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Convolvulus pluricaulis (Shankhapushpi) ameliorates human microtubule-associated protein tau *(hMAPτ)* induced neurotoxicity in Alzheimer's disease *Drosophila* model

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ABSTRACT

Convolvulus pluricaulis (Shankhapushpi) has long been used as traditional herbal medicine in India as nerve tonic. We studied the neuroprotective effects of *C. pluricaulis* extract (aqueous) against human microtubule-associated protein tau *(hMAPτ)* induced neurotoxicity in Alzheimer's disease (AD) *Drosophila* model. We analysed the lifespan, locomotor activity, τ protein level, reactive oxygen species (ROS), lipid peroxidation (LPO), catalase (CAT), superoxide dismutase (SOD) and acetylcholinesterase (AChE) activities in 10th, 20th and 30th days old control (wild type), τ control tauopathy *Drosophila* reared on *C. pluricaulis* supplemented with regular food or regular standard food. *C. pluricaulis* significantly offsets hMAPτ induced early death and extends the lifespan and diminishes the level of τ protein in tauopathy *Drosophila*. *C. pluricaulis* also enhances the antioxidant enzyme activities and ameliorates the τ-induced oxidative stress and restore the depleted AChE activity in the fly model. This study provides the first evidence that supplementation of *C. pluricaulis* along with the regular standard food ameliorate the neurotoxic effect of *hMAPτ* in AD *Drosophila* model and also reveals that it is a potent neuroprotective agent.

1. Introduction

Alzheimer's disease (AD) is a devastating neurodegenerative disease and a major form of dementia. Currently 46.8 million people worldwide are living with dementia and this number will reach 131.5 million till 2050 (Prince et al., 2016). AD is pathologically defined by severe neuronal loss, aggregation of amyloid-β (Aβ) as extracellular senile plaques, and formation of intraneuronal neurofibrillary tangles of hyperphosphorylated τ protein (Van Cauwenberghe et al., 2016). Numerous drugs are being designed for the treatment of AD, and different therapeutic strategies have been applied to suppress the AD pathogenesis (Folch et al., 2016). Available drugs temporarily relieve symptoms, does not prevent, but delay the onset, slow the progression, or improve the symptoms of AD (Cummings et al., 2014). As there is no suitable drug to cure for AD till date; identification of specific active compounds that prevent the formation or degradation of senile plaques and neurofibrillary tangles makes an appealing therapeutic and preventive strategy in the development of drugs. Medicinal plants have historically proven their value as a source of molecules with therapeutic potential, and an important pool for the identification of novel drug leads. Medhya plants are mentioned in Ayurveda known for their antioxidant and neuroprotective properties and have been proposed as an alternative choice for the treatment of AD.

Convolvulus pluricaulis (CP) also known as Shankhapushpi (Family: Convolvulaceae) is a perennial that grows in the plains of India. 'Charak Samhita' has described shankhapushpi as one of the best nerve tonic (medhya rasayana) to treat nervous disorders and aging (Chunekar and Pandey, 2002). The nerve tonic retards brain aging and help in the regeneration of neural tissues besides producing antistress, adaptogenic and memory enhancing effect (Singh et al., 2008). Even though Ayurvedic practitioners have been using shankhapushpi for centuries, there is no rigid scientific evidence for the positive effects of this herb.

Drosophila melanogaster is used as a valid *in vivo* model system to study many human diseases, including neurodegenerative disorders because of its short lifespan, ease of maintenance, and the availability of genetic tools, mutants, large homogeneous populations and the striking number of parallels between *Drosophila* and human (Jennings, 2011). *Drosophila* genetic model of tauopathies have been created by overexpression of human mutant copies of microtubule-associated protein tau (hMAPτ) to specific neuronal cells in flies using the UAS-

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GAL4 expression system (Whittmann et al., 2001). These hMAPτ overexpressed flies show the various easily-quantifiable phenotypes, such as eye degeneration, developmental defects, shortened lifespan, locomotor defects, elevated oxidative stress, learning and memory defects (Sun and Chen, 2015). The selective accumulation of abnormal τ in the areas of neuron leads to degeneration claim that the mechanisms of τ neurotoxicity are conserved between flies and humans (Whittmann et al., 2001). These homologies and quantifiable phenotype are amenable to rapid screening, the fly tauopathy model can be used to identify the molecular mechanisms that underlie τ neurotoxicity and evaluate possible candidate drugs for AD.

In view of this, the present investigation is aimed at to study the neuroprotective effects of *C. pluricaulis* (shankhapushpi) against the hMAPτ induced neurotoxicity in the *Drosophila* AD model system *in vivo* and identify it as a future therapeutic agent against AD.

2. Materials and methods

2.1. Convolvulus pluricaulis root collection and preparation of aqueous extract

The authenticated *Convolvulus pluricaulis* (CP) roots were a generous gift from the Ayurvedic physician Dr. Jayagovinda, SahasrakshaVaidya Shala, Ukkinadaka, Kasaragod, Kerala, India. Roots of the plant were triturated in a blender until a finely granulated powder was obtained. Aqueous extract was made from 100 g of this root powder by adding distilled water and soaking it overnight. After filtration the extract was lyophilised and stored at 4 °C for experimental use. The yield was 10.5 g (8.6%, w/w).

