3%, $P = 0.0008$) and 50 µM (74.6%, $P = 0.0081$) as compared to 100% control [Figure 4b]. The result suggests that dietary supplementation of DMF notably decreased the level of protein carbonyl content in wild-type and transgenic *C. elegans*, thereby attenuating the oxidative stress and protecting against AD.

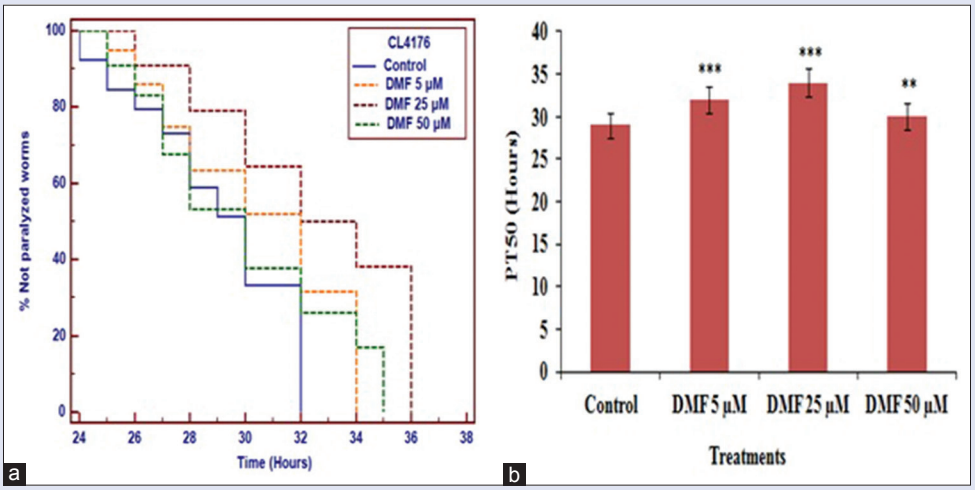

Figure 2: (a) Effect of 5,7-dihydroxy-4-methoxyflavone on Aβ induced paralysis in CL4176 transgenic strain. Age synchronized CL4176 worms were fed with 5,7-dihydroxy-4-methoxyflavone (5, 25 and 50 µM) or vehicle control on nematode growth medium plate containing OP-50 as food source for 48 h prior to temperature up shift and scored for paralysis. To induce the transgene expression temperature was up shifted from 16°C to 25°C. 5,7-dihydroxy-4-methoxyflavone (5, 25 and 50 µM) treated CL4176 worms were found to be non-paralyzed in comparison to control worms. The data was processed using the Kaplan–Meir survival analysis in Medcalc 12.7.7.0 software. (b) PT_{50} values in hours (the time at which 50% of worms were paralyzed or dead after temperature upshift) for all 5,7-dihydroxy-4-methoxyflavone treatments (5, 25 and 50 µM) were calculated from paralysis curve as compared to untreated control. Differences between the data were considered significant at $P \leq 0.05$. Error bars represent means ± standard error of the mean. $^{**}P \leq 0.0001$

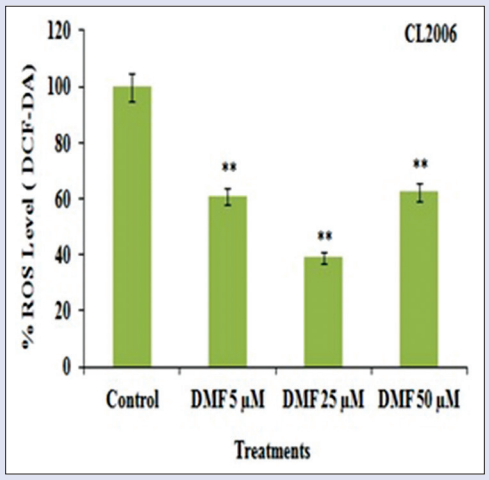

Figure 3: Measurement of reactive oxygen species levels in 5,7-dihydroxy-4-methoxyflavone treated (5, 25 and 50 µM) CL2006 strain. The age synchronized day 4 treated and non-treated live worms were utilized to measure intracellular reactive oxygen species using 2,7-dichlorofluorescein diacetate dye at 37°C. The result exhibited low level of intracellular reactive oxygen species in 5,7-dihydroxy-4-methoxyflavone supplemented CL2006 worms as compared to vehicle control. The graph was plotted as relative change in reactive oxygen species compared to control at 100%. The data is statistically analyzed using ANOVA in ASSISTAT 7.7 beta statistical assistance software. Error bars represent means ± standard error of the mean. $^{**}P \leq 0.0001$

