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Amyloid-β induced neuropathological actions are suppressed by *Padina gymnospora* (Phaeophyceae) and its active constituent α-bisabolol in Neuro2a cells and transgenic *Caenorhabditis elegans* Alzheimer's model

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ABSTRACT

The inhibition of Aβ peptide development and aggregation is a hopeful curative approach for the discovery of disease modifying drugs for Alzheimer's disease (AD) treatment. Recent research mainly focuses on the discovery of drugs from marine setting due to their immense therapeutic potential. The present study aims to evaluate the brown macroalga *Padina gymnospora* and its active constituent α-bisabolol against $A\beta_{25-35}$ induced neurotoxicity in Neuro2a cells and transgenic *Caenorhabditis elegans* (CL2006 and CL4176). The results of the *in vitro* study revealed that the acetone extract of *P. gymnospora* (ACTPG) and its active constituent α-bisabolol restores the $A\beta_{25-35}$ induced alteration in the oxidation of intracellular protein and lipids. In addition, ACTPG and α-bisabolol inhibited cholinesterase and β-secretase activity in Neuro2a cells. Moreover, the intracellular reactive oxygen species (ROS) and reactive nitrogen species (RNS) production was reduced by ACTPG and α-bisabolol in Neuro2a cells. The decrease in the expression level of apoptotic proteins such as Bax and caspase-3 in ACTPG and α-bisabolol treated group indicates that the seaweed and its bioactive compound have anti-apoptotic property. Further, the *in vivo* study revealed that the ACTPG and α-bisabolol exerts neuroprotective effect against Aβ induced proteotoxicity in transgenic *C. elegans* strains of AD. Moreover it altered the Aβ mediated pathways, lifespan, macromolecular damage and down regulated the AD related gene expression of *ace-1*, *hsp-4* and *Aβ*, thereby preventing Aβ synthesis. Overall, the outcome of the study signifies the neuroprotective effect of ACTPG and α-bisabolol against Aβ mediated AD pathology.

1. Introduction

Alzheimer's disease (AD) is considered as an inevitable neurodegenerative disease that mainly occurs in elderly people, characterized by loss of thinking skills, cognitive dysfunction, and neuropsychiatric damage. The abnormal level of amyloid beta (Aβ) accumulation and tau protein play a key role in the interruption of synaptic cleft and cognitive failure of patients with AD. Generally, the amyloid hypothesis postulates that Aβ induced neurotoxicity is the leading cause of synaptic impairment and subsequent brain deterioration that accentuates the development of AD pathology. The assembly of Aβ peptide states that under systematic conditions, Aβ peptides are monomeric in nature, while in AD condition Aβ peptides are transformed into oligomers and neurotoxic mature fibrils [1]. The Aβ monomers are harmless to brain neurons, but the fibril formation is associated with misfolding of proteins which makes the Aβ peptide to induce neurotoxicity and synaptotoxicity [2]. Further, the Aβ mature fibrils may possibly aid tau phosphorylation, mitochondrial dysfunction, calcium homeostasis, synaptic damage and cognitive decline. The extracellular aggregated Aβ peptide deposition was detected in patients with AD, the major one being $A\beta_{1-40}$ or $A\beta_{1-42}$ peptides. But they also comprise of peptides with smaller sequences such as $A\beta_{25-35}$ peptide made up of a stretch of 11amino acid residues which produces similar neurotoxic effects as those created by its full length Aβ peptides. The neurotoxic Aβ peptide gets detached from amyloid precursor protein (APP) through the sequential cleavage by both β-secretase and γ-secretase cleaving enzymes [3–6].

In the present scenario scientific evidence exemplified that the aggregated Aβ peptides have been projected as a cause of inducing oxidative stress in the cells and play a substantial role in AD pathology.

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The Aβ peptide induced oxidative stress play a crucial role in protein and lipid oxidation which in turn diminish cellular function and induce necrosis, that results in causing cognitive impairment in AD patients [7,8]. The aggregated Aβ peptides are considered as harmful neurotoxins which also affect the synaptic plasticity in brain cortex regions. It indicates that the inhibition of Aβ fibril formation is a hopeful approach for the treatment of AD patients [9]. Although various promising curative agents from natural and synthetic backgrounds are in clinical trials, among the different therapeutic drugs, only few cholinesterase inhibitors (ChEIs) including donepezil, rivastigmine, galantamine and N-methyl-D-aspartate (NMDA) receptor antagonist memantine have revealed satisfactory neuroprotection against AD. These restorative agents are mainly used for short-term relief of cognitive defects in patients with AD [10]. However, the neuropathological, biological and heritable research evidence on Aβ peptide undoubtedly suggest that aiming to target Aβ induced neurotoxicity might be appropriate for the treatment of AD patients. In the search of neuroprotective agents against Aβ induced neurotoxicity, natural derived small molecules are generally analyzed using neuronal cell lines and transgenic animals as model system [11,12]. In addition to the Neuro2A (N2a) cells which are considered as suitable cell lines for Aβ toxicity research, the renowned AD model of *C. elegans* which expresses the human neurotoxic Aβ peptide in the body wall muscles are also used as drug screening systems for understanding the disease mechanism of AD. Moreover this *in vivo* AD model has aided to identify the relationship between the extension lead of lifespan pathways and Aβ proteotoxicity in neurodegenerative diseases [13]. Furthermore, the whole genome sequence of *C. elegans* confirmed that approximately 38% of genes from *C. elegans* have a human ortholog (τ protein and APP) [14]. Hence, *C. elegans* has unique advantages as an *in vivo* model for the investigation of AD and other neurodegenerative disorders [15].

Presently bioactive molecules derived from natural background are increasingly used in the exploration of innovative drugs for the treatment of AD. In general, marine macroalgae or microalgae hold massive amount of secondary metabolites and these small molecules have been used as nutraceuticals, cosmeceuticals and pharmaceutical products [16]. Hence the present study is focused on finding the neuroprotective efficacy of *Padina gymnospora* and its bioactive molecule α-bisabolol, which are identified to hold an extensive range of priceless therapeutic action such as anti-bacterial, anti-quorum sensing, anti-coagulant, anti-oxidant, anti-cholinesterase, anti-cancer, anti-angiogenic, anti-adhesive and anti-amyloidogenic activities [17–19]. The acetone extract of *P. gymnospora* (ACTPG) has been previously reported to possess anti-amyloidogenic property, when studied through cell free *in-vitro* systems [7,20]. However the hopeful neuroprotective effect of *P. gymnospora* and α-bisabolol against Aβ induced neurotoxicity in *in-vitro* cellular models and *in-vivo* transgenic animal systems like *C. elegans* is not available. The positive results of the cell free preliminary investigation of ACTPG and α-bisabolol encouraged us to extent the study by confirming the anti-amyloidogenic property against $A\beta_{25–35}$ peptide induced toxicity in both N2a cells and transgenic *C. elegans*. Over all the present study for the first time undoubtedly shows the neuroprotective potentials of ACTPG and α-bisabolol, which can be used to combat Aβ mediated AD.

2. Materials and methods

2.1. Chemicals and antibodies

MTT (3-(4, 5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide), nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3′-indolyphosphate (BCIP) was purchased from Himedia (Mumbai, India). DCFH-DA (2′,7′ Dichlorodihydrofluorescin diacetate), Rhodamine 123, Thioflavin S and Anti-rabbit IgG secondary antibody were purchased from Sigma (St. Louis, MO, USA). Antibodies against β-actin, caspase-3, β-amyloid, Bcl-2 and Bax were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). $A\beta_{25–35}$ peptide was purchased from GenScript USA Inc. α-bisabolol was obtained from Alfa Aesar. All the other reagents and chemicals used were of analytical grade.

2.2. Evaluation of neuroprotective efficacy of ACTPG and α-bisabolol against $A\beta_{25-35}$ induced neurotoxicity in N2a cells

2.2.1. Cell culture conditions of N2a cells

The mouse neuroblastoma N2a cells were procured from National Centre for Cell Sciences, Pune, India. The N2a cells were cultured in Dulbecco's Modified Eagle Medium in 25-cm^2 flasks containing 10% fetal bovine serum (FBS) and 1X penicillin-streptomycin antibiotic with the supplementation of 5% CO_2 in a moisturized incubator maintained at 37 °C.

2.3. The dose fixing of $A\beta_{25-35}$ peptide, ACTPG and α-bisabolol in N2a cells

For fixing the neurotoxic dosage of Aβ peptide, the N2a cells (2×10^5 cells/ml) were co-incubated with different concentration of $A\beta_{25–35}$ peptide ranging from 10 to 50 μM in 96 well plates for 24 h. Subsequently, to assess the neuroprotective efficiency of ACTPG and α-bisabolol, the cells were pre-treated with different concentration of ACTPG (10–50 μg/ml), α-bisabolol (2–10 μg/ml) and the standard drug donepezil (50 μg/ml) for 2 h, followed by the treatment with fixed concentration of 50 μM of $A\beta_{25-35}$ peptide. After treatment, cells, washed with PBS, were treated with 1 mg/ml of MTT and incubated for 3 h at 37 °C. After incubation, MTT was replaced with an equal volume of dimethyl sulphoxide and was read at 570 nm using a spectrophotometer (Molecular Device Spectramax M3, equipped with Softmax Pro V 5.4.1 software) [21]. The results are displayed as percentage of viable cells when compared with vehicle control.

$$\% \text{ Cell survival} = \frac{\text{Mean absorbance in test wells}}{\text{Mean absorbance in control wells}} \times 100$$

2.4. Treatment schedule and preparation of cell homogenate

The N2a cells were grown in both glass cover slips and 96 well plates for adherence and the cells were co-incubated with fixed concentration of ACTPG (25 and 50 μg/ml) and α-bisabolol (5 and 10 μg/ml) for 2 h and then treated with 50 μM of $A\beta_{25-35}$ peptide for 24 h. For preparing the cell homogenate, after the incubation period, the cells were collected, washed with ice cold PBS buffer and then lysed with lysis buffer (10 mM Tris, 20 mM EDTA and 0.25% Triton X-100; pH-8.0). The cell lysates were subjected to centrifugation at 4000g for 30 min at 4 °C and the supernatant was stored in −80 °C until further use. The protein concentration of all the incubated samples were quantified by Bradford's method.

2.5. Assessment of intracellular reactive oxygen species (ROS) production

The ROS production was assessed by spectroflourimetric and confocal microscopic study [21], by incubating the cells in 96 well plate and coverslip respectively. After the treatment period of 24 h, the growth medium was removed from the incubated samples and subsequently washed with PBS. Then, 10 μM of 2′,7′ Dichlorodihydrofluorescin diacetate (DCFH-DA) was added and kept at 37 °C for 30 min in dark. After incubation, extracellular DCFH–DA was removed by washing the cells again with PBS. Finally, lysis buffer (pH 8.0) was added to the cells and the cell lysate was measured at excitation and emission wavelength of 480 and 535 nm, respectively using SpectraMax M3 Microplate Reader. The Aβ induced ROS production by N2a cells (which were cultured in coverslips) were also examined in confocal microscope (LSM 710, Carl Zeiss, Oberkochen, Germany).

2.6. Determination of reactive nitrogen species (RNS)

For the measurement of RNS [22], the cell homogenate with 100 μg of protein from all the samples was mixed with Griess reagent and incubated at room temperature under dark condition for 30 min. Finally the total reaction mixture was centrifuged (1008 g) for 10 min, and the absorbance of the supernatant was read at 548 nm in comparison with 5–25 μM of sodium nitrite as standard. The results were expressed as mmoles of nitrite/mg of protein.