2.2. Drosophila strains and fly husbandry

Drosophila melanogaster (Oregon K) strain was obtained from the *Drosophila* stock center, University of Mysore, Karnataka, India. Oregon K (OK) flies were used as wild type controls.Transgenic *Drosophila* tauopathy model (V337 M/FM7a;GMR/CyO) was generously gifted by Dr. Upendra Nongthomba, Molecular Reproduction and Developmental Genetics, Indian Institute of Science, Bengaluru, India. All the *Drosophila* stocks were maintained in the Department of Animal Science, Central University of Kerala on standard wheat cream agar media supplemented with dry yeast granules at 22 ± 1 °C and 70–80% relative humidity, and 12 h light/12 h dark in a vivarium (Anupama et al., 2016). Isogenic line of OK and transgenic tauopathy flies were reared on regular standard *Drosophila* food medium. The 7 days old virgin flies were allowed to mate and lay eggs on grape juice medium (containing 3% agar-agar, 1.2% sucrose, 2% ethanol, 1% acetic acid, and 27.2% grape juice without any preservative) for 2 h. The collected eggs from the isogenic fly lines were used for all the experimental studies.

2.3. Preparation of C. pluricaulis supplemented food media and treatment

Most of the Ayurvedic therapies are through oral method, flies were fed on standard wheat cream agar food media supplemented with *C. pluricaulis* extract. *C. pluricaulis* was added in desired concentration (weight/volume) to freshly prepared 5 ml of fly food while preparing and was thoroughly mixed. The mixed food was poured into experimental vials and allowed to cool and solidify before use. In all *C. pluricaulis* supplemented feeding experiments, eggs were collected from fly stocks reared on regular standard food. For each experiment, the regular and the *C. pluricaulis* supplemented foods were prepared from the same batch; likewise all adults for a given experiment were derived from a common pool of eggs of the desired genotype and reared in parallel with the regular or the supplemented food.

2.4. Toxicity analysis and selection of C. pluricaulis concentrations for experiments

LC_{50} of *C. pluricaulis* was determined for selecting concentrations to treat *Drosophila* for the experiments; i.e., the concentration at which 50% of eggs fail to reach the adulthood. For this experiment, 100 eggs were transferred to the medium containing different concentrations of 0.01-10% *C. pluricaulis* and allowed to hatch. Hatched larvae were maintained in the same media. Emerged flies were counted every day from the day of eclosion to the last day of emergence in each treated group. From this, percentage of viability of emerging flies was recorded. Data for each treated and control groups were considered to determine the LC_{50} by Probit method (Finney, 1971). *C. pluricaulis* showed a concentration dependent reduction in emergence of adult flies. While above 0.075% of *C. pluricaulis* supplement food showed more toxicity. The calculated LC_{50} was 0.05%. Hence, less than half of the LC_{50} sublethal concentrations of 0.01% and 0.02% *C. pluricaulis* were selected for feeding hMAPτ induced Alzheimer's disease model to understand the neuroprotective role of *C. pluricaulis* in a concentration dependent fashion.

The detailed experimental groups are as follows:

Control group − Oregon K (OK) wild type *Drosophila* reared on regular standard food.

τ control −Tauopathy *Drosophila* reared on regular standard food.

τ + 0.01% CP −Tauopathy *Drosophila* reared on 0.01% *C. pluricaulis* supplemented with regular standard food and;

τ + 0.02% CP- Tauopathy *Drosophila* reared on 0.02% *C. pluricaulis* supplemented with regular standard food.

To understand the age dependent changes in the climbing performance, τ protein level, ROS generation, LPO level, antioxidant and acetylcholinesterase enzyme activities were analysed in 10^{th}, 20^{th} and 30^{th} days old adult flies with replicates.

2.5. Gustatory assay

For confirming the intake of *C. pluricaulis* containing food, we performed feeding assay (Lee et al., 2010). Flies were reared for 20 days in media supplemented with 0.01% and 0.02% *C. pluricaulis*. The feeding assay was performed after starving flies for 3 h. Twenty flies from each group were transferred into vials containing *C. pluricaulis* diet with orange-red synthetic food dye (Tiger, Manju chemicals pvt. ltd). Feeding was continued for 10 min and the fed flies were collected. Flies were washed with phosphate buffered saline (PBS) (pH 7.2), eyes were removed from the body to avoid the interference of eye pigment with the absorbance spectrum. Flies were homogenised in 1 ml of distilled water, centrifuged at 12000 rpm for 10 min. The diluted (100 times) supernatant was used to measure the absorbance at 595 nm using spectrophotometer. There was no significant difference in food intake observed between *C. pluricaulis* supplemented regular standard food compared to tauopathy and control (OK) flies reared on the regular standard food (without the *C. pluricaulis* supplementation). Hence, we conclude that *C. pluricaulis* is not an antifeedent for the *Drosophila*.