5,7-dihydroxy-4-methoxyflavone confers resistance against acute and chronic aldicarb

Aldicarb is acetylcholine (ACh) esterase inhibitor which causes accumulation of ACh at the synapse which overstimulates muscles leading to paralysis in the worms.[31] To investigate the protective effect of DMF on acute and chronic aldicarb (Ald) resistance in wildtype and CL2006 worms, we conducted the aldicarb assay with or without DMF treatments (5, 25 and 50 µM). The result exhibited percentage of nonparalyzed AldiDMF treated wildtype worms in 5 µM (45.5%, $P = 0.0175$), followed by 25 µM (73.3%, $P = 0.0009$) and 50 µM (56.6%, $P = 0.002$) as compared to untreated control (0.05% DMSO) [Figure 5a]. Similarly, we observed maximum percentage of nonparalyzed DMF treated CL2006 worms in 25 µM (68.3%, $P = 0.0003$) followed by 5 µM (43.33%, $P = 0.0009$) and 50 µM (30%, $P = 0.021$) in the presence of 1 mM aldicarb as compared to untreated control [Figure 5b].

Table 1: Delay of paralysis in *Caenorhabditis elegans* transgenic strain CL4176

Treatments	Mean±SE (h)	Minimum (h)	Maximum (h)	PT_{50} (h)	*P* value
Control	29.06±0.305	24.0	32.0	29.0	
DMF 5 µM	30.49±0.354	24.0	34.0	32.0	<0.0001**
DMF 25 µM	32.44±0.402	26.0	36.0	34.0	<0.0001 **
DMF 50 µM	29.92±0.391	25.0	35.0	30.0	<0.0001 **

The PT_{50} values in hours were calculated from paralysis curve after temperature upshift for all DMF treatments with respect to untreated control. Differences between the data were considered significant at $P \leq 0.05$. Error bars represent means±SEM. SEM: Standard error of mean; $^{**}P \leq 0.0001$; DMF: 5,7-dihydroxy-4-methoxyflavone; SE: Standard error

The DMF treated or untreated wildtype and CL2006 worms were exposed from embryos with or without aldicarb treatment (0.1 mM) to investigate the involvement of ACh in the lifespan extension. The worms treated with aldicarb became slightly contracted, and these worms died earlier than the DMF treated worms. The mean lifespan of AldDMF treated wildtype worms was observed in 5 µM (4.34 ± 0.19), 25 µM (5.14 ± 0.17) and 50 µM (4.47 ± 0.17) as compared to untreated control (0.05% DMSO) (3.60 ± 0.13) [Figure 5c and Table 2]. Whereas, AldDMF treated CL2006 worms exhibited mean lifespan in 5 µM (3.08 ± 0.12), 25 µM (3.65 ± 0.14) and 50 µM (3.20 ± 0.14) with respect to untreated control (2.46 ± 0.09) [Figure 5d and Table 2]. Furthermore, the DMF was able to delay the sensitivity of wildtype and CL2006 worms in response to aldicarb treatment. Therefore, the results suggest that DMF provide resistance to the worms against acute and chronic aldicarb, thereby DMF modulating the ACh mediated neurotransmission.