2.7. Determination of oxidative lipid and protein damage

Cell homogenate containing 100 μg of protein was used for both the assays. For the assessment of lipid peroxidation, the cell homogenate from each experimental group was mixed with the equal volume of ice-cold 15% trichloroacetic acid (TCA) and the mixed solution was subjected to centrifugation at 1008g for 15 min. After centrifugation, 0.37% TBA and 15% TCA was added to the supernatant and then the reaction mixture were incubated at 100 °C for 20 min. After incubation, the samples were read at 532 nm along with malondialdehyde (MDA) as the standard and the results were displayed as μmol of MDA/mg of protein. The protein oxidation was assessed according to previously reported method [23,24]. In this method the cell homogenate from each experimental group was mixed with 0.2% Di-nitro phenyl hydrazine (DNPH) in 2.5 N HCl and then the samples were kept in dark for 1 h followed by precipitation with 10% TCA. Subsequently the precipitated samples were washed three times with 1:1 ratio of ethanol and ethyl acetate and then the samples were centrifuged at 1372 g for 20 min. Finally, the pellet was dissolved in 2 ml of 2 M guanidine HCl and the absorbance was read at 360 nm using SpectraMax M3 Microplate Reader. The results were represented as μmoles of free carbonyl content/mg of protein.

2.8. Assessment of mitochondrial membrane potential (MMP)

The MMP was assessed using fluorescent dye Rhodamine 123 (Rh 123). After 24 h of the treatment period in 96 well plates, the culture medium was aspirated from the cells, and the cells were washed twice with PBS and then incubated for 30 min with 5 μM of Rh 123 fluorescent dye. The fluorescence content of the wells was measured at excitation and emission wavelength of 480 and 535 nm, respectively using SpectraMax M3 Microplate Reader. The cells grown in coverslip were used for taking the representative confocal image (LSM 710, Carl Zeiss, Oberkochen, Germany) [25].

2.9. Evaluation of cholinesterase inhibitory activity

Acetylcholinesterase (AChE) and Butyrylcholinesterase (BuChE) inhibitory activity was assessed in the cell homogenate (containing 100 μg of protein) by previously reported method [26]. DTNB (3 mM) was allowed to react with the cell homogenate from each experimental group and the final volume was made up to 1 ml with Tris-HCl buffer (pH 8.0). After that, 1 mM of the substrate acetylthiocholine iodide (ATCI) and butyrylthiocholine iodide (BTCI) was added to initiate the enzyme action of AChE and BuChE, respectively. The 5-thio-2-nitrobenzoate anion was noticed by the formation of yellow color. The absorbance was measured at 405 nm using SpectraMax M3 Microplate Reader and the specific activity (U/mg of protein) of AChE and BuChE was calculated as below

$$\text{Specific activity (SA)} = \frac{\Delta A \text{ (change in OD/min)} \times \text{reaction volume} \times 1000}{\varepsilon\lambda \times \textit{protein concentration} \times \textit{sample volume}}$$

2.10. Morphological assessment of apoptosis by Acridine orange/Ethidium bromide (AO/EtBr) dual staining method

The AO/EtBr dual-staining is mainly used to assess cell death to discriminate the live from apoptotic and necrotic cells [27]. The differentiation of live and apoptotic cells are based on the membrane integrity of the cell, depending on which it uptakes the dye. The fluorescent dye Acridine orange (AO) stains DNA in bright green color and allows for the visualization of the nuclear chromatic pattern of the cells. Apoptotic cells have reduced chromatin that is evenly stained. Ethidium bromide (EtBr) stains DNA in red color, but in the case of viable cells, the EtBr dye is not allowed to enter into the cells. In this assay, confocal microscopic (Zeiss LSM 710, Germany) investigation was performed to assess the morphological alteration of apoptotic cells using AO/EtBr dual fluorescent staining method. After 24 h of treatment period, the cells grown in coverslips were washed with PBS and then stained with 10 μg/ml of AO/EtBr solution (1:1 v/v). The samples were incubated at room temperature for 10 min in dark condition and finally the images of the stained cells were taken in a confocal microscope.

2.11. Measurement of Caspase-3 activity

The presence of caspase-3 in the cell lysate was measured by acetyl-Asp-Glu-Val-Asp p-nitroanilide (substrate) hydrolyzing activity [28]. In brief, the reaction mixture (cell lysate (100 μg of protein), Ac-DEVD-pNA(200 μM) and caspase-3 assay buffer) was incubated at 37 °C for 1 h under dark. Upon incubation, caspase-3 cleaves the substrate and liberates p-nitroaniline, which was read at 405 nm. p-Nitroaniline was used as reference to compare the unknown sample. The results were displayed as mmol of pNA/min/mg of protein. Quantitative measurement of caspases-3 enzyme activity was calculated using the formula

$$\text{Specific activity} = \frac{\mu\text{mol pNA} \times \text{dilution factor}}{\text{Time} \times \text{volume of sample} \times \text{Protein concentration}}$$

2.12. Assessment of the expression level of apoptosis related proteins

N2a cells were maintained in 6 well plates and after the treatment period, the cells from the different groups were collected, washed with PBS buffer and then added radio immunoprecipitation assay lysis buffer for the procurement of proteins from the cells. Protein (100 μg) from each group were electrophoresed on 12% sodium dodecyl sulfate (SDS) gels for separation and transferred to polyvinylidene difluoride (PVDF) membranes. The PVDF membrane was blocked overnight with 5% skim milk. After that, the PVDF membrane was dunked in corresponding primary antibodies (1:1000 dilution) such as β-actin (sc-130,657), Bcl-2 (sc-783), Bax (sc-493) and Caspase-3 and incubated for 6 h followed by addition of anti-rabbit IgG-alkaline phosphatase conjugated secondary antibody (Sigma; A3687; 1:2000 dilution), which was incubated for 3 h. The chromogenic substrate nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3′-indolyphosphate (BCIP) were used for the development of bands [28].

2.13. β-Secretase (BACE1) assay

β-secretase activity in the Aβ treated N2a cells was assessed using a β-Secretase Fluorometric Assay kit (Biovision Inc., Milpitas, CA, USA) in 96-well black polystyrene microplates [29]. To each well of the 96-well plate, 50 μl of samples from different groups were added, followed by addition of 50 μl of 2X reaction buffer and 2 μl of β-secretase substrate. The plate was incubated in the dark at 37 °C for 1 h and then the fluorescence was detected at excitation and emission wavelengths of 335 nm and 495 nm respectively, using a multi label reader (Molecular Device Spectramax M3). β-secretase activity was expressed as relative fluorescence units per μg of protein samples.

2.14. In vivo neuroprotective efficiency assessment of ACTPG and α-bisabolol against $A\beta_{25-35}$ induced neurotoxicity in transgenic Caenorhabditis elegans

2.14.1. C. elegans strains and its maintenance

CL4176 (*dvIs27* [*myo-3p*:A-Beta (1–42):*let-851* 3′ UTR) + *rol-6*(su1006)]) and CL2006 (*dvIs2* [pCL12(*unc-54*/human Aβ peptide 1–42 minigene) + pRF4]) nematodes were obtained from CGC (*Caenorhabditis* Genetics Centre, University of Minnesota, USA) and maintained at 15 °C on Nematode growth media (NGM) plates seeded with *E. coli* OP50 (as a food source). The transgenic CL2006 stain which produces $A\beta_{3-42}$ in their body-wall muscles was maintained at 15 °C throughout the lifespan. On the other hand, the expression of $A\beta_{1-42}$ in the muscle cells of the transgenic strain CL4176 is temperature inducible, which depends on temperature upshifting from 15 °C for 36 h to 23 °C [30]. Both the transgenic mutants were incorporated with roller marker and homogenous populations were obtained by worm picking method. Age-synchronized animals were prepared by transferring them to fresh NGM plates seeded with *E. coli* OP50 for 3 days to get adult stage and permissible to lay the eggs. Soon after obtaining the proper adult stage with eggs, the nematodes were washed with M9 buffer several times and the mixture of sodium hypochlorite along with 5 M KOH (1:1) was used to rupture the nematode cell body to obtain eggs. The bleached eggs were transferred immediately to fresh NGM plate seeded with *E. coli* OP50 and kept at 15 °C for hatching. Once the nematodes were hatched, exactly roller mutant L4 stage nematodes were taken for behavioural and molecular studies.

2.15. Liquid survival assay

Age synchronized CL2006 and CL4176 nematodes were washed with M9 buffer (3.0 g KH_2PO_4, 6.0 g Na_2HPO_4, 0.5 g NaCl and 1.0 g NH_4Cl) and accurately 10 nematodes were transferred to the wells (24 well plate) containing 450 µl of M9 buffer and 50 µl of ~0.5 OD grown bacterial culture. Habitually, laboratory food source of *E. coli* OP50 was used as control in both the mutants. Worms were examined for death on uninterrupted exposure to the different concentration of ACTPG (25, 50 and 100 µg/ml) and α-bisabolol (25, 50 and 100 µg/ml) at12 h time interval using stereo microscope (Nikon SMZ1000, Japan). Worms were considered as dead upon the failure of response by touching them with worm picker (platinum wire) and by the absence of pharyngeal movement [31].

2.16. Chemotaxis assay

Chemotaxis assay of the ACTPG and α-bisabolol treated nematodes were performed according to the recent scientific evidence [31]. Age synchronized transgenic strains CL2006 and CL4176 were treated with ACTPG (25 µg/ml) and α-bisabolol (25 µg/ml) for 24 h. The exposed nematodes were then washed with M9 buffer 3–4 times and 20 µl of odorant (0.1% benzaldehyde in 100% ethanol) along with 20 mM sodium azide, was added to the "attractant" spot marked in the plate (2 cm from the edges of the 10 cm plate). Exactly, on the opposite side (2 cm of the edges) of the attractant spot, 20 µl of control odorant (100% ethanol) along with 20 mM sodium azide was added. Immediately then, about 20 nematodes were transferred to the centre of the plate loaded with attractant and control odorant. The assay plates were incubated at 23°C and the number of nematodes in each quadrant was scored after 1 h. The Chemotaxis index (CI) was calculated using the formula: CI = (number of nematodes in attractant quadrant–number of nematodes in control quadrant)/total number of initial nematodes assayed.

2.17. Roller moment assay

The CL2006 and CL4176 strains have a roller co-injected marker. Due to this, the transgenic nematodes roll relatively than sinusoidal moment. The roller moment directly conveys that the nematode is active phenotypically. Henceforth, the rolling movement of CL4176 and CL2006 after 24 h exposure of ACTPG (25 µg/ml) and α-bisabolol (25 µg/ml) was measured as number of rolls made by the nematodes per 20s [32].

2.18. ROS assay in C. elegans

Intracellular levels of Reactive Oxidative Species (ROS) were qualitatively visualized in *C. elegans* using 2,7-dichlorofluorescein diacetate (DCF-DA; SIGMA) stain. Both the mutants were exposed individually to ACTPG (25 µg/ml) and α-bisabolol (25 µg/ml) for 24 h in 450 µl M9 buffer added with 50 µl of *E. coli* OP50. At the end of 24 h treatment with ACTPG (25 µg/ml) and α-bisabolol (25 µg/ml), the nematodes were collected in micro centrifuge tubes and washed with M9 buffer 3–4 times. Finally, DCFDA (100 µM) was added to the tubes containing nematodes and incubated for 15 min at dark condition. The nematodes were then subjected for confocal laser scanning microscopy (LSM 710, Carl Zeiss, Oberkochen, Germany) by placing them in the glass slides along with 2 mM sodium azide [33].

2.19. In vivo fluorescence staining of β-amyloid

Age synchronized individual CL2006 and CL4176 transgenic nematodes were exposed (as mentioned in the liquid survival assay) to the protective dose of ACTPG (25 µg/ml) and α-bisabolol (25 µg/ml) for 24 h, and then washed with M9 buffer 3–4 times followed by 1X PBS (pH 7.5) 2 times. Subsequently, the washed nematodes were stained with 0.125% Thioflavin S in 50% ethanol for 2 min followed by 1X PBS wash and finally mounted on glass slides along with 2 mM sodium azide for microscopy. Fluorescence images were acquired using a CLSM (LSM 710, Carl Zeiss, Oberkochen, Germany) at 20X magnification. Wild type nematodes were used as a negative control. The Thioflavin S-reactive deposits at the pharyngeal bulb region in distinct animals were recorded [34].