2.6. Life span (Longevity) assay

Lifespan assay was carried out with freshly eclosed flies reared from the embryonic stage, on a regular (control) or supplemented food (0.01% and 0.02% *C. pluricaulis*) at 22 ± 1 °C. Newly emerged flies were collected and separated on the basis of sex within 4 h after eclosion from the respective experimental groups. In order to keep the flies stress free, 10 male and 10 female flies were kept in each food vial (20/vial). Flies were transferred, without anesthetization to fresh vials after every 3 days and the number of surviving flies was recorded on a daily basis until the death of flies after eclosion. The survival of adults was recorded by subtracting the number of dead flies from the total population, and the result is expressed in percentage. All vials were kept

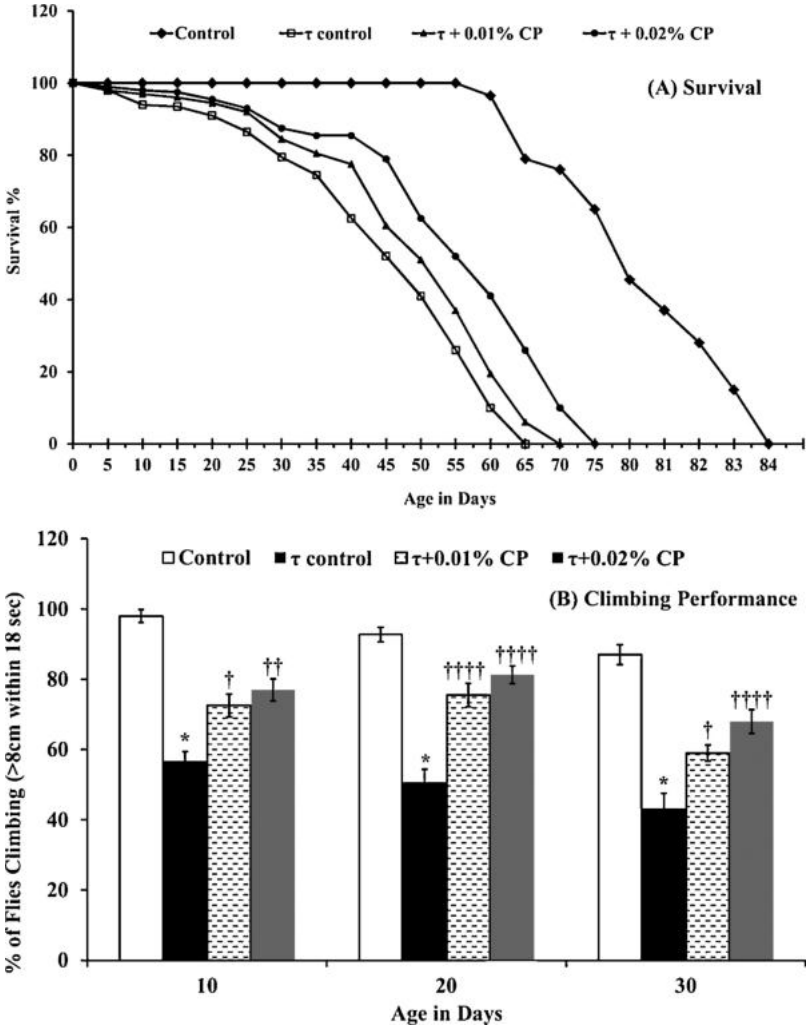

Fig. 1. Survival of the fly after eclosion (A) and climbing performance (B) measured at different age (days) in control, τ control reared on standard food and tauopathy *Drosophila* reared in 0.01% and 0.02% concentrations of *C. pluricaulis* supplemented food. Control: Oregon K- wild type *Drosophila*; τ control: Tauopathy *Drosophila*; CP: *Convolvulus pluricaulis*. Values are mean survival on respective days (n = 200/experimental group); Values are mean ± SE climbing performance (n = 20/assay, assay repeated 20 times/experimental group). *Significant against control at $p < 0.0001$; †significiacnt against τ control at $p < 0.05$; ††significant against τ control at $p < 0.01$; ††††significant against τ control at $p < 0.0001$.

under constant observation and the lifespan of each fly was noted by simply noting the survivability of flies which includes the number of days from the recorded birth to death. The mean lifespan (Xm = ΣXi/n), where Xm is the mean lifespan, Xi is the life span of the i^{th} fly and 'n' is the total number of flies in the experimental group. n = 200 from 10 vials for each experimental feeding regime. Survival curves in Fig. 1A is based on observations of 200 adult flies per group. Even though surviving flies were counted on daily basis, the data are presented for 5 day intervals for convenience.

2.7. Negative geotaxis assay for motor activity

Negative geotaxis assay was to assess the motor activity of tauopathy *Drosophila* reared on regular standard food and in *C. pluricaulis* supplemented regular standard food. Test flies were cold anesthetized and placed in a vertical glass column (length 25 cm; diameter 1.5 cm) sealed at one end. After a brief recovery period, flies were gently tapped to the bottom of the column. Following 18 s observation of their climbing ability along the wall of the vertical glass column by visual method, flies that reached the top of the column and the flies that remained at the bottom were counted separately. Data were expressed as percent flies escaped beyond a minimum distance of 8 cm in 18 s of interval. Twenty adults (n = 20) per replication were used for each assay and the assay was repeated twenty times. The scores for each replication was an average of trials for each group of flies including control (Feany and Bender, 2000).