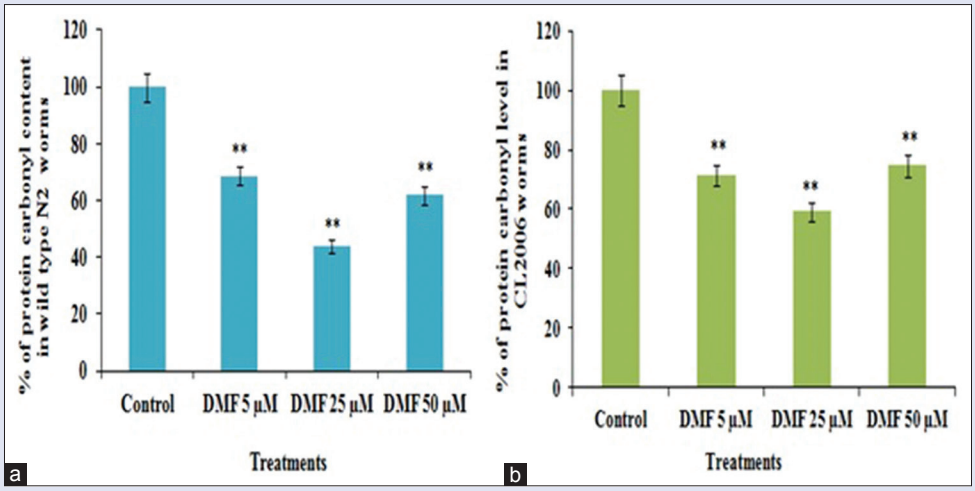

Figure 4: (a) Effect of 5,7-dihydroxy-4-methoxyflavone treatments (5, 25 and 50 µM) on protein carbonyl contents in N2 worms. Age synchronized wild type worms were grown on OP-50 seeded nematode growth medium plate with or without of 5,7-dihydroxy-4-methoxyflavone. The 5,7-dihydroxy-4-methoxyflavone treated or nontreated day 5 N2 worms were used for the protein carbonylation assay. The 5,7-dihydroxy-4-methoxyflavone treatments were found to decrease the level of protein carbonyl contents in treated N2 worms as compared with control. Error Bars represent means ± standard error of the mean. $^{**}P \leq 0.0001$. (b) Effect of 5,7-dihydroxy-4-methoxyflavone treatments (5, 25 and 50 µM) on protein carbonyl contents in CL2006 worms. 5,7-dihydroxy-4-methoxyflavone treated (5, 25 and 50 µM) CL2006 worms were found to decrease the protein carbonyl content in comparison to control worms. The graph was plotted as relative change in protein carbonyl content in comparison to control at 100%. The data is statistically analyzed using ANOVA in ASSISTAT 7.7 beta statistical assistance software. Differences between the data were considered significant at $P \leq 0.05$ error bars represent means ± standard error of the mean. $^{**}P \leq 0.0001$

Table 2: Lifespan analysis of wildtype and CL2006 worms in the exposure of chronic aldicarb at 20°C

Strains	Treatments (µM)	Mean lifespan	±SD	±SE	Sample size (*n*)	Percentage change	*P* value
Wildtype (N2)	Control + aldicarb	3.60	1.71	0.13	160		
	DMF 5 µM + aldicarb	4.34	2.40	0.19	160	20.5	<0.0001
	DMF 25 µM + aldicarb	5.14	2.27	0.17	160	42.7	<0.0001
	DMF 50 µM + aldicarb	4.47	2.24	0.17	160	24.1	<0.0001
CL2006	Control + aldicarb	2.46	1.26	0.09	160		
	DMF 5 µM + aldicarb	3.08	1.56	0.12	160	25.2	<0.0001
	DMF 25 µM + aldicarb	3.65	1.86	0.14	160	48.37	<0.0001
	DMF 50 µM + aldicarb	3.20	1.81	0.14	160	30.8	<0.0001

The wildtype N2 and CL2006 worms were treated with different concentrations of DMF (5 µM, 25 µM and 50 µM) and exposed with 0.1 mM aldicarb. The mean lifespan was calculated as the average number of days the worms survived in each test concentration. The data were processed using the Kaplan–Meir survival analysis in Medcalc 12.7.7.0 software. DMF: 5,7-dihydroxy-4-methoxyflavone; SE: Standard error; SD: Standard deviation

5,7-dihydroxy-4-methoxyflavone modulates the mRNA expression level of stress-responsive and Alzheimer's disease-related genes