2.20. Assessment of the expression level of AD related genes by real time PCR

To study the expression level of *Aβ*, *dnj-14, hsp-4* and *ace-1* genes (Table 1), total RNA was isolated from CL4176 and CL2006 nematodes that have been exposed to the protective dose of ACTPG (25 µg/ml) and α-bisabolol (25 µg/ml) for 24 h. After incubation, the nematodes were washed 2–3 times with M9 buffer and washed 2 times with freshly prepared DEPC water (0.1%). The total RNA was then isolated using

Table 1
Genes used for qPCR analysis and their primer sequences.

S.No	Gene	Forward Primer	Reverse Primer
1	*ace-1*	GGAGATCCGAACAAAAACGA	TGACGATTCAACGGTCATGT
2	*rpb-12*	CGCCGAAAATGAAATCAAAC	GGGCGTCGTACACCATCA
3	*hsp-4*	GATGAAGCAGATTGCCGAAT	CATCCTTGGTGGCTTGTTTT
4	*dnj-14*	GTGGATGTTGAAGCCTTGGT	TTGCAACAGCATTGACAACA
5	*Aβ*	CCGACATGACTCAGGATATGAAGT	CACCATGAGTCCAATGATTGCA

RNA-Xpress™ (Invitrogen Inc.) reagent. cDNAs were generated from the total RNA samples using MultiScribe™ Reverse Transcriptase enzyme kit (Applied Biosystems Inc.) according to the manufacturer's protocol. The amplified genes were normalized with reference gene (*rpb-12*) and $2^{-\Delta\Delta Ct}$ method was used to calculate relative fold changes in the treated samples.

2.21. Assessment of β−amyloid level by Western blot analysis

After treating the CL2006 and CL4176 strains with protective dose of ACTPG (25 μg/ml) and α-bisabolol (25 μg/ml), the nematodes were lysed using lysis solution (7 M urea, 2 M thiourea, 4% CHAPS and 30 mM Tris-HCl (pH 8.5) and protease inhibitor), sonicated and centrifuged at 11,200 g for 10 min. Proteins (100 μg) were separated on 12% SDS polyacrylamide gels electrophoresis. The separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes using transferring unit for 3 h. The membranes were incubated overnight with 5% skim milk to prevent the nonspecific binding of antibodies. After incubation, blocked membranes were washed and incubated with primary antibodies β-actin (Santa Cruz; sc-130,657) and β-amyloid (Santa Cruz; sc-9129) [1:1000 dilution] for 6 h and then with 3 h incubation of anti-rabbit IgG-alkaline phosphatase conjugated secondary antibody (A3687; 1:2000 dilution). The bands were developed using 5-bromo-4-chloro-3′-indolyphosphate and nitro-blue tetrazolium as substrates.

2.22. Statistical analysis

Statistical analysis was performed using SPSS 17.0 software package. The results of all the experiments were represented as mean ± SD of triplicates employed. Analysis of variance was performed by one-way ANOVA. For the Real time PCR analysis Student's t-test has been performed. Significant differences between control and treated groups were determined by Duncan's multiple range tests and P value ≤ 0.05 were regarded as significant.

3. Results and discussion

3.1. Neuroprotective efficacy of ACTPG and α-bisabolol against $A\beta_{25\text{-}35}$ induced neuronal cell death in N2a cells

The senile plaques (SPs) accumulation in the hippocampal region of the brain generally comprises of the neurotoxic $A\beta_{25\text{-}35}$ peptide, which is considered as a key neuropathological hallmark of AD. The level of neurotoxic Aβ peptide in the brain cortex region is intensified through the repeated breakdown of APP by two proteolytic enzymes such as β and γ-secretases [35]. Though the key player of SPs is the full length of neurotoxic Aβ peptide containing 40–42 amino acid residues, the 11 stretch amino acid residue of smaller $A\beta_{25\text{-}35}$ fragments is also extensively revealed to form β-sheet assembly by itself. It also creates similar effects to those made by its parent $A\beta_{1\text{-}42}$ fragments in acting as an active peptide and inducing neurotoxic effect to the brain cells [36]. Therefore the current study deals with the neuroprotective competency of ACTPG and α-bisabolol against Alzheimer's toxic $A\beta_{25\text{-}35}$ peptide in N2a cells.

3.2. Evaluation of neuroprotective efficiency of ACTPG and α-bisabolol against neurotoxic $A\beta_{25\text{-}35}$ peptide in N2a cells

For fixing the optimum neurotoxic dose, the N2a cells were initially treated with different dosage of $A\beta_{25\text{-}35}$ peptide (10–50 μM). After 24 h of incubation, reduced viability was observed in $A\beta_{25\text{-}35}$ treated N2a cells in a concentration dependent manner as shown in Fig. 1A. At 50 μM of $A\beta_{25\text{-}35}$ treated N2a cells, 50% of viability was observed, which was fixed for further studies. In a concentration dependent manner, the pre-treatment of ACTPG (10–50 μg/ml) and α-bisabolol (2–10 μg/ml) safeguarded the cells from Aβ induced neurotoxicity and restored the viability of cells over 93% (Fig. 1B). Morphological assessment revealed that diminishing cell to cell communication and apoptosis was observed in $A\beta_{25\text{-}35}$ alone treated group (50 μM). Whereas the pre-treatment of N2a cells with different dosage of ACTPG (10–50 μg/ml) and α-bisabolol (2–10 μg/ml) restored the alteration in cell morphology, cell to cell contact when compared to control group, as displayed in Fig. 1C. Based on the observed results, the neuroprotective dose of ACTPG (25 and 50 μg/ml) and α-bisabolol (5 and 10 μg/ml) was fixed for further assays.

3.3. ACTPG and α-bisabolol attenuates $A\beta_{25\text{-}35}$ induced ROS and RNS in N2a cells

Numerous hypotheses have proposed that oxidative stress is a noticeable feature in AD pathology, which possibly relates to the impairment of neuronal cell functions and brain cell death in AD patients. Research evidence elucidates that, the increased level of ROS production in the cortex region of brain leads to the activation of Aβ peptide accumulation and disease progression of AD. The formation of RNS has also been notably involved in AD pathology. The generation of free radicals by Aβ peptide makes the cells prone to toxic effects like chromosomal damage, protein and lipid oxidation and neuronal destruction [37]. Hence the regulation or scavenging of ROS and RNS production level is considered to be a commanding strategy for preventing the brain cells from Aβ induced neurotoxicity. The generation of ROS in N2a cells was detected by DCFH-DA fluorescent dye. The N2a cells treated with the neurotoxic $A\beta_{25\text{-}35}$ peptide for 24 h displayed increased fluorescence intensity when compared to untreated cells, which is an indication for the Aβ induced ROS production. Two hour pre-treatment of ACTPG (25 and 50 μg/ml), α-bisabolol (5 and 10 μg/ml) and standard drug donepezil followed by Aβ treatment prevented the upsurge of intracellular ROS production which was measured quantitatively (Fig. 2A), moreover it was further validated by the decrease in the fluorescent intensity by confocal microscope (Fig. 2B). Fig. 2C further exemplifies the levels of nitrate produced in the cell lysates derived from the N2a cells treated with $A\beta_{25\text{-}35}$ alone and pre-treatment with ACTPG and α-bisabolol. An increase in the production of nitrite level was observed in $A\beta_{25\text{-}35}$ treated group (0.13 ± 0.007 mmol/mg of protein), whereas the nitrite levels were greatly reduced in pre-treatment with ACTPG (0.03 ± 0.001and 0.03 ± 0.002 mmol/mg of protein) and α-bisabolol (0.02 ± 0.002 and 0.03 ± 0.001 mmol/mg of protein). Prevention of oxidation stress by ACTPG and α-bisabolol implies that the drugs have the ability to prevent Aβ induced neuronal dysfunction and synaptic loss, which are implicated in oxidative stress induced AD pathogenesis [38].

3.4. Effect of ACTPG and α-bisabolol on $A\beta_{25\text{-}35}$ induced oxidative damage to lipids and proteins in N2a cells

Lipid peroxidation (LPO) is a primary product of free radicals which cause damage to the tissues and also act as a key player in deterioration of brain cells of AD patients. The accumulation and aggregation of Aβ peptide in the brain cortex region intensified the level of ROS which is interconnected to the oxidation of lipids and consequently makes the neurotoxic byproduct aldehyde 4-hydroxynonenal. It is also involved in the change of membrane carriers, alters calcium homeostasis and prompts the formation of neurofibrillary tangles [39]. Similarly, during the early stages of cognitive impairment, the oxidative stress markers for protein like the protein carbonyl content (PCC) were found to be elevated in the AD patients [37]. In the current study, the exposure of N2a cells to 50 μM Aβ raised the level of thiobarbituric acid reactive substances (TBARS) 71.09 ± 1.111 μM whereas pre-treatment with ACTPG (25 and 50 μg/ml) and α-bisabolol (5 and 10 μg/ml) showed a reduction in the level of TBARS (53.37 ± 1.476, 53.33 ± 0.344, 39.93 ± 0.468, 35.13 ± 1.191 μM of TBARS/mg of protein) as shown in Fig. 3A. An increase in the level of PCC (44.21 ± 0.367 μM of free

