



# Protective effect of *Capsosiphon fulvescens* on oxidative stress-stimulated neurodegenerative dysfunction of PC12 cells and zebrafish larva models

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## Abstract

Reactive oxygen species (ROS) at high concentrations induce oxidative stress, an imbalanced redox state that is a prevalent cause of neurodegenerative disorders. This study aimed to investigate the protective effect of *Capsosiphon fulvescens* (CF) extract on oxidative stress-induced impairment of cognitive function in models of neurodegenerative diseases. CF was extracted with subcritical water and several solvents and H<sub>2</sub>O<sub>2</sub> (0.25 mM) or aluminum chloride (AlCl<sub>3</sub>; 25 μM) as an inducer of ROS was treated in PC12 neuronal cells and zebrafish larvae. All statistical analyses were performed using one-way analysis of variance and Dunnett's test using GraphPad Prism. H<sub>2</sub>O<sub>2</sub> and AlCl<sub>3</sub> were found to significantly induce ROS production in PC12 neuronal cells and zebrafish larvae. In addition, they strongly affected intracellular Ca<sup>2+</sup> levels, antioxidant enzyme activity, brain-derived neurotrophic factor (BDNF) and tropomyosin receptor kinase B (TrkB) signaling, acetylcholinesterase (AChE) activity, and hallmarks of Alzheimer's disease. However, treatment of H<sub>2</sub>O<sub>2</sub>-induced PC12 cells or AlCl<sub>3</sub>-induced zebrafish larvae with CF subcritical water extract at 90°C and CF water extract effectively regulated excessive ROS production, intracellular Ca<sup>2+</sup> levels, and mRNA expression of superoxide dismutase, glutathione peroxidase, glycogen synthase kinase-3 beta, β-amyloid, tau, AChE, BDNF, and TrkB. Our study suggested that CF extracts can be a potential source of nutraceuticals that can improve the impairment of cognitive function and synaptic plasticity by regulating ROS generation in neurodegenerative diseases.

**Keywords:** *Capsosiphon fulvescens*, Oxidative stress, Cognitive function, Neurodegenerative diseases

## Introduction

Oxidative stress is a common cause of neurodegenerative diseases

like Alzheimer's diseases (AD), which are the leading causes of death worldwide (Singh et al., 2019). Excessive quantities of reactive oxygen species (ROS), a cellular metabolism consequence,

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play a critical role in neurodegeneration (Zorov et al., 2014). The brain uses a large amount of oxygen to produce energy; owing to the low antioxidant levels and many polyunsaturated fatty acids in the brain tissue, it is vulnerable to ROS-induced oxidative damage (Uttara et al., 2009). Increased oxidative stress in the brain has been reported to be closely associated with cognitive decline in aged mice, where ROS modifies amyloid beta ( $A\beta$ ) and hyperphosphorylation of tau (p-Tau) protein, two critical biomarkers of AD brains (Liu et al., 2015; Logan et al., 2019). Many studies have reported glycogen synthase kinase-3 beta (GSK3 $\beta$ ) activity to be directly related to increased levels of  $A\beta$ , p-tau, and synaptic damages in AD patients and in rodent models (DaRocha-Souto et al., 2012) where p-tau, increased by GSK3 $\beta$ , modulates the level of acetylcholinesterase (AChE) (Cortés-Gómez et al., 2021). Presence of large amounts of AChE is a common feature of AD brain and is associated with  $A\beta$  and neurofibrillary tangles (NFT).  $A\beta$  aggravation and abnormal p-tau levels influence AChE expression and increase neurotoxicity (García-Ayllón et al., 2011). Activity of AChE in blood and plasma can be used to assess AD pathophysiology, since plasma AChE activity is increased in patients with AD (Carvajal & Inestrosa, 2011). In AD, memory and learning are impaired due to the suppression of brain-derived neurotrophic factor (BDNF)/tropomyosin receptor kinase B (TrkB) signaling (Zhang et al., 2016). In addition, some data shows that  $A\beta$  reduces BDNF activation via reducing phosphorylated cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) protein, and phosphorylation of GSK3 at serine-129 leads to CREB deactivation which causes dysregulation in BDNF/TrkB signaling (Amidfar et al., 2020; Grimes & Jope, 2001).

*Capsosiphon fulvescens* (CF) is a green seaweed that has been traditionally used as functional food material to cure gastrointestinal problems and hangovers in Korea (Critchley et al., 1998). CF approximately contains 13% ash, 1% lipids, 30% proteins and 55% polysaccharides along with essential macro and micro minerals, such as Ca, Fe, Mg, Na, K, and P. It exhibits strong 2,2-diphenyl-1-picrylhydrazyl (DPPH) and nitrite scavenging activities (Asanka Sanjeeva et al., 2018). Fang et al. (2012) had reported nine compounds from 95% EtOH extract of CF that exhibited AChE inhibitory activity associated with neuroprotective properties (Fang et al., 2012). Oh & Nam (2019) had reported that hydrophilic glycoproteins of CF protect aging-induced spatial memory impairment in rat dorsal hippocampus (Oh & Nam, 2019). However, very few studies on the neuroprotective mechanism of CF have been conducted till

date. The objective is to determine the neuroprotective properties of CF.