The protection against Aβ toxicity and oxidative stress in the transgenic *C. elegans* of AD model confer due to antioxidant property of DMF. Therefore, to investigate protective effects of DMF treatment against Aβ toxicity and oxidative in transgenic worms, the quantitative real-time PCR was performed. The DMF (25 µM) treatment significantly upregulated the mRNA expression level of *sod-1* (2.68-fold, $P = 0.0006$), *sod-2* (3.17 fold, $P < 0.0001$), *sod-3* (4.26 fold, $P < 0.0001$), *hsp-16.2* (2.95 fold, <0.0001), *ctl-1* (2.20 fold, $P = 0.0009$), *gst-4* (3.01 fold, $P < 0.0001$), *unc-17* (1.43, $P = 0.0016$), *unc-50* (3.38, $P < 0.0001$) and *cha-1* (3.22, $P = 0.0076$) as compared to endogenous control *β-actin* [Figure 6]. The up-regulation of these stress-responsive, ACh synthesis and transporter-related genes suggest that DMF (25 µM) treatment might be contributed to the protection against Aβ toxicity and oxidative stress in the transgenic worms of AD.

DISCUSSION

The increasing occurrence of neurodegenerative diseases has become serious public health problems in the modern society. However, these deleterious changes due to neurodegenerative diseases have direct impact on physical, structural, and biological components of an organism that ultimately cause disease and death. These changes adversely affect liveliness of human's life and enhance the mortality rate as a function of time. To overcome this problem, it is important to search for bioactive molecules which can be utilized in the prevention of neurodegenerative diseases. Moreover, various pharmacologically bioactive compounds from natural resources have become potential candidates to develop effective drug against age-related diseases.[32] The maintenance of protein homeostasis plays major role in the healthspan and longevity promotion of organisms. The increased oxidative stress disrupts protein homeostasis with aging which leads to aggregation of misfolded proteins Aβ causes AD. The neurodegeneration in AD is characterized by progressive loss of memory and behavior in elderly population.[33] The major causative agents of neurodegeneration in AD are cerebral degeneration, neuronal