2.8. ELISA hMAPτ assay

In order to establish *C. pluricaulis* moderates or degrades the neurofibrillary tangles of τ *in vivo*; τ protein was quantified in *Drosophila* using the ELISA hMAPτ assay. The sample was prepared by homogenizing the heads of 30 flies on a respective age of fly in 200 µl of ice cold PBS (0.01 M; pH 7.4), centrifuged at 3000*g* for 10 min at 4 °C. The supernatant was filtered and used as sample. The assay was performed following the hMAPτ assay kit manufacturer's instructions (Elabscience, UK). The micro ELISA plate provided in the kit has been pre-coated with an antibody hMAPτ. Standard or samples were added to the appropriate micro ELISA plate wells combined with the specific antibody. Then a biotinylated detection antibody specific for hMAPτ and avidin-horse raddish peroxidise (HRP) conjugate was added to each well and incubated for 1 h at 37 °C. Free components were washed and the substrate solution was added to each well. Only those wells that contain hMAPτ, biotinylated detection antibody and avidin HRP conjugate appeared blue in colour. The enzyme-substrate reaction was terminated by the addition of a sulphuric acid solution and the colour turns yellow. The optical density (OD) was measured spectrophotometrically at a wavelength of 450 nm. The OD value was proportional to the concentration of hMAPτ. The concentration of hMAPτ from unknown samples was obtained as pg/ml by comparing the OD of the samples to the standard curve.

2.9. Biochemical assays

Sample for biochemical analysis was prepared by homogenizing the heads of 30 flies in 200 µl of ice cold sodium phosphate buffer (0.1 M; pH 8.0), centrifuged at 3000*g* for 10 min at 4 °C. The supernatant was filtered and used as sample for assays.

2.9.1. Reactive oxygen species (ROS) generation

Dihydrofluorescein diacetate (DCF-DA) was used to measure the ROS generation in the fly heads. DCF-DA is a non-polar compound that after conversion to its polar derivative by intracellular esterases rapidly reacts with ROS to form a highly fluorescent dichlorofluorescein (Driver et al., 2000). An aliquot of head sample (50 µl) was dispensed into tubes containing Locke's buffer solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 5 mM HEPES, 2 mM $CaCl_2$ and 10 mM glucose pH 7.4) to which 10 µl of DCF-DA (5 µM) was added and incubated for 30 min at room temperature. The fluorescence was measured with excitation and emission wavelengths at 480 and 530 nm.

2.9.2. Lipid peroxidation (LPO)

The extent of LPO was measured by the thiobarbituric acid reactive substances (TBARS) in the fly head homogenate (Ohakawa et al., 1979). The reaction mixture contained 500 µl fly head homogenate, 1.5 ml acetic acid (pH 3.5, 20%v/v), 1.5 ml of thiobarbituric acid (TBA) (0.8% w/v), 200 µl sodium lauryl sulphate (SDS) (8% w/v). The mixture was heated in a boiling water bath for 45 min and adducts formed were extracted into 3 ml of 1-butanol and the colour intensity was measured at 532 nm and quantified as malondialdehyde equivalents using 1,1,3,3- tetramethoxy propane as standard.

2.9.3. Catalase (CAT) and superoxide dismutase (SOD)

CAT activity was determined using standard method (Aebi, 1984). The rate of H_2O_2 (final concentration 8.8 mM) decomposition by the enzyme was monitored by the addition of an aliquot (equivalent to 10 µg protein, 20 µl) from fly head homogenate. The decrease in H_2O_2 was monitored for 3 min at 240 nm and expressed as µmol of H_2O_2 decomposed/min/mg protein (ε-H_2O_2 44.1 mM^{-1} cm^{-1}). SOD activity was determined by monitoring the inhibition of quercetin autooxidation (Kostyuk and Potapovich, 1989). Total volume of 1 ml reaction mixture contained 3–5 µg protein; 0.016 M sodium phosphate buffer (pH 7.8), 8 mM N,N,N,N-tetra methyl ethylene diamine (TEMED) and 0.08 mM ethylene diamine tetra acetic acid (EDTA). The reaction was started by adding 0.15% quercetin dissolved in dimethyl formamide. The rate of quercetin auto oxidation was monitored for 3 min at 406 nm. Following the addition of sample (5–10 µg protein), the decrease in absorbance was monitored. The amount of protein that inhibits quercetin oxidation by 50% was defined as one unit. The result is expressed as units/mg protein.

2.9.4. Acetylcholinesterase (AChE)

AChE activity was determined by the method of Ellman et al. (1961). To 300 µl of reaction mixture containing 290 µl sodium phosphate buffer (0.1 M; pH 8.0), 5 µl 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) (10 mM) and 3 µl of head homogenate sample, 2 µl of acetylthiocholine (78 mM) was added. Changes in the absorbance at 412 nm were monitored for 1 min and expressed as nmoles of substrate hydrolyzed/min/mg protein.