## Materials and Methods

### Reagents

Materials, such as 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium-12-Bromide (MTT), 2',7'-dichlorofluorescein diacetate (DCFH-DA), 10-nonyl bromide acridine orange hydrochloride solution, Triton X-10, ethylenediaminetetraacetic acid (EDTA), aluminum chloride ( $AlCl_3$ ), and hydrogen peroxide, were from Sigma-Aldrich (St. Louis, MO, USA). Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum, phosphate-buffered saline (PBS), trypsin, and penicillin-streptomycin were obtained from Hyclone (Logan, UT, USA). Sodium phosphate ( $NaHPO_4$ ), sodium chloride (NaCl), methanol (MeOH), and ethanol (EtOH) were obtained from DUKSAN (Ansan, Korea).

### Preparation of *Capsosiphon fulvescens* (CF) extracts

Fresh CF was collected from Jeollanam-do province, South Korea. A voucher specimen was deposited in the herbarium of the Department of Nutraceutical Resources, Mokpo National University, Republic of Korea. CF extracts were produced with subcritical water and several solvents (water, MeOH, and EtOH) to investigate the neuroprotective potential. Fresh CF was thoroughly washed with tap water to remove excess salt. For solvent extraction, 6 g of CF were dissolved in 200 mL of 100% MeOH, 200 mL of 100% EtOH, and 200 mL of water separately. The extracts were filtered, evaporated under vacuum, then freeze-dried for up to 72 h to yield 0.9 g of dry MeOH extract (CFM; 15% yield), 0.05 g of dry EtOH extract (CFE; 0.83% yield), and 1.12 g of water extract powder (CFW; 18.7% yield). For the subcritical water extract preparation, hydrolysis of CF was performed at various temperatures (90 °C, 150 °C, and 210 °C), labelled as CF90, CF150, and CF210. For each temperature, 6 g of CF were soaked in an extraction cell with 250 mL of distilled water, and the botanical extraction equipment (TPR-1, Taiatsu Techno, Tokyo, Japan) was maintained at a constant reaction pressure of 3 MPa for 1 min. The extracts were then filtered and freeze-dried for up to 72 h to yield 1.43 g of CF90 (23.8% yield), 2.09 g of CF150 (34.8% yield), and 2.82 g of CF210 powder (47% yield).

### Cell culture

PC12 neuronal cells were obtained from the American Type

Culture Collection (Manassas, VA, USA) and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37°C under 5% CO<sub>2</sub>. During the experiment, the medium was replaced every two days. When the cells achieved 75%–90% confluence, they were seeded in culture plates.

#### Determination of cell cytotoxicity

PC12 cells were seeded in 96-well plates at a density of  $2 \times 10^4$  cells/well. They were treated with different concentrations of CF extract for 2 h and further treated with H<sub>2</sub>O<sub>2</sub> and incubated for 24 h at 37°C and 5% CO<sub>2</sub>. Cell cytotoxicity was observed using the MTT solution (250 µg/mL). After 3 h, the MTT solution was removed followed by addition of dimethyl sulfoxide, to dissolve the purple formazan product formed by living cells. The optical density was then measured at a wavelength of 540 nm using a spectrophotometer (Immuno Mini NJ-2300, Biotech, Tokyo, Japan).

#### Measurement of cellular reactive oxygen species (ROS) production

The effect of H<sub>2</sub>O<sub>2</sub> on intracellular ROS production was evaluated by culturing PC12 cells in black 96-well plates for 48 h at a density of  $2 \times 10^4$  cells/well, and then treated with CM-H2DCFDA (10 µM) in growth medium for 30 min. The cells were then washed with PBS and treated with CF extracts for 2 h, followed by treatment with H<sub>2</sub>O<sub>2</sub> (0.25 mM) for 24 h. The cells were subsequently washed with PBS solution, and fluorescence intensity was measured at excitation/emission wavelengths of 485 nm/520 nm using a fluorescence microplate reader (Perkin-Elmer, Waltham, MA, USA).

#### Measurement of acetylcholinesterase (AChE) enzyme activity

PC12 cells were seeded in 6-well plates at a density of  $0.5 \times 10^4$  cells/well for 24 h. They were treated with CF extracts for 2 h, followed by treatment with H<sub>2</sub>O<sub>2</sub> (0.25 mM) for 24 h at 37°C and 5% CO<sub>2</sub>. The cell extracts were prepared by adding high-ionic strength buffer (10 mM NaHPO<sub>4</sub>, 1 M NaCl, 10% Triton X-10, and 1 mM EDTA) and centrifuged at 12,000 rpm for 20 min 4°C. The collected supernatant was used to determine the AChE activity.

#### Measurement of intracellular calcium

PC12 cells were cultured in black 96-well plates at a density of  $2 \times 10^4$  cells/well for 48 h. They were treated with CF extracts for 2 h, followed by treatment with H<sub>2</sub>O<sub>2</sub> (0.25 mM) for 24 h. Thereafter,

the culture medium was removed, and the calcium-sensitive fluorescent dye Fluo-3 AM (5 µM) was added by mixing with fresh culture medium for 30 min. After incubation, the cells were washed with PBS, and the intensity was measured at excitation/emission wavelengths of 490 nm/520 nm using a fluorescence microplate reader (Perkin-Elmer, Waltham, MA, USA).