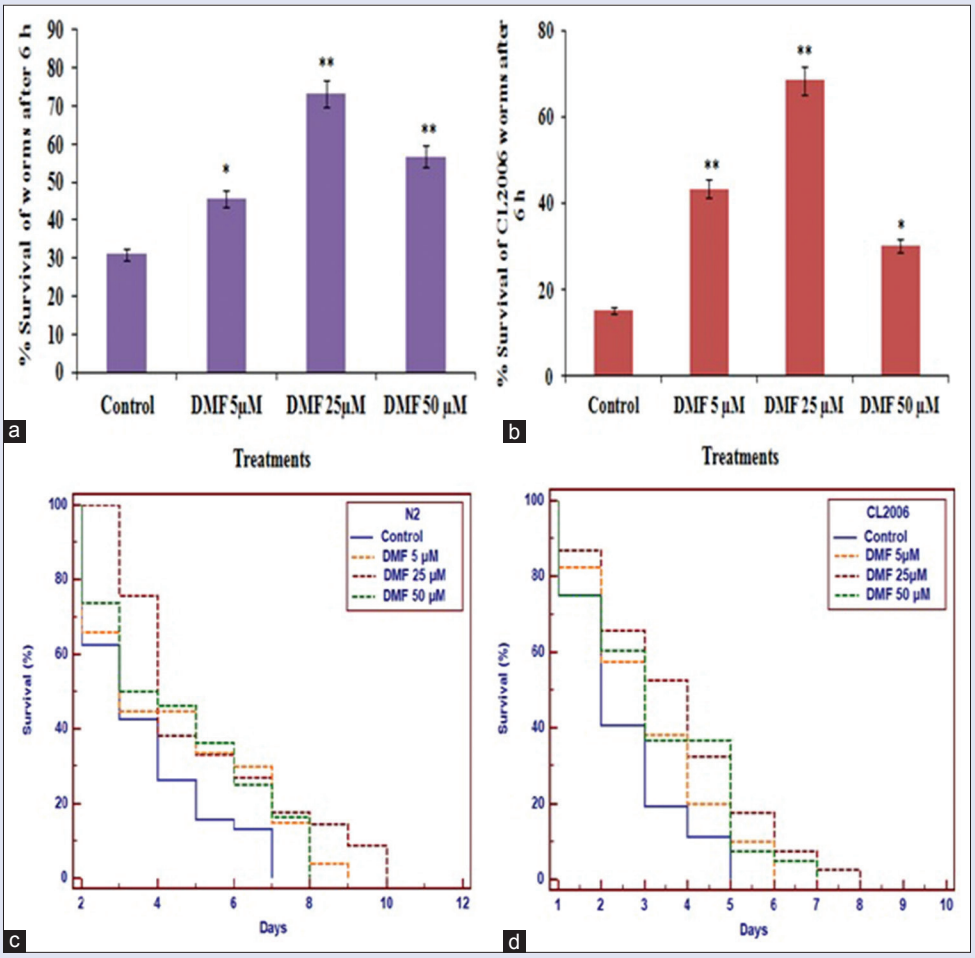

Figure 5: (a) Acute aldicarb resistance in 5,7-dihydroxy-4-methoxyflavone supplemented wild type worms. The age synchronized 5,7-dihydroxy-4-methoxyflavone treated or untreated wildtype worms were exposed to aldicarb (1 mM) for 6 h. 5,7-dihydroxy-4-methoxyflavone treatments (5, 25 and 50 μM) delay the effect of aldicarb on wildtype worms. Thus, 5,7-dihydroxy-4-methoxyflavone provides resistance to the worms in the presence of aldicarb treatment as compared with control. Differences between the data were considered significant at $P \leq 0.05$. Error Bars represent means ± standard error of the mean. *$P \leq 0.05$, **$P \leq 0.0001$. (b) Acute aldicarb resistance in 5,7-dihydroxy-4-methoxyflavone supplemented CL2006 worms. The age synchronized 5,7-dihydroxy-4-methoxyflavone treated or untreated CL2006 worms were exposed to aldicarb (1 mM) for 6 h. 5,7-dihydroxy-4-methoxyflavone treatments (5, 25 and 50 μM) delay the effect of aldicarb on CL2006 worms. Thus, 5,7-dihydroxy-4-methoxyflavone provides resistance to the worms in the presence of aldicarb treatment as compared with control. Differences between the data were considered significant at $P \leq 0.05$. Error bars represent means ± standard error of the mean. *$P \leq 0.05$, **$P \leq 0.0001$. (c) In chronic aldicarb assay, the age synchronized N2 wildtype worms were exposed to different concentrations of 5,7-dihydroxy-4-methoxyflavone (5, 25 and 50 μM) in the presence of aldicarb treatment (0.1 mM). 5,7-dihydroxy-4-methoxyflavone treatments exhibited significant increase in the lifespan of N2 worms in comparison to control in the presence of aldicarb. The lifespan data were processed using the Kaplan–Meir survival analysis in Medcalc 12.7.7.0 software. (d) The age synchronized CL2006 worms were exposed to different concentrations of 5,7-dihydroxy-4-methoxyflavone (5, 25 and 50 μM) in the presence of aldicarb treatment (0.1 mM). 5,7-dihydroxy-4-methoxyflavone treatments exhibited significant increase in the lifespan of CL2006 worms in comparison to control in the presence of aldicarb. The lifespan data were processed using the Kaplan–Meir survival analysis in Medcalc 12.7.7.0 software