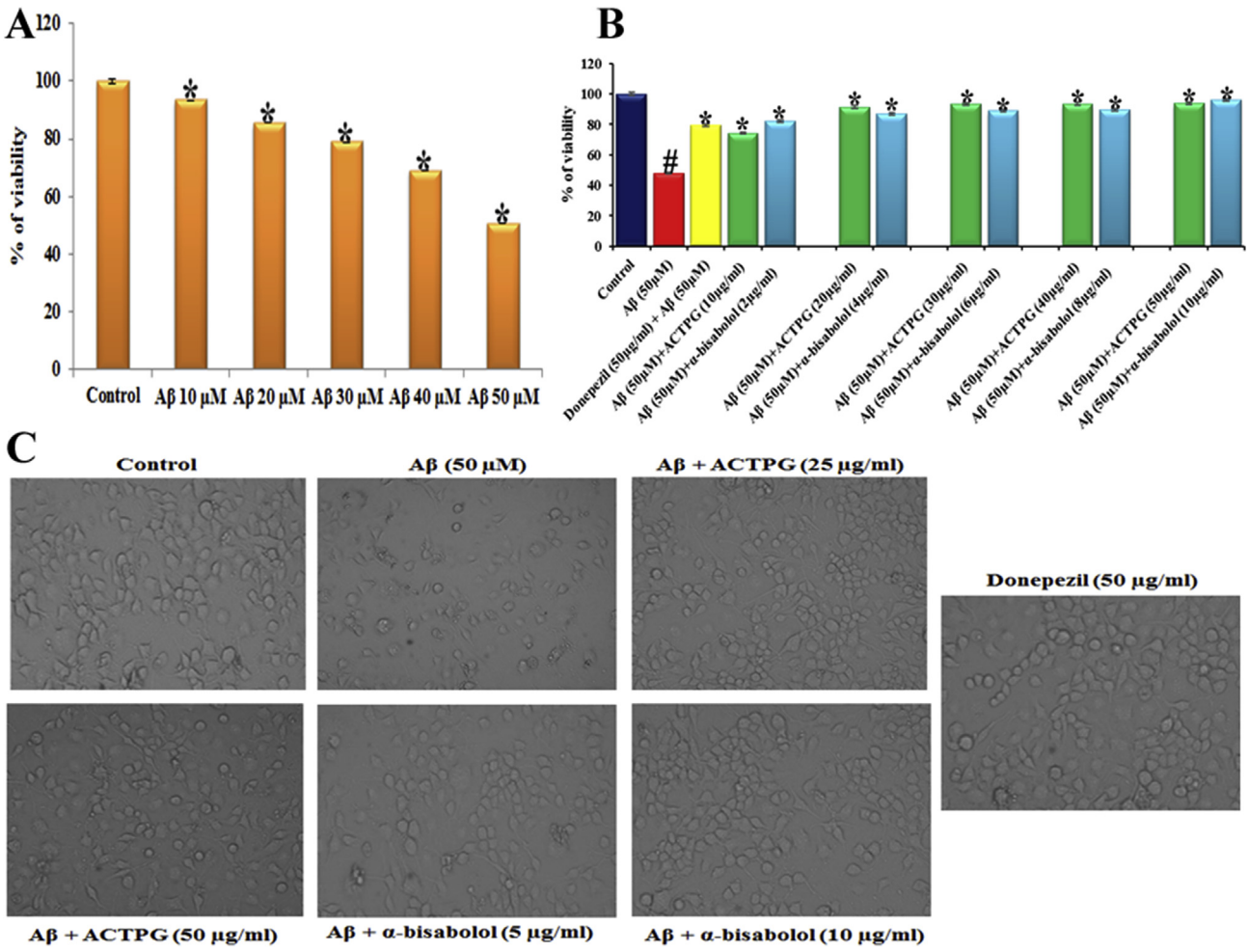

Fig. 1. Protective effects of ACTPG and α-bisabolol on $A\beta_{25\text{-}35}$ induced cytotoxicity in Neuro2a cells (A) Dose dependent cytotoxicity was exerted by $A\beta_{25\text{-}35}$ peptide (10–50 μM) in Neuro2a cells for 24 h. (B) Pre-treatment of ACTPG (10–50 μg/ml) and α-bisabolol (2–10 μg/ml) significantly protected Neuro2a cells from $A\beta_{25\text{-}35}$induced toxicity in a concentration dependent manner for 24 h (ANOVA; Statistical significance was at$P \leq 0.05$; #Control versus $A\beta_{25\text{-}35}$; *$A\beta_{25\text{-}35}$ versus treated). (C) Light microscopic image of $A\beta_{25\text{-}35}$treated Neuro2a cells which showed cell shrinkage, reduced cell density and apoptosis. Whereas, the pre-treatment of ACTPG and α-bisabolol restored the changes in cell morphology.

carbonyl content/mg of proteins), was observed in $A\beta_{25\text{-}35}$ treated N2a cells which clearly suggest that Aβ activated the oxidation of proteins in N2a cells. Whereas the pretreatment with ACTPG (25 and 50 μg/ml) and α-bisabolol (5 and 10 μg/ml) showed the reduced level of PCC (23.12 ± 1.084, 23.09 ± 0.481, 21.69 ± 1.168 and 22.15 ± 0.189 μM of free carbonyl content/mg of protein, respectively). Donepezil treatment (50 μg/ml) also exhibited a similar effect in reducing the PCC (21.33 ± 0.189 μM of free carbonyl content/mg of protein) as shown in Fig. 3B. Hence the study projected that ACTPG and α-bisabolol will be very helpful to either treat or delay the progression of AD, since they avert the oxidative stress related macromolecular damage.

3.5. Effect of ACTPG and α-bisabolol on neurotoxic $A\beta_{25\text{-}35}$peptide induced mitochondrial membrane potential loss in N2a cells

The dysfunction in mitochondria lead to the development of reactive oxygen species, diminishing in the respiratory chain and degeneracy of mitochondrial membrane potential and also play a crucial role in neuronal cell death [40]. Moreover, it was observed that the increased level of neurotoxic Aβ peptide affect the enzyme activity in mitochondria, particularly the enzymes involved in mitochondrial complex I [41]. As apparent to the previous studies, the Aβ peptide plays a key role in the activation of apoptotic proteins in mitochondria. In the present study confocal microscopic investigation (Fig. 4A–B) clearly shows that the rhodamine 123 signals are high in untreated N2a cells. However, the N2a cells treated with neurotoxic Aβ peptide showed decrease in rhodamine signals which noticeably identifies the MMP loss in N2a cells. Two hour pre-treatment of ACTPG (25 and 50 μg/ml), α-bisabolol (5 and 10 μg/ml) and donepezil with N2a cells reestablished the MMP from Aβ toxicity. The results indicated that the Aβ treated N2a cells showed the MMP loss, while the pretreatment of ACTPG, α-bisabolol and donepezil with N2a cells was active to restore the MMP. Restoration of MMP loss clearly validated the antioxidant potential of ACTPG and α-bisabolol and further its ability in preventing Aβ mediated AD pathology.

3.6. Anti-ChE activity of ACTPG and α-bisabolol in N2a cells treated with $A\beta_{25\text{-}35}$ peptide

AChE and BuChE enzymes are mainly involved in neurotransmission for regulating the neurotransmitter (ACh) level in synaptic cholinergic neurons. In AD condition both the enzymes play a crucial role for upholding the deposition and aggregation of neurotoxic Aβ peptide, which is considered as a key factor for AD pathology. The inhibition of ChE enzymes is an alternative strategy to control the neurotransmission of brain cells and also avert the deposition of neurotoxic Aβ peptide in synaptic cleft. Consequently, the ChE inhibition is a positive curative

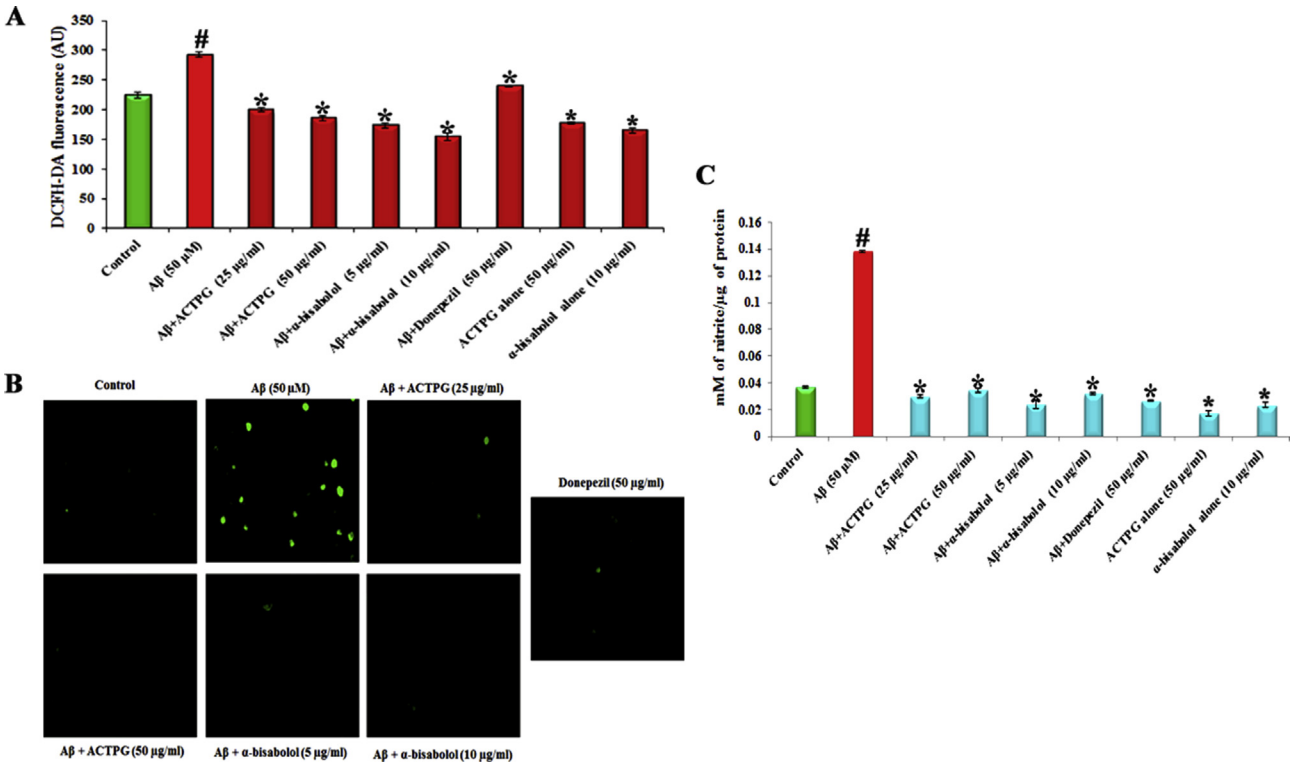

Fig. 2. Effect of ACTPG and α-bisabolol on scavenging of intracellular ROS and RNS in Neuro2a cells treated with $A\beta_{25\text{-}35}$. (A) Quantitative analysis of ROS production in Neuro2a cells upon treatment with $A\beta_{25\text{-}35}$ and pretreatment with ACTPG and α-bisabolol in a fluorescent microplate reader. (B) Confocal microscopic image of DCF shows increase in fluorescent intensity in $A\beta_{25\text{-}35}$treated group and a reduction in the fluorescent intensity was observed in ACTPG and α-bisabolol in Neuro2a cells for 24 h (C) Effect of ACTPG and α-bisabolol in reducing the nitrite levels in $A\beta_{25\text{-}35}$treated Neuro2a cells (ANOVA; Statistical significance at $P \leq 0.05$; #Control versus $A\beta_{25\text{-}35}$; *$A\beta_{25\text{-}35}$ versus treated).

approach for AD patients, in the meantime they could act on therapeutic targets such as the alteration in APP processing and accumulation of neurotoxic Aβ plaques [20]. Therefore in the present study, inhibitory efficiency of ACTPG and α-bisabolol on both AChE and BuChE enzymes was evaluated in N2a cells. The action of AChE and BuChE enzymes was enhanced in 50 μM of $A\beta_{25\text{-}35}$ treated group, while pre-treatment of ACTPG (25 and 50 μg/ml) and α-bisabolol (5 and 10 μg/ml) showed an extensive reduction in the level of AChE and BuChE enzymes as shown in Fig. 5A–B. Treatment with the standard drug donepezil at 50 μg/ml also caused a reduction in the ChE enzyme levels. The results suggest that ACTPG and α-bisabolol protects the N2a cells from neurotoxic $A\beta_{25\text{-}35}$ induced modifications of cholinergic neurons.

3.7. Anti-apoptotic efficacy of ACTPG and α-bisabolol against neurotoxic $A\beta_{25\text{-}35}$ peptide in N2a cells

AD is a caused by different factors like oxidative stress, deficit of acetyl choline, cell death and apoptosis, which contribute for the progression of AD [42]. During the oxidative stress condition which is induced in AD, the lysosomes lose the degradative ability on the amyloid peptide, which causes the overload of the Aβ peptide in the cells, thus inducing apoptosis [43,44]. The protective effect of ACTPG and α-bisabolol against neurotoxic Aβ peptide induced apoptosis was assessed by confocal laser scanning microscope (CLSM) analysis using AO/EtBr dual staining for the detection of live and apoptotic cells [35]. During imaging, the live cells appear with green nucleus, while the nucleus of live apoptotic cells are displayed as orange in color and dead cells are shown in red color. Fig. 6A exemplifies that apoptotic and dead cells were noticed in $A\beta_{25\text{-}35}$ treated cells which were detected by irregular nucleus, displaying the orange-red staining of the cells. In contrast, N2a cells pre-treated with ACTPG (25 and 50 μg/ml) and α-bisabolol (5 and 10 μg/ml) were alive during the treatment period (which can be identified from the green fluorescent staining of the cells), with organized structure and protected from Aβ induced neurotoxicity.