#### Zebrafish husbandry and toxicity assay

Adult Zebrafish (*Danio rerio*, AB line) were in-house cultured in a 14-h light/10-h dark cycle with  $28 \pm 0.5^\circ\text{C}$  and pH 7.0 in a continuously flow-through system (ZebTEC, Tecniplast, Buguggiate, Italy) in the laboratory at Mokpo National University. According to their developmental stage, GEMMA Micro feeds (Skretting; Tooele, UT, USA) of various sizes were supplied to the zebrafish. Adult male and female zebrafish were placed in mating tanks at 1:1 ratio, and embryos were collected the following day. Embryos were cultured in a 96-well cultural plate with E2 embryo medium (15 mM NaCl, 0.5 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgSO<sub>4</sub>, 0.15 mM KH<sub>2</sub>PO<sub>4</sub>, 0.05 mM Na<sub>2</sub>HPO<sub>4</sub>, and 0.7 mM NaHCO<sub>3</sub>, pH 7.1–7.4) until 6 days' post fertilization (dpf). At 6 dpf, the zebrafish larvae were exposed to the different concentrations of CF extracts and incubated for 24 h. Viability and morphological changes of the larvae in response to AlCl<sub>3</sub> and CF extract exposure were observed under a LEICA MZ10F microscope (Leica, Wetzlar, Germany) by confirming the relative shape and size of the eye, yolk sac, jaw, tail, etc., compared to the normal group, and the observed malformations and mortality rates were calculated.

$$\text{Mortality} = \left[ \frac{(\text{Total number of larvae} - \text{Number of alive larvae})}{\text{Total number of larvae}} \right] \times 100$$

$$\text{Malformation} = \left[ \frac{(\text{Total number of larvae} - \text{Number of normal larvae})}{\text{Total number of larvae}} \right] \times 100$$

#### Oxidative stress-induced model

AlCl<sub>3</sub> was used as an inducer of ROS production in zebrafish larvae, as AlCl<sub>3</sub> exposure increases the production of free radicals, disrupts the mitochondria, and ultimately leads to oxidative stress and neurodegeneration (Kaur et al., 2022). The latter were exposed to different concentrations of AlCl<sub>3</sub> (12.5, 25, 50, 100, and 250 µM) after 4 dpf. AlCl<sub>3</sub> powder was dissolved in distilled water, and the final concentration was prepared by diluting with E2 medium. The treatment was continued up to 8 dpf, after which, the AlCl<sub>3</sub> solution was treated every 2 d until the experiments were completed.

### Measurement of reactive oxygen species (ROS) production in zebrafish larvae

Based on the method proposed by Park et al. (2021), DCFH-DA reagent was used to stain nucleic acids for measuring intracellular ROS production (Park et al., 2021). After 8 dpf, the solution of DCFH-DA (20 µg/mL) was added to each well of the culture plate and allowed in the dark for 1 h at 28 °C, respectively. When the experiment was completed, the zebrafish larvae were washed twice or thrice with embryo medium and used 0.5% MS-222 to anesthetized. Images were obtained using a fluorescence microscope (Leica). Fluorescence intensity of each zebrafish larva was quantified using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

### Quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted from pools of 40–50 larvae using a Bullet Blender homogenizer and TRIzol reagent (Molecular Research Center, Cincinnati, OH, USA). RNA primers were purchased from Integrated DNA Technologies (Coralville, IA, USA). Quantitative PCR (qPCR) assays were conducted with the Applied Biosystems StepOne™ Real-Time PCR System Thermal Cycling Block (ABI, Los Angeles, CA, USA) using a SYBR Green Real-Time qPCR Kit (BIOFACTM, Daejeon, Korea). Sequences of the oligonucleotide primers were as follows: glutathione peroxidase (GPx)–forward: 5'-TGAGAAGGGTTTACGCATCC-3' and reverse: 5'-GCTGCTCCTTCATCCACTTC-3'; superoxide dismutase (SOD)–forward: 5'-ATGGTGAACAAGGCCGTTTG-3' and reverse: 5'-AAAGCATGGACGTGGAAACC-3'; GSK3β–forward: 5'-GCCACAGAAACCAAGAGAGC-3' and reverse: 5'-GAATGCAAACGGAAGTGGAT-3'; AChE–forward: 5'-GGGCACCAGGTTTTCAGTAAA-3' and reverse: 5'-CTTTTGGCCGTTGTTCTCAT-3'; BDNF–forward: 5'-CGAGGAATAGACAAGCGGCA-3' and reverse: 5'-ATCCGTATAAACCGCCAGCC-3'; Trkβ–forward: 5'-TCACCTATGGCAAGCAACCC-3' and reverse: 5'-CTTTGGGGCAAGTACGAGGT-3'; Aβ–forward: 5'-AGCAGCTGGTGGAGACTCAT-3' and reverse: 5'-TAGTGGCGGATAGTGTGCTG-3'; and Tau (tau)–forward: 5'-ACTGAGTTCTGGGAGCGA AA-3' and reverse: 5'-CAAATGGGAATGTTGCTGTG-3'. The qPCR reaction conditions were: 15 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 45 s at 60 °C, followed by plate analysis. In each run, the positive and negative controls were included. The number of gene copies was calculated using the Ct value and standard curve and

the significance analysis was performed on the log2 transformed values that were obtained via analysis of variance (ANOVA).