2.10. Statistical analysis

All the analysis was made in respective replicate and values are represented as mean ± SEM. The significant difference between the experimental groups was obtained by one-way analysis of variance (ANOVA) and Tukey HSD post hoc was used to test the significance level between τ controls (tauopathy *Drosophila* reared on regular standard food), tauopathy *Drosophila* reared on the *C. pluricaulis* supplemented food and control (OK wild type *Drosophila* reared on regular standard food) groups. The significant difference between survival curves was analysed using the Kaplan Meier log-rank test, and followed Tukey HSD post hoc test using SPSS (version 16.0) (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. C. pluricaulis extends the life span (longevity) of tauopathy Drosophila

The overexpression of the hMAPτ caused a marked reduction in survival as compared to the flies without a transgene. The flies overexpressing hMAPτ reared on regular standard food have a shorter life span compared to wild type control (Fig. 1A). Mean life span of tauopathy flies is 46.25 ± 0.479 days paralleled to 76.25 ± 1.315 days in control flies (OK) ($p < 0.001$). The concentration dependent significant increase in mean life span was observed in hMAPτ overexpressing *Drosophila* reared on 0.01% and 0.02% *C. pluricaulis* supplemented regular standard food than that of food medium without *C. pluricaulis* supplementation ($p < 0.001$). The mean life span of hMAPτ overexpressing flies reared on 0.01% and 0.02% *C. pluricaulis* supplemented regular standard food is 51.63 ± 0.239 and 57.75 ± 0.323 days respectively.

3.2. C. pluricaulis protects hMAPτ induced locomotor deficits

Data obtained in the negative geotaxis assay in tauopathy flies revealed the severe age related locomotor dysfunctions. More number of tauopathy flies (43–57%) had a tendency to stay at the bottom of the vial. Among controls, more flies about 87–98% were able to reach to the top of the vial within 18 s, tauopathy flies reared on standard regular food exhibited a significant decrease in climbing ability with aging of the flies ($p < 0.0001$; Fig. 1B). This reduction of climbing activity was compared to control flies reared on the regular standard food is 42.09%, 45.28% and 50.29% analysed on 10^{th}, 20^{th} and 30^{th} day respectively. In contrast, flies overexpressing hMAPτ showed rapid and much decline in climbing behaviour, clearly suggesting the induction of locomotor deficits by τ neurotoxicity. In parallel experimental groups, tauopathy flies reared on the 0.01% and 0.02% *C. pluricaulis* supplemented regular standard food showed significantly improved climbing performance than the tauopathy flies reared on regular standard food ($p < 0.0001$). This amelioration is 60.10% and 57.23% on 20^{th} and 30^{th} day tauopathy flies reared on 0.02% *C. pluricaulis* supplemented regular standard food compared to tauopathy flies reared on regular standard food. *C. pluricaulis* showed significant improvement of tauopathy fly locomotor deficits caused by overexpressed τ indicating its neuroprotective effect.

3.3. Effect of C. pluricaulis on expression levels of τ protein

The significant age related increased level of τ protein was found in tauopathy flies compared to the control flies reared on the regular standard food ($p < 0.0001$; Fig. 2A). There is a significant reduction of τ protein in tauopathy flies reared on the *C. pluricaulis* supplemented food compared with the same type of tauopathy flies reared on regular standard food ($p < 0.0001$; Fig. 2A). This reduction of τ protein in tauopathy flies reared on 0.01% and 0.02% *C. pluricaulis* supplemented food compared with the tauopathy flies reared on regular standard food is 25.62%, 30.80% and 29.97%; and 31.59%, 38.36% and 37.96% measured on 10^{th}, 20^{th}, and 30^{th} day of flies age.

3.4. Ameliorative effects of C. pluricaulis on hMAPτ induced oxidative stress

There is a significant age related increased level of LPO and elevated ROS generation in the tauopathy flies than the control flies reared on