Caspase-3 enzyme activation prompts the Aβ induced apoptotic process [45]. The neurotoxic $A\beta_{25\text{-}35}$ peptide treated with N2a cells revealed an upsurge in Caspase-3 enzyme activity (0.143 ± 0.015 μmolpNA/min/mg of protein). Whereas, the N2a cells pre-treated with ACTPG (25 and 50 μg/ml), α-bisabolol (5 and 10 μg/ml) and donepezil (50 μg/ml) showed a reduction in Caspase-3 activity 0.064 ± 0.009,0.067 ± 0.013, 0.071 ± 0.007,0.072 ± 0.009 and 0.060 ± 0.004 μmolpNA/min/mg of protein respectively, which are displayed in Fig. 6B. Neuronal apoptosis which plays a major role in the neuronal injury caused in AD, is influenced by the expression of different proteins like Bcl-2, Bax and Caspase-3 [46]. The anti-apoptotic efficacy of ACTPG and α-bisabolol against neurotoxic $A\beta_{25\text{-}35}$ peptide was evaluated by assessing the expression of anti-apoptotic protein (Bcl-2) and apoptotic proteins (Bax and Caspase-3) by western blotting technique (Fig. 6C). Treatment of the neurotoxic $A\beta_{25\text{-}35}$ peptide (50 μM) to the N2a cells, intensified the expression of apoptotic proteins (Bax and Caspase-3) showing the triggering of apoptotic pathways. In contrast, pre-treatment of ACTPG (25 and 50 μg/ml), α-bisabolol (5 and 10 μg/ml) and standard drug donepezil (50 μg/ml) repressed the expression of Bax and Caspase-3 and induced the expression of the anti-apoptotic protein Bcl-2. The relative protein expression of bcl-2, bax and caspases-3 was quantified by ImageJ software analysis (Fig. 6D). The results were in agreement with previous study, where the treatment with oligomeric Aβ activated the protein expression of Bax and Caspase-3 and alleviated the anti-apoptotic protein Bcl-2 expression in hippocampal slice culture [44].

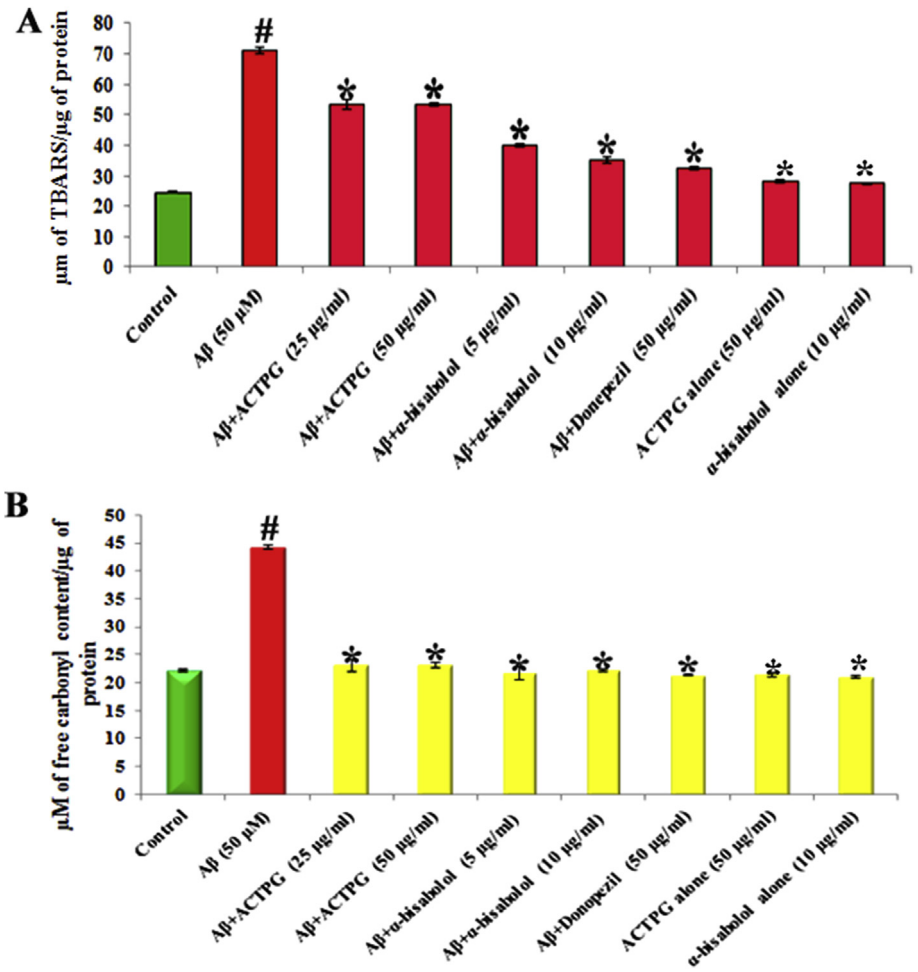

Fig. 3. Effect of ACTPG and α-bisabolol on $A\beta_{25\text{-}35}$induced macromolecular damages in Neuro2a cells (A) Lipid peroxidation was suppressed by ACTPG and α-bisabolol, which was confirmed by the reduction in MDA content. (B) Protein carbonyl levels were also decreased by ACTPG and α-bisabolol, which was measured from the amount of free carbonyls present in Neuro2a cells (ANOVA; Statistical significance at $P \leq 0.05$; #Control versus $A\beta_{25\text{-}35}$; *$A\beta_{25\text{-}35}$ versus treated).

3.8. Assessment of the ability of ACTPG and α-bisabolol to inhibit BACE1 activity in N2a cells treated with $A\beta_{25\text{-}35}$ peptide

The several cellular dysfunction observed in AD are either directly or in-directly related with the release of Aβ peptide by the key enzyme BACE1 (β-secretase βAPP-cleaving enzyme 1), which necessitates the inhibition of BACE1 for AD treatment [47,48]. Hence in the current study, the BACE1 inhibitory efficacy of ACTPG (25 and 50 µg/ml) and α-bisabolol (5 and 10 µg/ml) was evaluated in cell lysate of N2a cells treated with neurotoxic Aβ peptide. The results of this study demonstrated an upsurge in BACE1 activity in Aβ treated N2a cells when compared to control (Fig. 6E). ACTPG and α-bisabolol treated groups displayed decrease in BACE1 activity similar to that of standard drug donepezil (10 µg/ml) when compared to Aβ treated group. The BACE1 inhibitory effect of ACTPG and α-bisabolol shows that they will be able to prevent the cellular damage, by reducing the generation of Aβ peptide.

3.9. Assessment of neuroprotective effect of ACTPG and α-bisabolol in transgenic Caenorhabditis elegans

3.9.1. ACTPG and α-bisabolol exposure enhances the life span of transgenic Caenorhabditis elegans CL4176 and CL2006

To assess the toxicity of compounds, the transgenic mutants CL4176 and CL2006 were exposed to ACTPG and its active compound α-bisabolol at the concentration of 25, 50 and 100 µg/ml. CL2006 strain which produces $A\beta_{3\text{–}42}$ in the muscles of body-wall was incubated at 15 °C throughout the life span assay. On the other hand, CL4176 is temperature dependent and upshifting from 15 °C for 36 h to 23 °C triggers the Aβ expression. So, the CL4176 was incubated at 23 °C. Both the mutant strains were completely killed at 228 h in the control setup. During treatment with ACTPG (25 µg/ml), there was no change in the lifespan in CL2006 strain, however in the CL4176 mutants, an extension in the lifespan of up to 240 h was observed. On other hand, when the CL2006 nematodes were exposed to α-bisabolol at 25 µg/ml concentration, the survival was increased up to 240 h compared to control and the lifespan decreased at increasing concentration (50 and 100 µg/ml). Similar kind of result was observed in CL4176 strain also, whereas complete killing was obtained at 252 h. Similar effect was exhibited by the indole alkaloid reserpine, which ameliorated the Aβ induced modification, by increasing the lifespan of proteotoxic models of transgenic *C. elegans* [49]. Therefore the observed results (Fig. 7A, B, C and D) of this study directly suggest that the ACTPG and α-bisabolol increased the lifespan at a concentration of 25 µg/ml without causing any toxic effect in transgenic mutants, hence this dose was fixed for further studies.

3.10. ACTPG and α-bisabolol decreases neuronal Aβ induced defect in chemotaxis behavior of CL2006 and CL4176

Various studies have reported that small oligomeric deposition of Aβ peptides are the important toxic substances to neuronal system

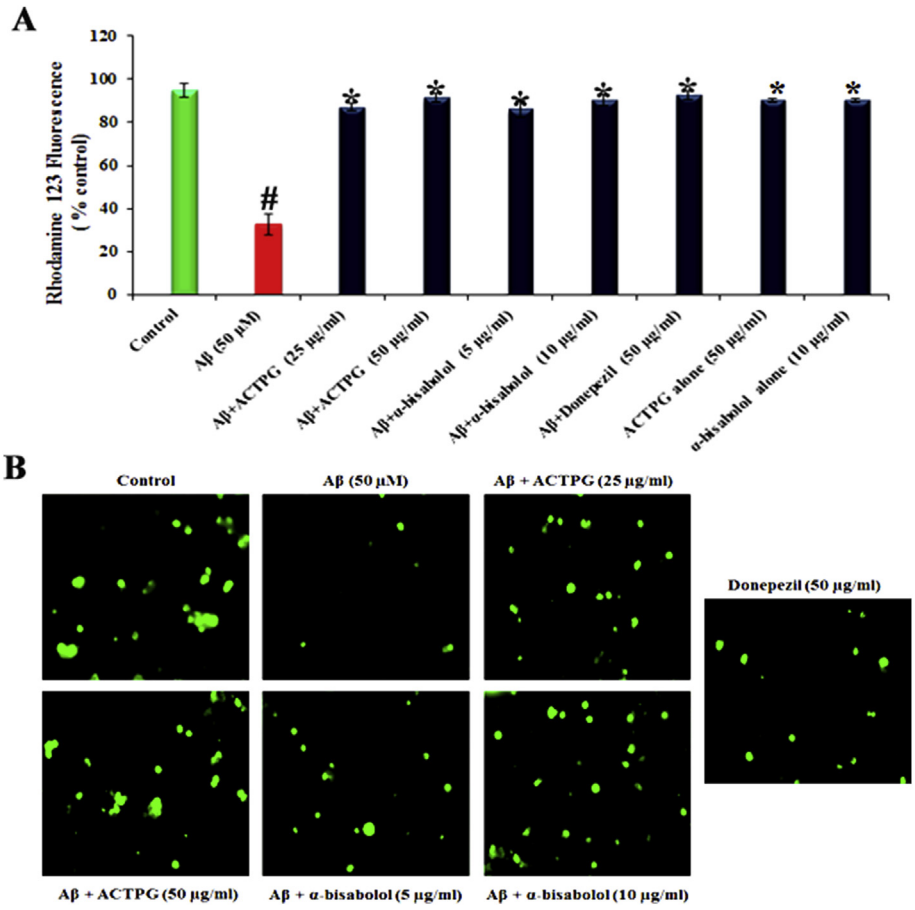

Fig. 4. Protective effects of ACTPG and α-bisabolol on decrease in mitochondrial membrane potential in Neuro2a cells treated with $A\beta_{25\text{-}35}$. (A) Quantitative measurement of MMP loss in a fluorescence microplate reader using Rhodamine 123 (ANOVA; Statistical significance at $P \leq 0.05$; #Control versus $A\beta_{25\text{-}35}$; *$A\beta_{25\text{-}35}$ versus treated). (B) Qualitative analysis of mitochondrial membrane potential by confocal microscopic examination of Rh123 fluorescence.