### Statistical analysis

Data are expressed as mean ± SEM. All statistical analyses were conducted with one-way ANOVA and Dunnett's test using GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant at  $p < 0.05$ .

## Results

### Effect of *Capsosiphon fulvescens* (CF) extracts on cytotoxicity of PC12 neuronal cells

Crude CF extracts at different concentrations (125, 250, and 500 µg/mL) were added to normal PC12 cells and incubated for 24 h; no cytotoxicity was observed, as shown in Fig. 1. Almost all CF extracts showed significant cell proliferation at lower concentrations than at higher concentrations compared to that in the normal group.

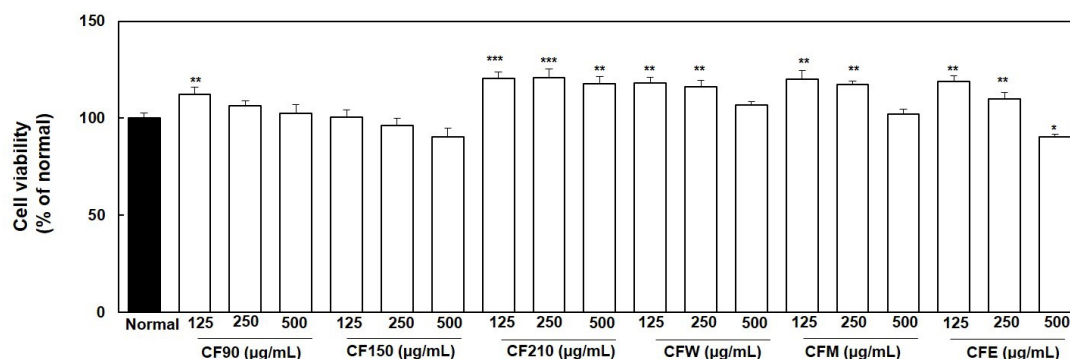
### Effect of *Capsosiphon fulvescens* (CF) extracts on death of H2O2-induced PC12 neuronal cells

As shown in Fig. 2A, PC12 cells were treated with different concentrations of H<sub>2</sub>O<sub>2</sub>. Based on the results of previous studies, we used concentrations ranging from 0.125, 0.5, 1, 2, and 4 mM to evaluate the cytotoxicity of H<sub>2</sub>O<sub>2</sub>-induced oxidative stress. Cytotoxicity, according to H<sub>2</sub>O<sub>2</sub> concentration, in PC12 cells was 81.6%, 49.4%, 36.4%, 36.1%, 7.2%, and 2.0% respectively. With reference to previous reports, we selected 0.25 mM concentration to analyze the neuroprotective effects of CF extracts; we found that CF90, CF150, CF210, and CFW extracts showed preventive effect against H<sub>2</sub>O<sub>2</sub> toxicity in PC12 cells (Fig. 2B). Among them, cell viability of PC12 cells was the highest with CF90 and CFW extracts. Therefore, only two extracts were used in subsequent experiments.

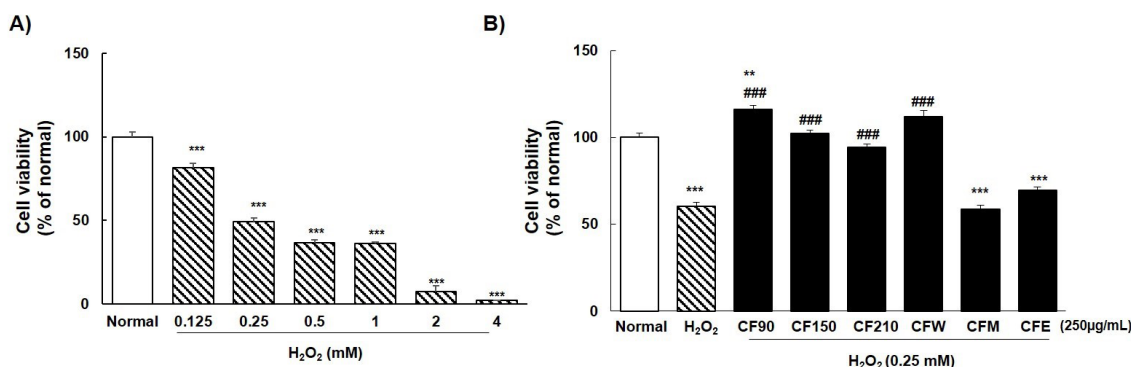
### Effect of *Capsosiphon fulvescens* subcritical water extract at 90°C (CF90) and water extract powder (CFW) extracts on reactive oxygen species (ROS) production, acetylcholinesterase (AChE) activity, and calcium Level in H<sub>2</sub>O<sub>2</sub>-induced PC12 neuronal cells

ROS generation in PC12 cells due to H<sub>2</sub>O<sub>2</sub> exposure was significantly ( $p < 0.001$ ) increased by approximately 76% compared to that in the normal group (Fig. 3A). It decreased





**Fig. 1. Effect of CF on viability of PC12 neuronal cells.** Data are presented as mean  $\pm$  SE ( $n = 12$ ) and analyzed by one-way analysis of variance followed by Dunnett test. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. untreated normal cells. CF, *Capsosiphon fulvescens*; Normal, untreated CF extracts; CF90, CF subcritical water extract at 90°C and 3 MPa; CF150, CF subcritical water extract at 150°C and 3 MPa; CF210, CF subcritical water extract at 210°C and 3 MPa; CFW, CF water extract; CFM, CF methanol extract; CFE, CF ethanol extract.