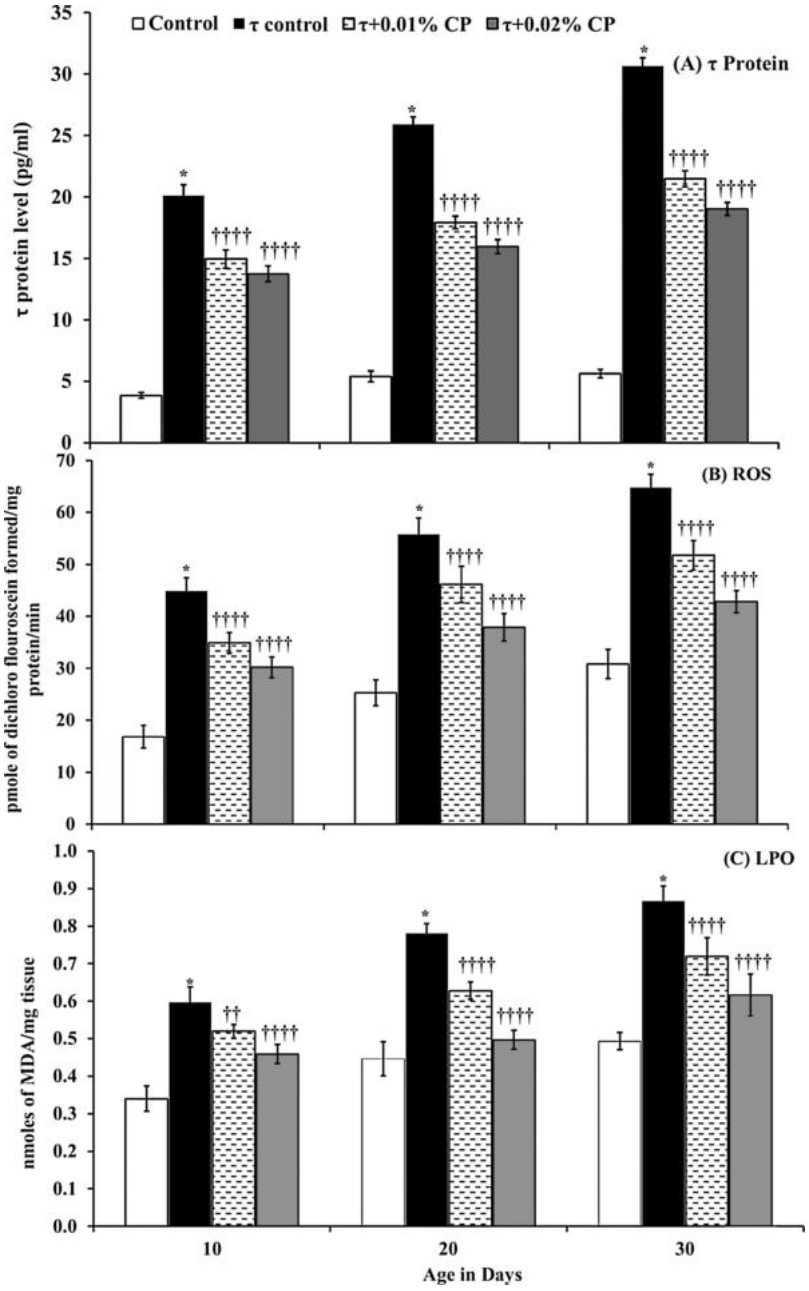

Fig. 2. Tau (τ) protein level (A), reactive oxygen species (ROS) generation (B) and lipid peroxidation (LPO) level (C) measured at different age (days) in control, τ control reared on standard food and tauopathy *Drosophila* reared in 0.01% and 0.02% concentrations of *C. pluricaulis* supplemented food. Control: Oregon K- wild type *Drosophila*; τ control: Tauopathy *Drosophila*; CP: *Convolvulus pluricaulis*. Values are mean ± SE (n = 30 fly heads/replicate, three experiments performed in triplicate). *Significant against control at $p < 0.0001$; ††significant against τ control at $p < 0.01$; ††††significant against τ control at $p < 0.0001$.

the regular standard food ($p < 0.0001$). The significant age related and concentration dependent reduction of LPO level and ROS generation, recorded in the tauopathy flies reared on the 0.01% and 0.02% *C. pluricaulis* supplemented regular standard food than the control ($p < 0.0001$). Fig. 2B and C shows *C. pluricaulis* significantly diminishes in the endogenous levels of oxidative stress markers in tauopathy fly induced by overexpression of τ indicating its ameliorative effect.

3.5. Effect of C. pluricaulis on antioxidant enzyme activities

There is a significant reduction in the activity of endogenous antioxidant enzymes, CAT and SOD in the tauopathy *Drosophila* reared on standard food compared to their control ($p < 0.0001$). The age related depletion of enzyme activities compared to control flies reared on the regular standard food is 44.64%, 45.83% and 48.99% CAT and 29.79%, 39.22% and 52.17% SOD on 10th, 20th and 30th day respectively. The tauopathy *Drosophila* reared on *C. pluricaulis* supplemented regular standard food showed the significant elevation of antioxidant enzyme activities. The significant neuroprotective role of *C. pluricaulis* was observed in the tauopathy flies reared on the 0.01% and 0.02% *C. pluricaulis* supplemented food. The significant restoration of enzyme activities in tauopathy flies reared on the 0.02% of *C. pluricaulis* supplemented with regular standard food compared to tauopathy flies reared on regular standard food is 41.35%, 47.34% and 60.26% CAT and 12.12%, 35.48% and 77.27% SOD (Fig. 3A and B). This restoration is age related and concentration dependent with *C. pluricaulis* supplementation.

3.6. Neuroprotective effects of C. pluricaulis on neurotransmitter enzyme activity

There is a decrease of AChE enzyme activity in the tauopathy flies than the control flies reared on the standard food ($p < 0.0001$; Fig. 3C). Interestingly, a significant enhancement in the activity of AChE in tauopathy flies reared on 0.01% and 0.02% *C. pluricaulis*