cell death, and abnormal accumulation of misfolded $A\beta_{1-42}$ peptides in the brain nerve cells.[33,34] The Aβ-induced toxicity causes neurotoxic effects in the organisms which lead to impairment in the neurological function. Previous studies have reported that bioactive molecules with longevity promoting and stress modulating properties also possess neuroprotective effect and reduce Aβ toxicity in *C. elegans*.[28,35,36] We have recently reported anti-aging and stress modulating potential of flavonoid DMF in wildtype *C. elegans*.[10] Therefore, the present article focused on the protective effects of DMF against AD by targeting Aβ toxicity and oxidative stress using transgenic *C. elegans*. To address this aspect, we utilized the unique transgenic strain CL4176 which express the human $A\beta_{1-42}$ in muscle tissues under temperature inducible system to study the anti-paralysis effect of flavonoid DMF. The present findings suggest that DMF treatments (5 μM, 25 μM and 50 μM) delayed Aβ-induced paralysis in transgenic worms as compared to untreated control indicating that DMF protects the worms against Aβ toxicity [Figure 2a]. The most significant anti-paralysis effect was found in the presence of 25 μM DMF as compared to other tested doses (5 μM and 50 μM) and untreated DMSO control. Previous investigations have reported that bioactive molecules might be beneficial at low concentrations and detrimental at high concentrations.[37,38] This biphasic dose response phenomenon is known as hormesis and caused by various bioactive molecules such as flavonoids, polyphenols, and others natural and synthetic molecules.[39] We also observed that 50 μM DMF exhibited less effective to *C. elegans* healthspan while better improvement in

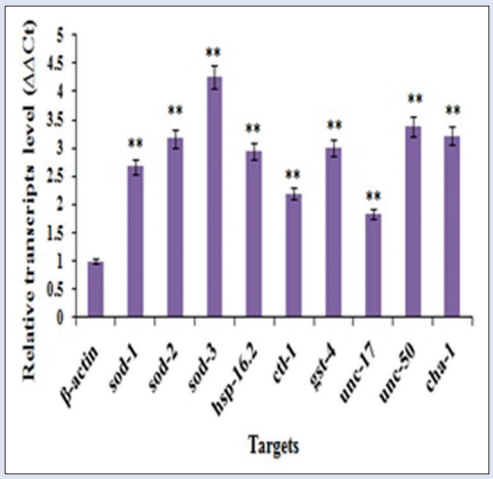

Figure 6: The relative quantification of stress responsive and AD related genes in 5,7-dihydroxy-4-methoxyflavone (25 μM) exposed CL2006 worms. 5,7-dihydroxy-4-methoxyflavone treatment upregulates the expression level of genes sod-1, sod-2, sod-3, hsp-16.2, ctl-1 gst-4, unc-17, unc-50 and cha-1. β-actin (act-1) was used as endogenous control and the relative expression level was quantified using real time polymerase chain reaction using Ct (ΔΔCt) method. The data were statistically analyzed using ANOVA in ASSISTAT 7.7 beta statistical assistance software. Error bars represent means ± standard error of the mean. $^{**}P \leq 0.001$