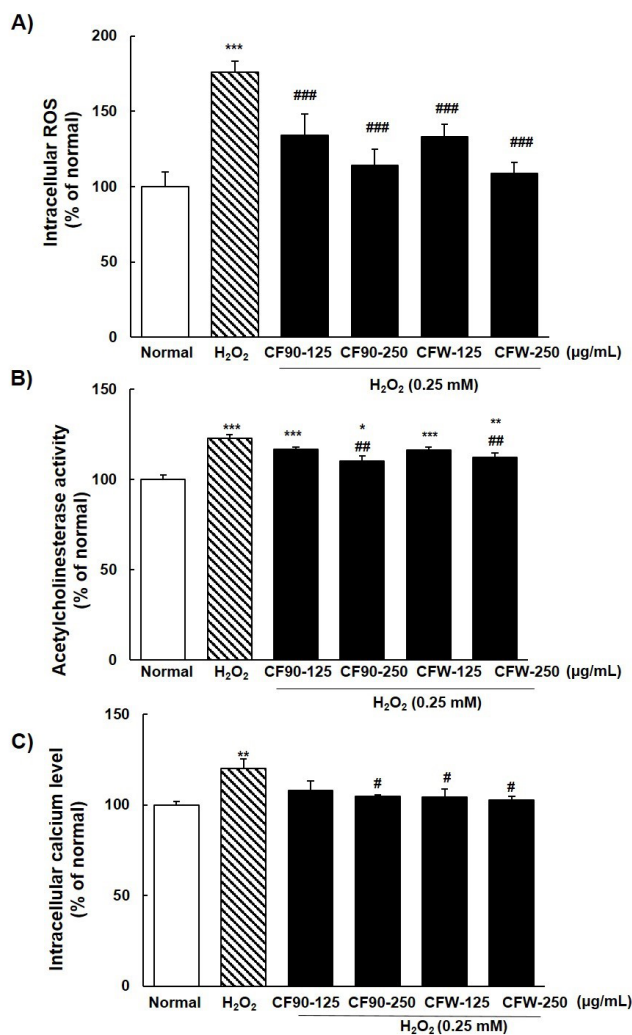


**Fig. 2. Effect of H<sub>2</sub>O<sub>2</sub> or CF on viability of PC12 neuronal cells.** Data are presented as mean  $\pm$  SE ( $n = 12$ ) and analyzed by one-way analysis of variance followed by Dunnett test. \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. untreated normal cells; ### $p < 0.001$  vs. H<sub>2</sub>O<sub>2</sub>-treated cells. CF, *Capsosiphon fulvescens*; Normal, untreated CF extracts; CF90, CF subcritical water extract at 90°C and 3 MPa pressure; CF150, CF subcritical water extract at 150°C and 3 MPa pressure; CF210, CF subcritical water extract at 210°C and 3 MPa pressure; CFW, CF water extract; CFM, CF methanol extract; CFE, CF ethanol extract.

effectively in a concentration-dependent manner by the addition of CF90 or CFW extract. However, there was no significant difference between the two groups (CF150 and CF210). Excess AChE enzyme activity and intracellular calcium levels, which are markers of neurodegeneration, were significantly increased to approximately 23% and 20% by 0.25 mM H<sub>2</sub>O<sub>2</sub> treatment compared to that in normal PC12 cells (Fig. 3B and 3C). Addition of CF90 and CFW extracts significantly reduced H<sub>2</sub>O<sub>2</sub>-stimulated AChE activity and calcium concentration in a concentration-dependent manner.

#### Effect of aluminum chloride (AlCl<sub>3</sub>) on survivability and reactive oxygen species (ROS) production in zebrafish larvae

To confirm the neuroprotective effects of CF, we designed an AlCl<sub>3</sub>-induced neurotoxicity model in zebrafish larvae (Fig. 4). When zebrafish larvae were treated with different concentrations of AlCl<sub>3</sub> (12.5, 25, 50, 100, and 250 µM) for 2 days, no toxicity was observed up to 250 µM AlCl<sub>3</sub> (Fig. 4A). At a concentration of 25 µM AlCl<sub>3</sub>, ROS production increased to approximately 19% than in the normal group (Fig. 4B and 4C), which was mainly observed in the liver and gastrointestinal region of the zebrafish larvae. The most suitable concentration for AlCl<sub>3</sub>-induced neurotoxicity in zebrafish larvae was 25 µM.



**Fig. 3. Effect of CF90 and CFW on reactive oxygen species production (A), acetylcholinesterase enzyme activity (B), and calcium level (C) in H<sub>2</sub>O<sub>2</sub>-induced PC12 neuronal cells.** The concentration of H<sub>2</sub>O<sub>2</sub> was 0.25 mM. Data are presented as mean  $\pm$  SE (n = 6) and analyzed by one-way analysis of variance followed by Dunnett test. \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001 vs. untreated normal cells; # $p$  < 0.05, ## $p$  < 0.01, ### $p$  < 0.001 vs. H<sub>2</sub>O<sub>2</sub>-treated cells. CF, *Capsosiphon fulvescens*; Normal, untreated CF extracts; CF90, CF subcritical water extract at 90°C and 3 MPa; CFW, CF water extract.