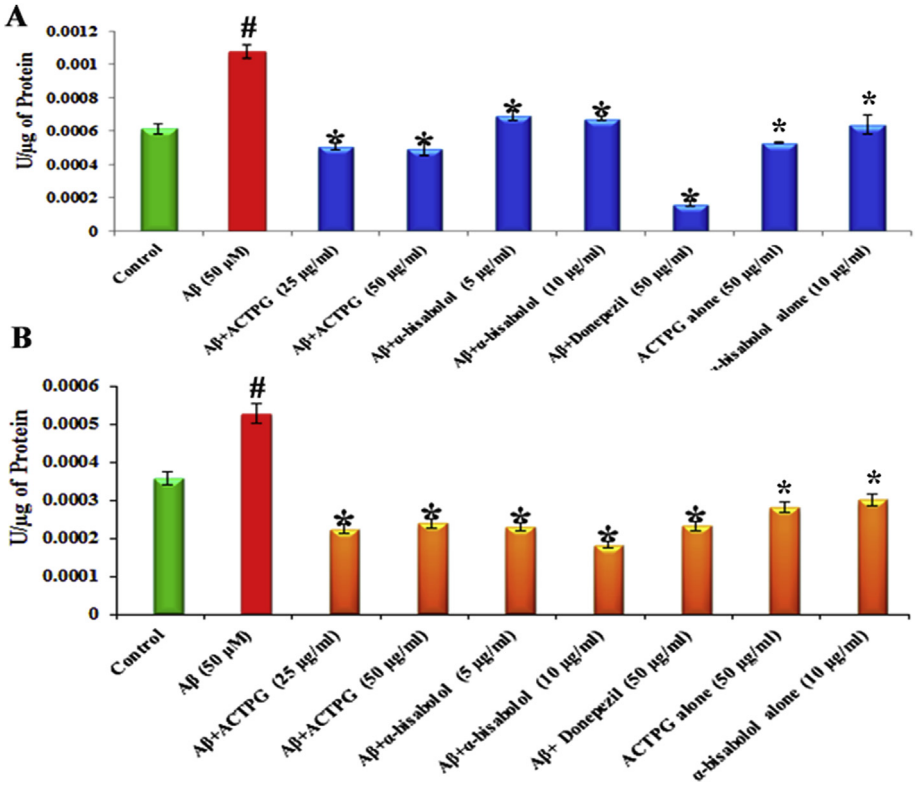

Fig. 5. Inhibitory potential of ACTPG and α-bisabolol on cholinesterase activity in $A\beta_{25\text{-}35}$ treated Neuro2a cells. (A) Acetylcholinesterase and (B) Butyrylcholinesterase (ANOVA; Statistical significance at $P \leq 0.05$ as significant; #Control versus $A\beta_{25\text{-}35}$; *$A\beta_{25\text{-}35}$ versus treated).

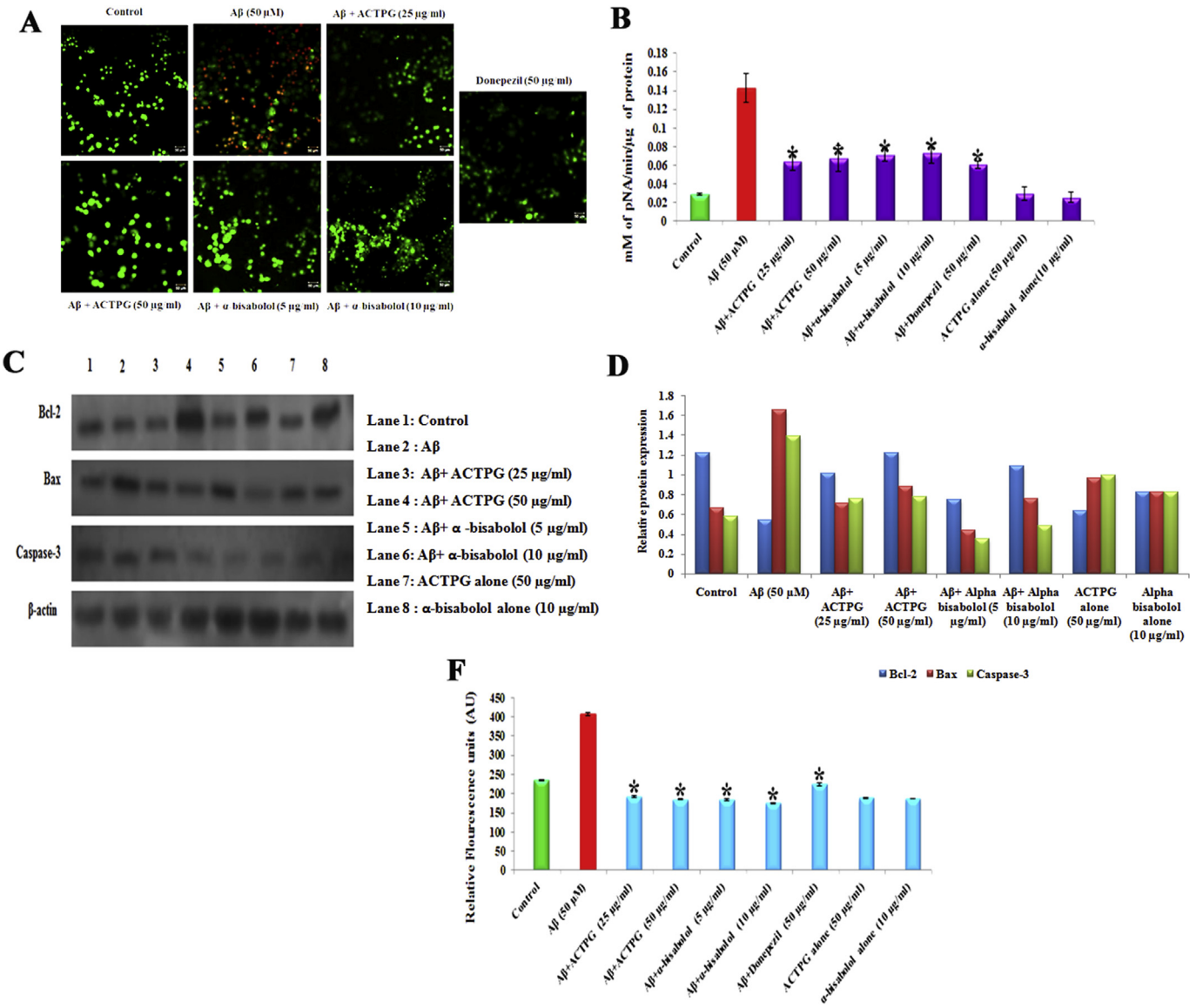

Fig. 6. (A) Confocal microscopic examination of the cells reveals the neuroprotective efficacy of ACTPG and α-bisabolol against $A\beta_{25-35}$induced neuronal apoptosis which was assessed using AO/EtBr double staining technique. (B) Inhibition of $A\beta_{25-35}$induced Caspase-3 activity by ACTPG and α-bisabolol. (C–D) Western blot analysis of anti-apoptotic protein Bcl-2 and apoptotic protein Bax and caspase-3 in N2a cells treated with ACTPG and α-bisabolol. The gel blots were cropped from different gels and the full length blots are given in the supplementary file. Quantification was done using ImageJ analysis (E) BACE1 inhibitory effect of ACTPG and α-bisabolol in N2a cells treated with neurotoxic $A\beta_{25-35}$peptide (ANOVA; Statistical significance at $P \leq 0.05$; #Control versus $A\beta_{25-35}$; *$A\beta_{25-35}$ versus treated).

[7–9]. In this study, we have used the transgenic mutants CL2006 and CL4176 to explore whether the effect of ACTPG and α-bisabolol on Aβ oligomeric deposition would protect and recover the neuronal chemotaxis. The Chemotaxis Index (CI) is a measure of the number of nematodes that are capable to reach at the attractant location [50]. Fig. 8A and B reveals that the CI value was increased in the transgenic strain CL2006 and CL4176. Both the transgenic mutants showed that the ACTPG (25 μg/ml) and α-bisabolol (25 μg/ml) exposed nematodes could differentiate the control odorant and the attractant. The control nematodes could not recognize the attractant in both transgenic *C. elegans*. Whereas, the ACTPG and α-bisabolol exposed nematodes could recognize the attractant and moved towards them. These results suggest that the ACTPG and α-bisabolol might be able to rescue the neuronal Aβ induced defects in chemotaxis behavior and also able to protect neuronal system against the Aβ induced toxicity.

3.11. Assessment of phenotypical variations in transgenic mutants CL2006 and CL4176

The CL2006 and CL4176 are roller conjugated transgenic mutants and the defects or any changes in their roller moment induced by the ACTPG and α-bisabolol was assayed by roller moment assay. The roller moment assay of CL2006 and CL4176 suggest that the CL2006 nematodes were very active in both control and also in treated conditions whereas in CL4176 the number of rolls was less when compared to CL 2006 (Fig. 9A–B). The number of rolls in CL4176 was less even at 0 h exposure and also at 24 h exposure of ACTPG and α-bisabolol. Therefore the results of roller moment assay suggest that there are no substantial changes in the behavior of transgenic mutants. Earlier study has reported that reserpine has the ability to enhance the life span and improve movement in transgenic *C. elegans* and was identified as a therapeutic drug for neurodegenerative disease [49]. Our results display that ACTPG and α-bisabolol surely can provide defense against the AD pathogenesis in *C. elegans*, in which ACTPG and α-bisabolol is able