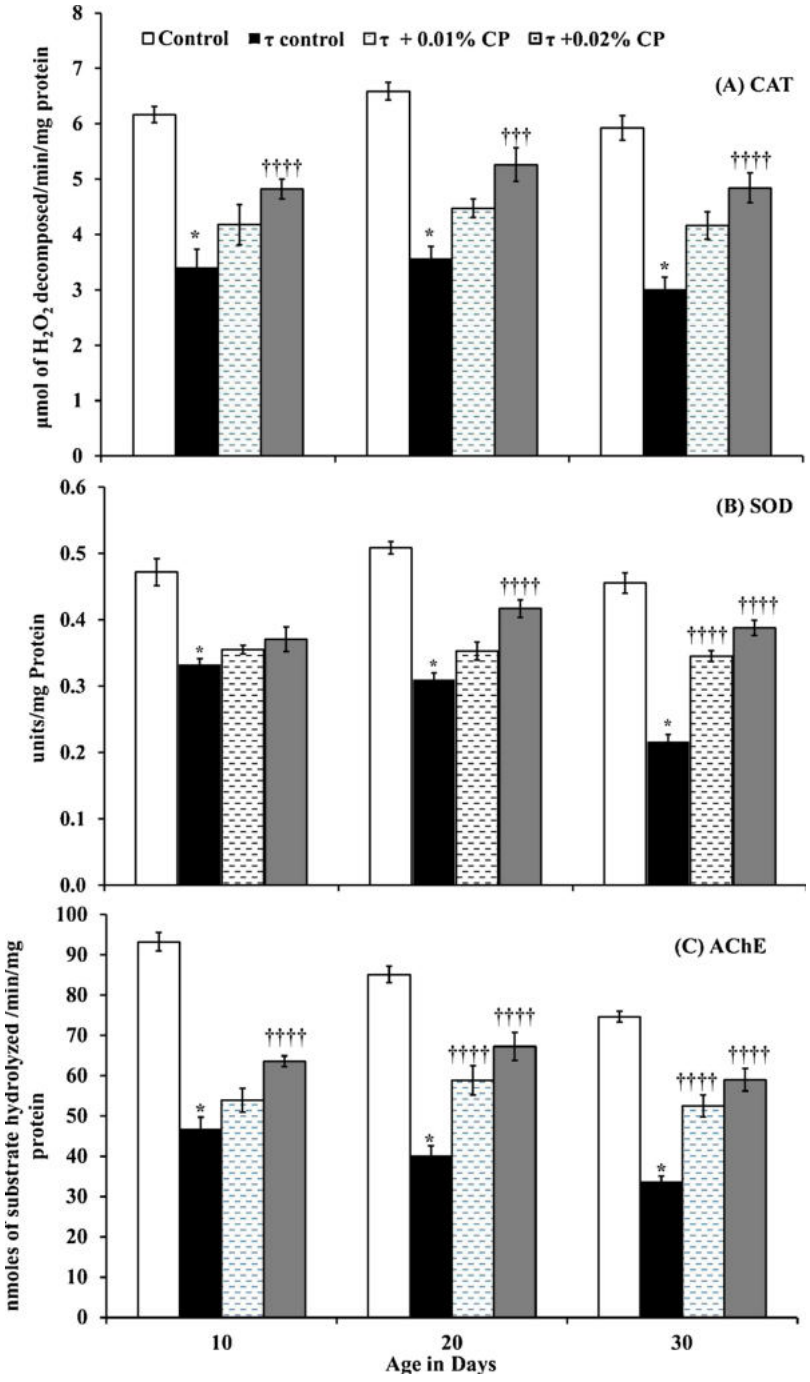

Fig. 3. Catalse (CAT) (A), superoxide dismutase (SOD) (B) and acetylcholinesterase (AChE) activity (C) measured at different age (days) in control, τ control reared on standard food and tauopathy *Drosophila* reared in 0.01% and 0.02% concentrations of *C. pluricaulis* supplemented food. Control: Oregon K- wild type *Drosophila*; τ control: Tauopathy *Drosophila*; CP: *Convolvulus pluricaulis*. Values are mean ± SE (n = 30 fly heads/replicate, three experiments performed in triplicate). *Significant against control at $p < 0.0001$; †††significant against τ control at $p < 0.001$; ††††significant against τ control at $p < 0.0001$.

supplemented regular standard food. There is a significant marked restoration of AChE activity in tauopathy flies analysed on 10th, 20th and 30th day is 15.12%, 46.28%, and 55.56% at 0.01% and 35.82%, 67.21% and 74.81% at 0.02% with *C. pluricaulis* supplemented food compared to tauopathy flies reared on the regular standard food without the *C. pluricaulis* supplementation ($p < 0.0001$).

4. Discussion

The hMAPτ overexpressing *Drosophila* reared on the *C. pluricaulis* supplemented food showed significant prolonged lifespan in the present study ($p < 0.001$; Fig. 1A). This might be due to the micronutrient present in the dietary supplement. The main phytochemical components present in the *C. pluricaulis* plant extract are alkaloids (shankhpushpine) and flavonoids (kampferol) of which convolvine and convolamine are the major constituents that are tropane alkaloids (Singh and Bhandari, 2000). The presence of the alkaloids and flavonoids in the *C. pluricaulis* plant extract act as powerful antioxidants there by increase the survival and longevity of flies in our study.

Neurodegenerative diseases are generally characterized by an age-dependent loss of locomotor or motor ability. The clinical criteria for the diagnosis of AD include onset and progressive impairment of memory, learning, and movement disorders (Kurlan et al., 2000). Tauopathy flies revealed a rapid decline in climbing behaviour, severe age related induction of locomotor deficits in the current study (Fig. 1B), which is analogous to reduced climbing ability as observed in human AD patients (Ali et al., 2012). This proves that locomotor defect in *Drosophila* is used as a representative phenotypic marker of the neurodegenerative aging disorder (Feany and Bender, 2000). The manifestations of neuronal dysfunction in *Drosophila*, such as locomotor

deficits, reduced lifespan, and increased levels of the τ, and which provide evidence that the aggregated τ is the primary determinant of the pathological behaviour in the *Drosophila* system (Whittmann et al., 2001). It is evident in the present study that the significant age related increased level of τ protein in tauopathy flies reared on the regular standard food ($p < 0.001$; Fig. 2A). The tauopathy flies reared on the 0.01% and 0.02% *C. pluricaulis* supplemented food showed significantly improved climbing performance than in the tauopathy flies reared on regular standard food ($p < 0.001$). *C. pluricaulis* showed a significant improvement of tauopathy flies locomotor deficits caused by over-expressed τ indicating its neuroprotective effect (Fig. 1B). Presence of polyphenols/flavonoids have impact on observed locomotor dysfunction and the results are consistent with previously demonstrated protective effects of flavonoids in *Drosophila* (Jimenez-Del-Rio et al., 2010; Anupama et al., 2016).