#### Effect of *Capsosiphon fulvescens* subcritical water extract at 90°C (CF90) and water extract powder (CFW) extracts on reactive oxygen species (ROS) production and oxidative stress in aluminum chloride (AlCl<sub>3</sub>)-induced zebrafish larvae

CF90 and CFW extracts treated with AlCl<sub>3</sub> (25  $\mu$ M) did not cause mortality or malformation in zebrafish larvae (Fig. 5A).

Intriguingly, the ROS production increased by AlCl<sub>3</sub> treatment was significantly suppressed by treatment with the CF90 and CFW extracts (Fig. 5B and 5C).

#### Effect of *Capsosiphon fulvescens* subcritical water extract at 90°C (CF90) and water extract powder (CFW) extracts on mRNA expression of antioxidant enzyme in aluminum chloride (AlCl<sub>3</sub>)-induced zebrafish larvae

We investigated the mRNA levels of antioxidant enzymes, such as SOD and GPx. Results showed that AlCl<sub>3</sub> significantly reduced the mRNA expression of SOD and GPx, and treatment with CF90 and CFW extracts (250  $\mu$ g/mL) significantly enhanced the activity of both enzymes (Fig. 6). Therefore, our data indicated that CF90 and CFW extracts can restore ROS production and oxidative stress through the regulation of antioxidant enzymes in AlCl<sub>3</sub>-induced zebrafish larvae.

#### Effect of *Capsosiphon fulvescens* subcritical water extract at 90°C (CF90) and water extract powder (CFW) extracts on mRNA levels related to neurodegenerative function in aluminum chloride (AlCl<sub>3</sub>)-induced zebrafish larvae

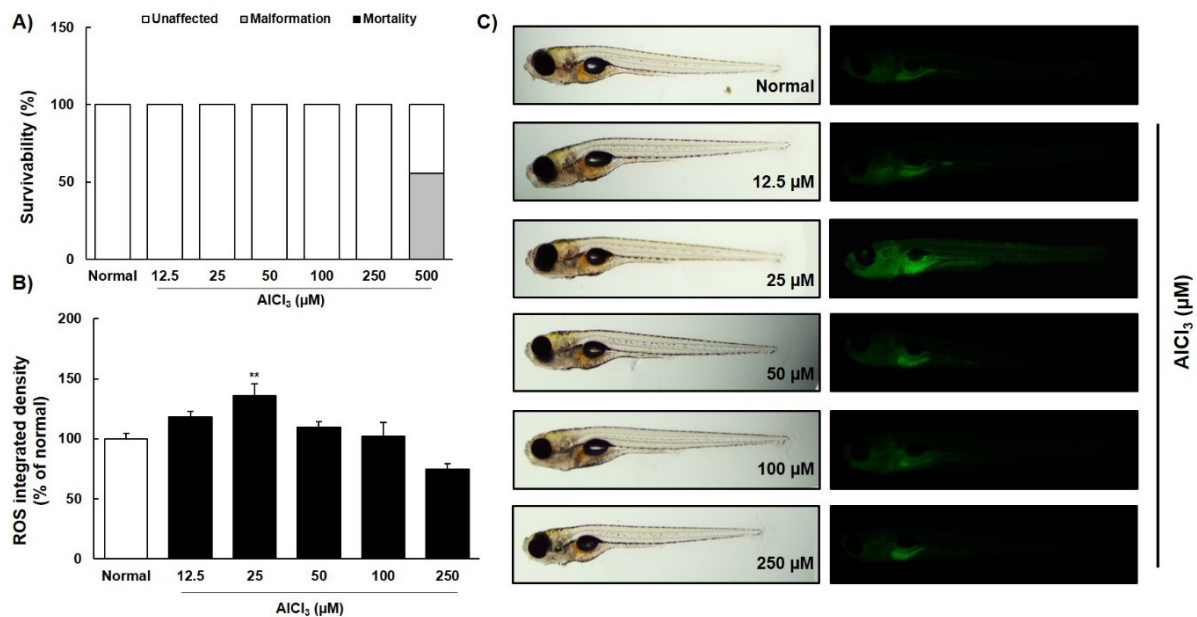
The excess-ROS group showed elevated GSK3 $\beta$  mRNA levels followed by an increase in  $\beta$ -amyloid, tau, and AChE, which cause memory deficits and are the main markers of neurodegeneration. CF90 and CFW extracts showed a significant preventive effect on AlCl<sub>3</sub>-induced neurodegeneration by downregulating the mRNA expression of GSK3 $\beta$ ,  $\beta$ -amyloid, tau, and AChE, hence suggesting their neuroprotective effects (Fig. 7).