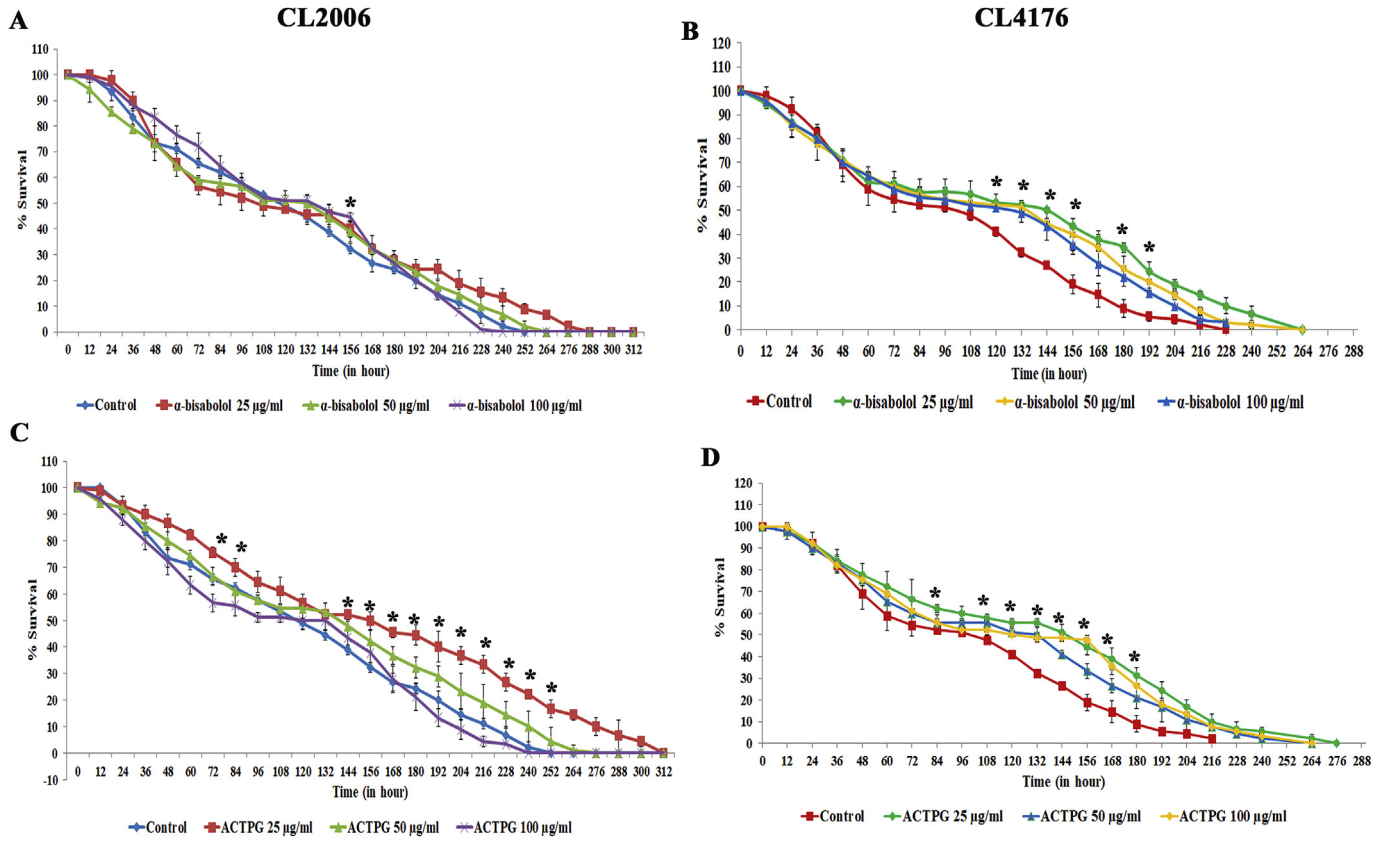

Fig. 7. ACTPG and α-bisabolol extends the life-span of transgenic *C. elegans* strain. Age synchronized CL2006 and CL4176 nematodes were placed at 15 °C and 23 °C on fresh NGM plates seeded with OP50 *E. coli* and, at adult stage, were fed with OP50 or with 25 µg/ml of ACTPG and α-bisabolol. The survival curves of CL2006 (**A–B**) and CL4176 (**C-D**) nematodes which were fed with presence and absence of ACTPG and α-bisabolol. The number of paralyzed worms (considered dead) was scored after treatment, at adult stage (day 0 in graph) and every consecutive day, until all worms were dead. Survival is expressed as a percentage of the initial population.*Represents Statistical significance (*P* value ≤ 0.05) between control and treated setup (n = 30worms/group).

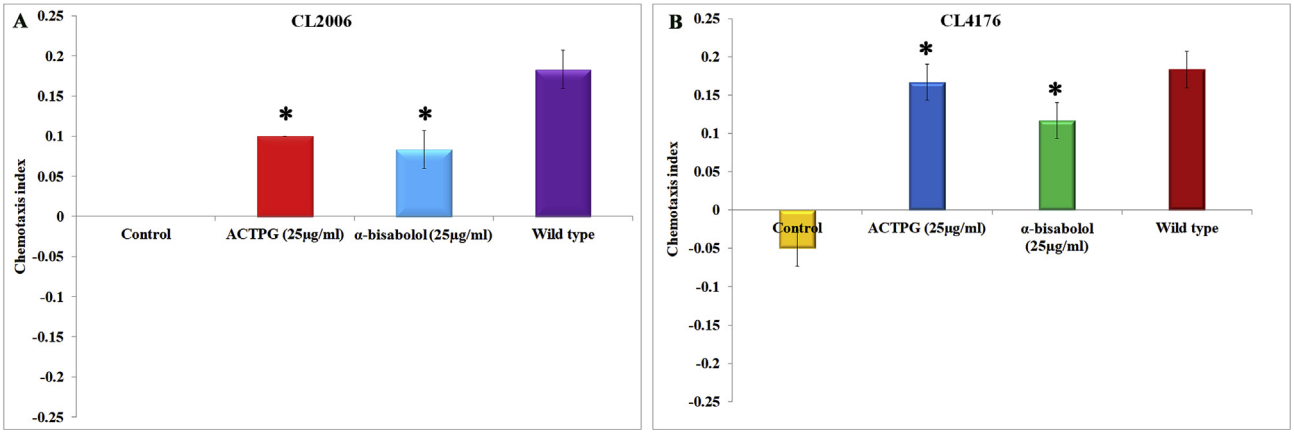

Fig. 8. Assays for chemotaxis behavior in neuronal Aβ expressing transgenic *C. elegans* strains (**A**) CL2006 (**B**) CL4176 which were fed with OP50 or with 25 µg/ml ACTPG and α-bisabolol at adult stage for 24 h. The chemotaxis behavior in neuronal Aβ strain CL2006, CL4176 and wild type was increased compared with control. The negative CI (Chemotaxis Index) reflects the sensing ability of nematodes towards the control odorant and positive CI reflects the sensing ability towards attractant. CI value = 0 denotes that the nematodes could not reach the control odorant and also the attractant. Wild type *C. elegans* were used as a control reference.*Represents Statistical significance (*P* value ≤ 0.05) between control and treated setup (n = 30worms/group).

to delay paralysis, and enhance the life span of Aβ expressing worms.

3.12. ACTPG and α-bisabolol reduces the formation of ROS in transgenic mutants CL2006 and CL4176

Generally, the transgenic *C. elegans* strains which were used in this study undergo oxidative stress in response to human$A\beta_{1-42}$ at normal conditions. The study on glycitein has suggested that it may suppress Aβ neurotoxicity through the antioxidant property and averting Aβ accumulation, thus having curative potential for the prevention of oxidative stress and Aβ mediated neurotoxicity in transgenic mutants [33]. In this study, ROS formation was evaluated in transgenic mutants CL2006 and CL4176 on ACTPG (25 µg/ml) and α-bisabolol (25 µg/ml) exposure and compared to the control nematodes (both CL2006 and

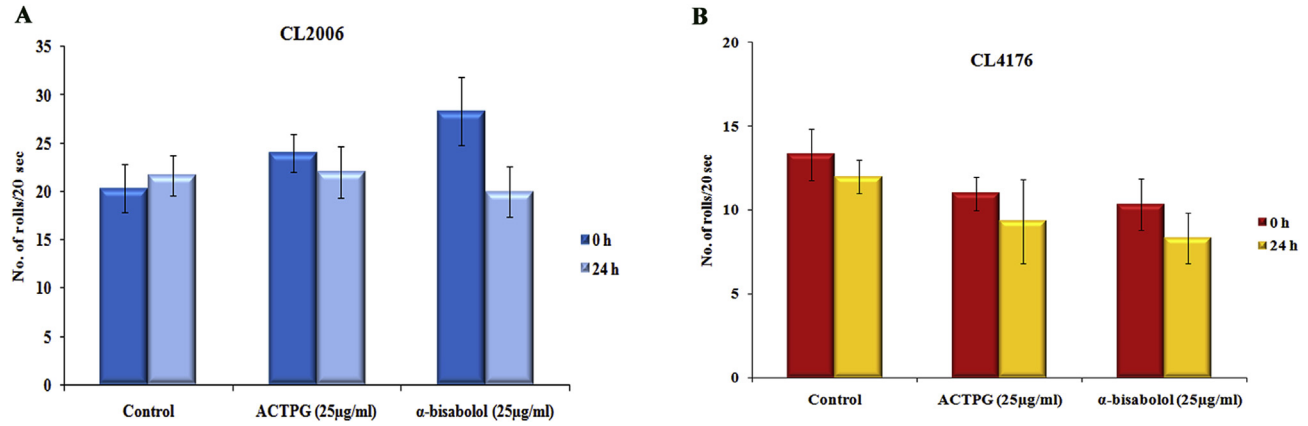

Fig. 9. Behavioural assessment of neuronal Aβ expressing transgenic strains (**A**) CL2006 (**B**) CL4176 which were fed with and without ACTPG and α-bisabolol (25 µg/ml) for 24 h by roller moment assay. ACTPG and α-bisabololimproved the locomotion of worms within 24 h compared to control untreated worms (n = 30 worms/group).

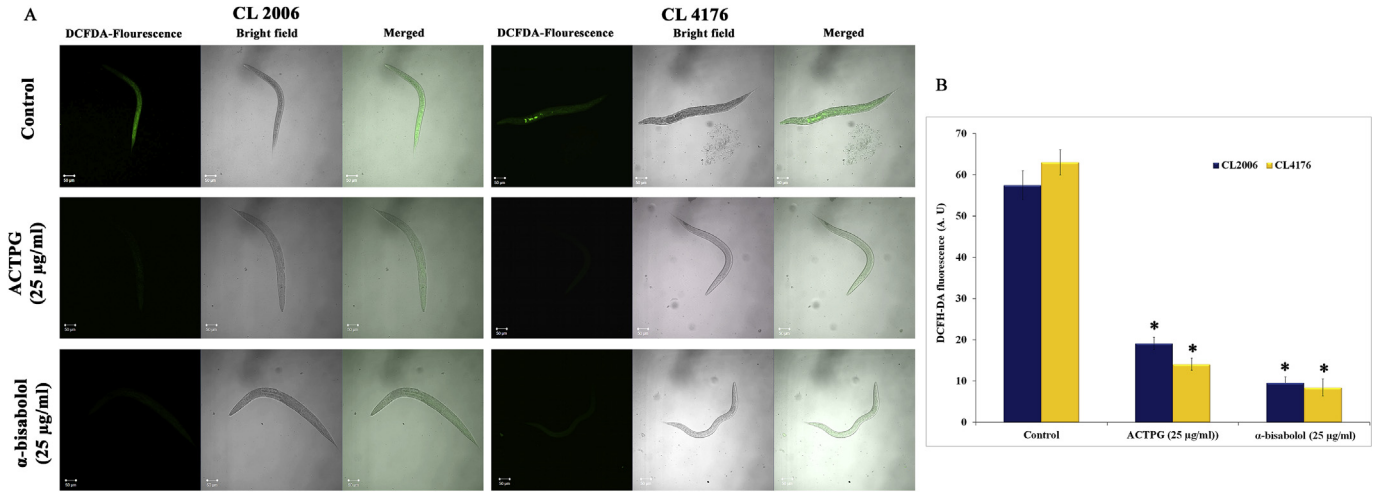

Fig. 10. Effect of ACTPG and α-bisabolol on ROS level in CL2006 and CL4176 transgenic *C. elegans* strains. **(A)** Age synchronized CL2006 and CL4176 nematodes were maintained at 15 °C for 24 h which were temperature upshifted to 23 °C on fresh NGM plates seeded with OP50 *E. coli* and then fed, at adult stage, with or without 25 µg/ml of ACTPG and α-bisabolol for 24 h followed by (B) the intracellular ROS which was measured in the *C. elegan s*through DCF-DA method. At least 30 animals (n = 30) from each group were analyzed for ROS level, and results are expressed as mean ± SD of relative fluorescence unit (RFU) *Represents Statistical significance (*P* value ≤ 0.05) between control and treated.

CL4176). The results clearly displayed that after 24 h exposure with the ACTPG and α-bisabolol ROS production were attenuated at the intra cellular levels in CL2006 and CL4176 mutants while in control nematodes, no ROS reduction was observed as shown in Fig. 10.

3.13. ACTPG and α-bisabolol suppresses the Aβ deposition in CL2006 and CL4176

Recently a study reported that the deposition of neurotoxic Aβ was reduced in worms (CL2006 and CL4176) fed with 40 µg/ml Scorpion venom heat-resistant peptide (SVHRP). Moreover, the study suggested that the defensive efficiency of SVHRP against Aβ induced neurotoxicity in both the mutants is through the inhibition of Aβ aggregation and deposition [34]. In the current study we have documented the Aβ reactive deposits in the head region of *C. elegans* transgenic mutant strains CL2006 and CL4176 to assess whether the effect of ACTPG (25 µg/ml) and α-bisabolol (25 µg/ml) on Aβ oligomerization would decrease the amount of amyloidosis or not. The result shows Aβ deposits (white arrows) which were detected in CL2006 and also in CL4176 in control nematodes but not in the wild type nematode (N2). The ACTPG and α-bisabolol has shown promising activity against the Aβ deposition in both the CL2006 and CL4176 mutant nematodes (Fig. 11). These results recommend that the inhibitory effect of ACTPG and α-bisabolol against Aβ induced toxicity in CL2006 and CL4176 may be due to the prevention of Aβ deposition and oligomerization.