C. elegans healthspan was found at 25 μM concentration of DMF as compared to untreated control. Based on previously reported dose-dependent responses in lifespan assay of DMF,[10] 25 μM DMF was exhibited as optimum concentration other than 5 μM and 50 μM. The protection by DMF against Aβ induced toxicity might be contributed due to its antioxidative properties. Previously, it was reported that soy isoflavone glycitein could delay Aβ-induced paralysis and provide protection against oxidative stress due to antioxidative properties of glycitein.[29] Similarly, it has also been reported previously that *Ginkgo biloba* extract EGb 761 protects against Aβ-induced paralysis and oxidative stress in transgenic *C. elegans*.[36] The Aβ-induced toxicity directly correlates with the oxidative stress in *C. elegans*. Oxidative stress has been postulated as major hallmark of aging-associated neurodegenerative diseases such as, AD.[14] The aggregation of Aβ protein causes elevation in free radical formation and ROS which create imbalance in cellular redox system. Furthermore, DMF supplementation also significantly attenuated intracellular ROS level in the transgenic CL2006 worms as compared to nontreated control worms [Figure 3]. The Aβ aggregates are produced by interaction of free radicals which have been reported as neurotoxic to the brain. Although, oxidative stress is assumed to occur before the Aβ aggregation suggesting that AD can be attributed to continuous exposure of oxidative stress, along with a destabilized cellular redox status.[40,41] The production of ROS due to Aβ peptide causes cellular and functional damage through protein carbonylation suggest that Aβ induced oxidative stress triggers Aβ-induced paralysis in transgenic *C. elegans*.[29,42] The accumulation of protein carbonyl content is the result of oxidative and cellular damage.[43] In addition, DMF also reduced the protein carbonyl content in DMF treated wildtype and transgenic worms as compared to untreated control [Figure 4a and b]. The reduced ACh level due to failure of cholinergic neuron is major cause of Alzheimer's disease.[44] Thus, modulation in synaptic ACh level might be contributed in developing strategy for managing age-related disease such as AD. Furthermore, the aldicarb sensitivity method was performed using wild-type N2 and CL2006 worms to understand the neurotransmission and modulation of synaptic ACh level in AD.[27] The present result based on this method indicates that DMF treatment modulated the ACh mediated neurotransmission at the presynaptic [Figure 5a-d]. In addition, DMF treatment conferred resistance against acute and chronic aldicarb exposure in the wild-type N2 and AD model CL2006 worms [Figure 5a-d]. Thus, DMF mediated protection against amyloid β toxicity is dependent on modulation of ACh level. The free radical theory of aging suggests that elevation in free radicals create imbalance between ROS and antioxidant defense system which disrupts the cellular key components. The group of antioxidant genes which encode antioxidant enzymes in the cellular defense system neutralize the adverse effects of ROS. The cellular antioxidant enzymes such as superoxide dismutase (*sod-1, sod-2 and sod-3*), catalase (*ctl-1*), and glutathione S transferase (*gst-4*) detoxify the cellular ROS and reduce the Aβ toxicity in the organisms.[45] The result exhibited up-regulation of genes, namely, *sod-1, sod-2, sod-3, hsp-16.2, ctl-1, gst-4 unc-17*, *unc-50*, and *cha-1* with respect to endogenous control *β-actin* in the DMF supplemented worms [Figure 6]. The casual relationship between ROS and Aβ toxicity has been long debated in the field.[31] These stress-responsive genes play significant role in cellular response against oxidative stress and provide protection to the organisms.[45] Therefore, elevation in expression of these genes suggests that DMF may provide protection against amyloid β toxicity and oxidative stress due to its antioxidative property. The accumulation of free radicals or insufficient antioxidant supply disrupts cellular redox balance which contributes to aging and age-related neurodegenerative diseases. The disturbance in cellular redox homeostasis leads to impaired cellular function and causes age-related neurodegenerative diseases. Thus, dietary interventions with increased antioxidative defence response will provide protection to cellular system against Aβ-induced toxicity and oxidative stress in the organisms. The *unc-17* gene encodes a synaptic vesicle ACh transporter which is highly conserved in *Drosophila* and humans.[44] UNC-17 plays key role in embryonic development and is involved in cholinergic neurons for loading ACh in synaptic vesicles to which UNC-17 is localized.[44] Whereas, *unc-50* gene encodes an integral membrane protein which is orthologous to Golgi components of *Saccharomyces cerevisiae*. UNC-50 regulates specific ionotropic ACh receptor trafficking to the cell surface and further required for normal synaptic neurotransmission at the neuromuscular junction.[44] The *cha-1* gene encodes a choline acetyltransferase enzyme that synthesize ACh is expressed in the neurons. This gene is necessary for viability, growth, locomotion, and sensitivity to ACh esterase inhibitor of the worms.[44] Therefore, up-regulation of *unc-17*, *unc-52*, and *cha-1* genes in DMF supplemented worms indicating that DMF treatment modulates ACh level and improves cholinergic neurons response in AD worms. Altogether, these findings suggest that the therapeutic potential of flavonoid DMF might be contributed due to its antioxidant and ACh modulating properties in the prevention of AD.

CONCLUSION

This study demonstrated the neuroprotective effects of flavonoid DMF against neurodegenerative disease like AD by reducing oxidative stress. DMF treatments (5, 25 and 50 μM) delayed Aβ-induced paralysis, reduced ROS level and protein carbonyl contents in transgenic worm models of AD. The flavonoid DMF also modulated ACh level and provided resistance against acute and chronic aldicarb. Furthermore, DMF also elevated the mRNA expression of antioxidant and ACh modulating genes in AD worms. This is the first study to report the neuroprotective effects of DMF in transgenic model of AD. Thus, future investigations are needed to develop useful strategies for natural drug-based prevention and treatment of neurodegenerative diseases.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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