#### Effect of *Capsosiphon fulvescens* subcritical water extract at 90°C (CF90) and water extract powder (CFW) extracts on mRNA levels related to cognitive function in aluminum chloride (AlCl<sub>3</sub>)-induced zebrafish larvae

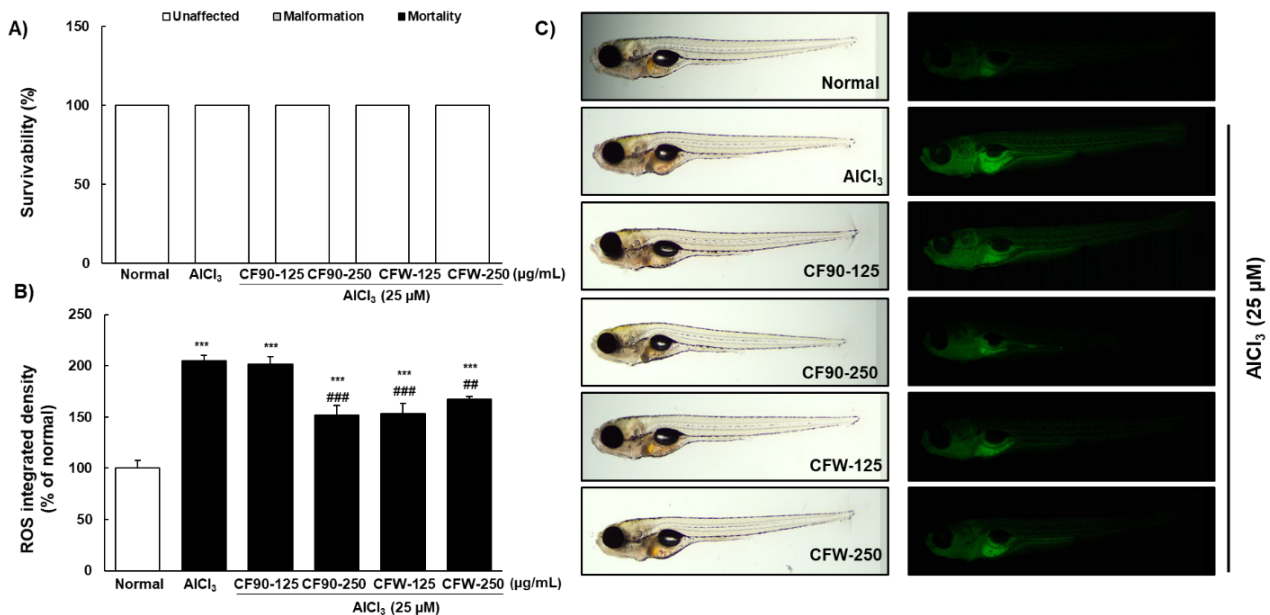
To investigate the neuroprotective effects of CF90 and CFW extracts against AlCl<sub>3</sub>-induced cognitive dysfunction, we measured the gene expression of BDNF and Trk $\beta$ . As shown in Fig. 8, BDNF and Trk $\beta$  mRNA expression was significantly decreased due to AlCl<sub>3</sub> treatment compared to that in the normal group, and was restored and protected by CF90 and CFW administration.

## Discussion

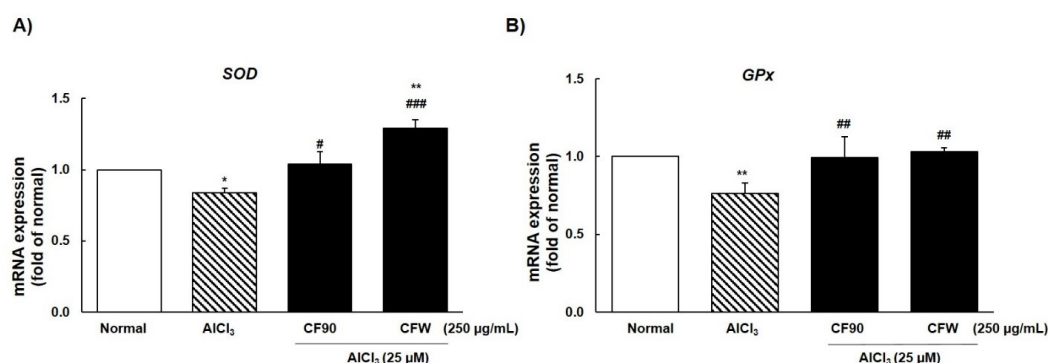
The current study investigated the protective effects of CF90 and CFW extracts on oxidative stress-stimulated neurodegenerative



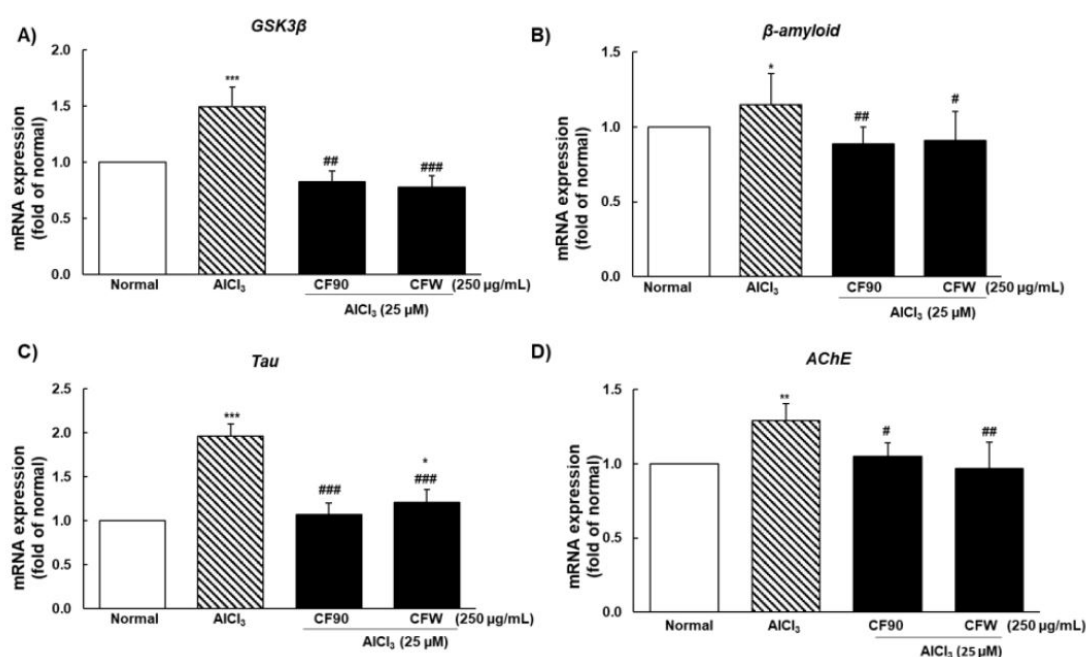
**Fig. 4.** Effect of  $\text{AlCl}_3$  on survivability (A) and reactive oxygen species production (B and C) in zebrafish larvae. Magnification was  $\times 20$ . Data are presented as mean  $\pm$  SE ( $n = 8$ ) and analyzed by one-way analysis of variance followed by Dunnett test. \*\* $p < 0.01$  vs. untreated normal zebrafish larvae.  $\text{AlCl}_3$ , aluminum chloride.