3.14. Quantitative assessment of candidate genes expression in CL2006 and CL4176 upon ACTPG and α-bisabolol treatment

The qPCR analysis was performed to analyze the expression level of *ace-1, hsp-4, dnj-14* and *Aβ* genes during ACTPG (25 µg/ml) and α-bisabolol (25 µg/ml) exposure. The *ace-1* acetylcholinesterase gene is responsible for cholinesterase activity and also plays a vital role in cholinergic nerves of *C. elegans*. Spontaneous production of Aβ in the untreated transgenic strains leads to an increased level of *ace-1* expression. A similar kind of result was observed previously, where the exposure of Aβ increases the expression of ChE enzymes in neuroblastoma cells [51]. However, the treatment with ACTPG and α-bisabolol decreased the expression level of *ace-1* gene in both transgenic mutants. The obtained results are in par with the *in vitro* cholinesterase inhibitory potential of ACTPG and α-bisabolol. Previous study has reported that alkaloids isolated from *Lycoris radiate* have increased the life span of transgenic *C. elegans* expressing Aβ by inhibiting the cholinesterase enzyme production [51]. The *hsp-4* gene is a heat shock

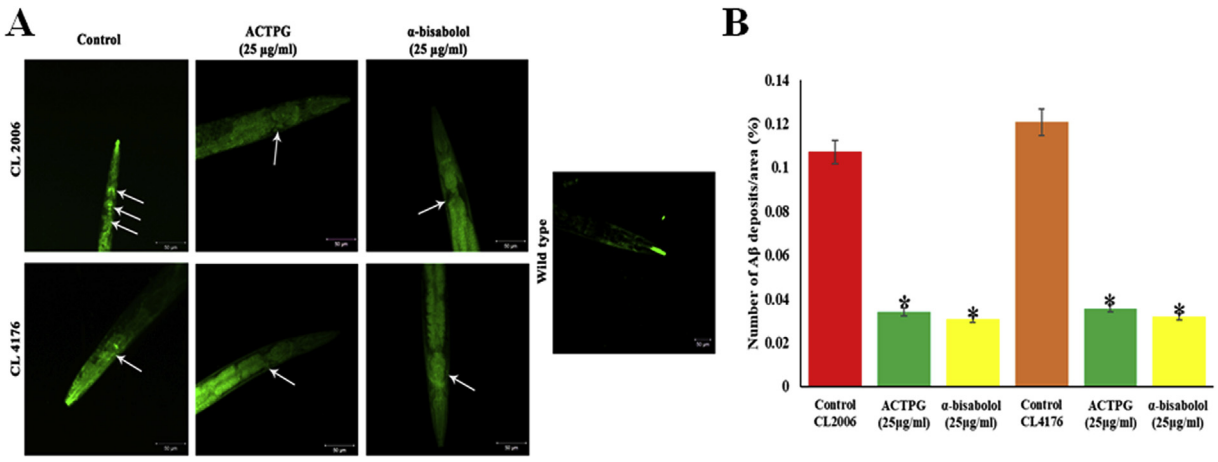

Fig. 11. ACTPG and α-bisabolol reduces Aβ deposition of CL2006 and CL4176 transgenic *C. elegans* strains which was assessed by Thioflavin S staining for 24 h. **(A)** The staining of amyloid plaques was done on whole-mount and the fixed samples were visualized. The white arrows indicate the Aβ deposition in representative control worms and reduction in deposition in the representative treated worms of 25 µg/ml of ACTPG and α-bisabolol, respectively (n = 30 worms/group). **(B)** The quantity is expressed as mean number of Aβ deposits in the head region/anterior area of the worm. Quantification was done using Image J software analysis. Error bars indicates SE. *$p < 0.05$.

transcription factor which is up regulated in response to endoplasmic reticulum stress associated with heat shock and protein misfolding in *C. elegans* [52]. During the exposure of ACTPG and α-bisabolol, the *hsp-4* gene is down regulated in both mutants when compared to control. It clearly suggests that there is no endoplasmic reticulum related stress in ACTPG and α-bisabolol treated *C. elegans*. In *C. elegans*, *dnj-14* mutants (*tm3223, ok237*), which are orthologous to human DNAJC5, showed impaired locomotion, reduced life-span, reduced neurotransmission and impaired chemosensation and mechanosensation; older *dnj-14* nematodes exhibited neuronal abnormalities like reduction in the number of visible neurites, loss of neuronal cell bodies, and the presence of contorted neuronal processes. Resveratrol, a natural stilbenoid ameliorated neurodegeneration in phenotypes of *C. elegans dnj-14* null mutants and increased their life span [53]. Similar to these results, *dnj-14* was also down regulated in untreated transgenic *C. elegans* strains, whereas a significant up regulation was observed in *dnj-14* expression upon ACTPG and α-bisabolol treatment. Moreover, this result validates the killing assay (enhanced lifespan) and recovery in chemotaxis behavior assay. Furthermore, the analysis of Aβ gene directly implies that ACTPG and α-bisabolol are significantly down regulated during the 24 h of exposure in both the mutants. The overall results (Fig. 12A, B, C and D) indicate that ACTPG and α-bisabolol enhance the life span of transgenic nematodes by reducing the Aβ induced toxicity.

3.15. ACTPG and α-bisabolol down regulates the Aβ protein expression in CL2006 and CL4176

To validate the behavioural and real time PCR analyses, the nematodes exposed to ACTPG (25 µg/ml) and α-bisabolol (25 µg/ml) were subjected for western blotting analysis. Both CL2006 and CL4176 transgenic mutants were exposed for 24 h and the protein level expression of Aβ was investigated using western blotting. The control nematodes of CL2006 and CL4176 showed up regulation of Aβ peptides. Exposure to ACTPG for 24 h was found to be enough for the down regulation of Aβ expression in both CL2006 and CL4176 nematodes. Similarly, the α-bisabolol exposed nematodes also showed significant inhibition of Aβ. From these results (Fig. 12 E and F), it is found that both the ACTPG and α-bisabolol are having promising inhibitory potential against Aβ deposition in the transgenic mutants of *C. elegans*. The possible mode of action for this effect could be the binding efficiency of ACTPG and α-bisabolol towards the active sites of Aβ peptides. We have previously reported the molecular docking analysis of α-bisabolol against the Aβ peptides [20]. According to the results in transgenic mutants, it is inferred that the Aβ deposition is segregated by the compounds by acting on the Aβ expression in *C. elegans* in both transcriptome and proteome levels. It suggests that the compound could possibly act in a multifaceted way to inhibit the Aβ expression.

4. Conclusion

In the present study, the neuroprotective efficacy of acetone extract of *P. gymnospora* (ACTPG) and its active constituent α-bisabolol was assessed against $A\beta_{25\text{-}35}$ induced neurotoxicity in N2a cells and transgenic mutants *Caenorhabditis elegans* (CL2006 and CL4176). The results of the *in vitro* study suggest that ACTPG and α-bisabolol restores from Aβ induced macromolecular damage such as lipid peroxidation and protein oxidation. Excitingly, the ACTPG and α-bisabolol was also found to shield the cellular mechanism from ROS and RNS mediated damage in N2a cells. Apart from these protective effects, ACTPG and α-bisabolol inhibits cholinesterase enzyme, β-secretase enzyme and apoptosis mediated cell death efficiently in N2a cells, which recommends that the seaweed and its active constituent might have vital role in improving cholinergic neurotransmission and thereby averting the neuronal loss that occurs due to Aβ induced cell death. In addition, *in vivo* study was performed in transgenic AD models of *C. elegans* strains for the validation of *in vitro* results. The results of the *in vivo* study displayed that the ACTPG and α-bisabolol holds neuroprotective efficacy against Aβ proteotoxicity by altering AD related pathways, lifecycle and macromolecular damage. Moreover, ACTPG and α-bisabolol down regulates AD related gene expression thereby averting the development of Alzheimer toxic Aβ peptide in both the transgenic mutants. Overall, the results of the study specify the neuroprotective effect of ACTPG and α-bisabolol against Aβ mediated AD pathology.

5. Author information

Balakrishnan Shanmuganathan and Sethuraman Sathya contributed equally to this work.

Conflicts of interest

The authors declare that they have no conflict of interests.

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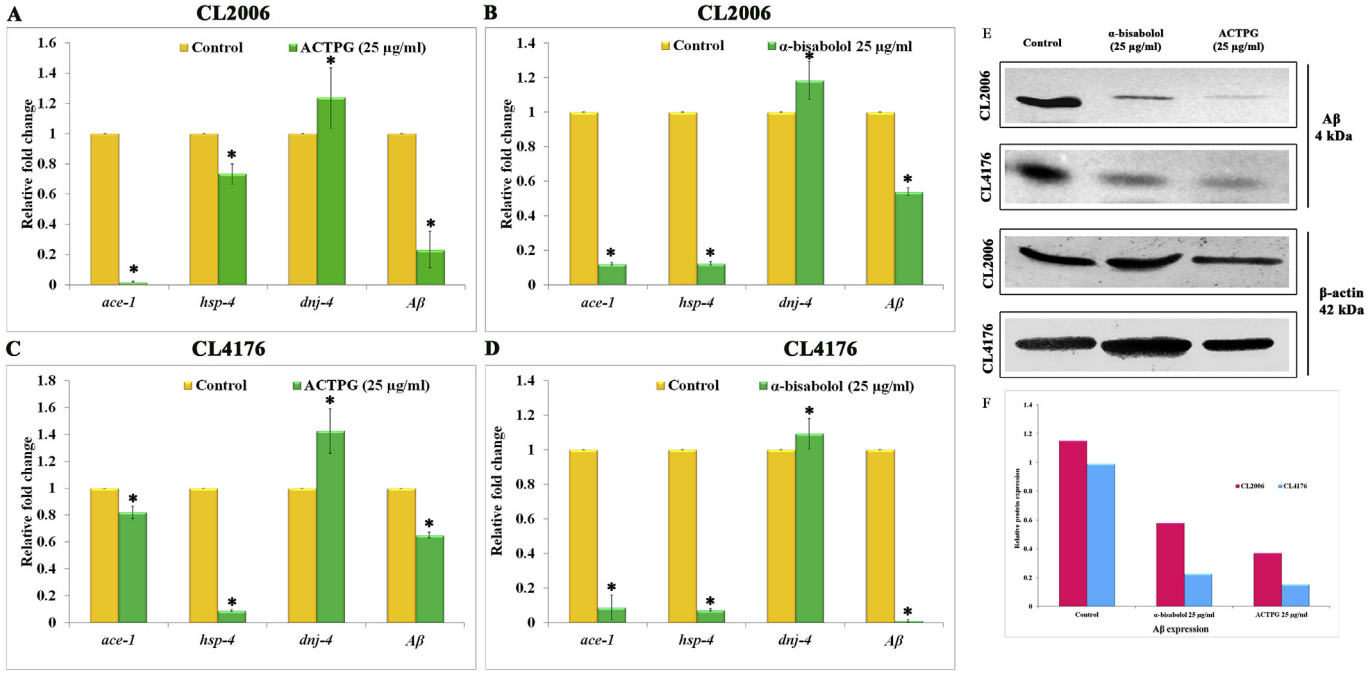

Fig. 12. Modulation of AD related gene expression in transgenic *C. elegans* strains (**A–B**) CL2006 and (**C–D**) CL4176 which were fed with or without 25 µg/ml ACTPG and α-bisabolol and measured by qRT-PCR analysis. The transcript level of AD related gene was significantly up and down regulated by ACTPG and α-bisabolol. The data was normalized to the expression of *rpb-12*. Student's t-test was used to compare the control and treated. * indicates significant at $P \leq 0.10$.**(E-F)**Inhibition of Aβ protein expression by ACTPG and α-bisabolol in CL2006 and CL4176 strains. The gel blots were cropped from different gels and the full length blots are given in the supplementary file. Quantification was done using ImageJ analysis.

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Appendix A. Supplementary data

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