The significant decrease of τ protein in the tauopathy flies in the range of 25 to 38% reared on the *C. pluricaulis* supplemented food ($p < 0.0001$; Fig. 2A) speculates that *C. pluricaulis* containing the polyphenol like flavonoids could be involved in the reduction of τ hyperphosphorylation in addition to attenuating several AD pathways, such as ROS generation, LPO, DNA fragmentation, caspase activation and Aβ accumulation. *C. pluricaulis* reduced the protein and mRNA levels of τ, amyloid-β precursor protein (AβPP) and Aβ production in rat (Bihaqi et al., 2012; Liu et al., 2012). Natural plant products are the rich source of τ-targeting components used as drugs for treating AD (Calcul et al., 2012). Several anti-τ natural products made by plants are polyphenols such as curcumin, from the turmeric (*Curcuma longa*) extract (Shytle et al., 2009). Hence, it can be suggested that natural products based anti-τ diets (diet aid in reducing τ) are the potential future treatments for AD.

The present study provides evidence on the age related increase in the ROS generation and level of LPO in the tauopathy flies than in the control (OK) flies reared on the regular standard food (Fig. 2B and C). This oxidative stress induced neurotoxicity is caused by the over-expression of the hMAPτ in the transgenic flies. Induction of oxidative stress in tauopathy flies is the result of the down-regulation of anti-oxidant enzyme in a *Drosophila* model expressing hMAPτ transgene (Dias-Santagata et al., 2007). The significant reduction in generation of ROS and LPO level in *C. pluricaulis* supplemented food fed tauopathy flies ($p < 0.001$); indicate that *C. pluricaulis* induced the production of antioxidants significantly and reduced the level of ROS and LPO. Thus, it is plausible that some phytoconstituents present in the *C. pluricaulis* with antioxidant activities scavenge the ROS and LPO produced by age related oxidative damage and τ protein production. The ability of *C. pluricaulis* to offset hMAPτ induced neurotoxicity in *Drosophila* suggests that polyphenolic flavonoids like component may be exploited as an agent in the management of oxidative stress mediated neurodegenerative pathologies.

The significant age related decrease in antioxidant enzyme activities accompanied by increased oxidative stress, promotes the neurodegeneration in elderly people (Fabian et al., 2012). Treatment with *C. pluricaulis*, improved the activities of antioxidants by scavenging superoxide and H_2O_2 produced by age related oxidative damage. The important factor noticed is that a concentration dependent increased level of antioxidant in the tauopathy flies reared on the *C. pluricaulis* supplemented food in their respective ages compared with flies reared on the regular standard food (control group) ($p < 0.01$; Fig. 3A and B). It is a clear indication that *C. pluricaulis* not only scavenges the oxidative stress but also boosts the activity of few antioxidant enzymes during normal physiological conditions. It is already proved that *C. pluricaulis* plant shankhpushpine, kampferol, convolvine and convolamine are the most notable constituent tropane alkaloids that act as powerful antioxidants (Singh and Bhandari, 2000).

It is evident in the present study that tauopathy flies exhibit deficit in the AChE enzyme compared to control (OK) flies reared on the regular standard food (Fig. 3C). The supplementation of *C. pluricaulis* along with the regular standard food rendered protection against the hMAPτ induced depletion of AChE level. The decline in AChE activity of the AD patient's brain when compared to healthy control individual acetylcholine and consequent cholinergic neurotransmission is one of the cognitive symptoms of AD (Rinne et al., 2003). Treatment using *C. pluricaulis* is enhancing the memory and increased AChE activity in hippocampal regions associated with the learning and memory functions in the young and old mice was reported (Sharma et al., 2010). The underlying mechanism of actions of *C. pluricaulis* may be attributed to cholinergic properties to be studied.

5. Conclusion

The present study provides the first evidence that supplementation of *C. pluricaulis* with the regular standard food prolongs lifespan and improves locomotor deficits by diminishing the level of τ protein in the *Drosophila* AD model. *C. pluricaulis* acts as an endogenous activator of the cellular antioxidant defences, ameliorate the τ-induced oxidative stress and restore the depletion of AChE activity in the fly model. Consumption of *C. pluricaulis* extract is likely to offer a therapeutic advantage in humans to prevent or delay the progression of oxidative stress mediated neurodegenerative disorder. This study suggests the neuroprotective effect of *C. pluricaulis* to minimise the τ induced neurotoxicity in AD model. Further studies are needed to evaluate the therapeutic efficacy and toxicity of isolated compounds of *C. pluricaulis* in various animal models.

Conflict of interest

The authors declare no conflict of interest.

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