**Fig. 5.** Effect of CF90 and CFW extracts on  $\text{AlCl}_3$ -induced reactive oxygen species production in zebrafish larvae. The concentration of  $\text{AlCl}_3$  was 25  $\mu\text{M}$ . Magnification was  $\times 20$ . Data are presented as mean  $\pm$  SE ( $n = 12$ ) and analyzed by one-way analysis of variance followed by Dunnett test. \*\*\* $p < 0.001$  vs. untreated normal zebrafish larvae; ## $p < 0.01$ , ### $p < 0.001$  vs.  $\text{AlCl}_3$ -treated zebrafish larvae. CF, *Capsosiphon fulvescens*; Normal, untreated CF extracts; CF90, CF subcritical water extract at 90°C and 3 MPa pressure; CFW, CF water extract;  $\text{AlCl}_3$ , aluminum chloride.



**Fig. 6. Effect of CF90 and CFW on mRNA expression of antioxidant enzyme in AlCl<sub>3</sub>-induced zebrafish larvae.** The concentration of AlCl<sub>3</sub> was 25 µM. Data are presented as mean ± SE (n = 3) and analyzed by one-way analysis of variance followed by Dunnett test. \**p* < 0.05, \*\**p* < 0.01 vs. untreated normal zebrafish larvae; #*p* < 0.05, ##*p* < 0.01, ###*p* < 0.001 vs. AlCl<sub>3</sub>-treated zebrafish larvae. CF, *Capsosiphon fulvescens*; Normal, untreated CF extracts; CF90, CF subcritical water extract at 90°C and 3 MPa pressure; CFW, CF water extract; AlCl<sub>3</sub>, aluminum chloride.

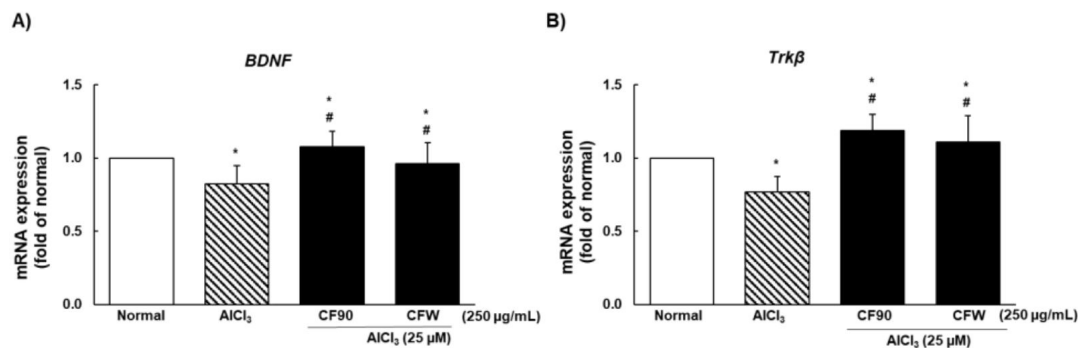


**Fig. 7. Effect of CF90 and CFW on mRNA levels related to neurodegenerative function in AlCl<sub>3</sub>-induced zebrafish larvae.** The concentration of AlCl<sub>3</sub> was 25 µM. Data are presented as mean ± SE (n = 3) and analyzed by one-way analysis of variance followed by Dunnett test. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001 vs. untreated normal zebrafish larvae; #*p* < 0.05, ##*p* < 0.01, ###*p* < 0.001 vs. AlCl<sub>3</sub>-treated zebrafish larvae. CF, *Capsosiphon fulvescens*; Normal, untreated CF extracts; CF90, CF subcritical water extract at 90°C and 3 MPa pressure; CFW, CF water extract; AlCl<sub>3</sub>, aluminum chloride.

dysfunction in PC12 cells and zebrafish larvae. We found H<sub>2</sub>O<sub>2</sub> and AlCl<sub>3</sub> to significantly elevate the excessive ROS production in both models, thereby inducing cell death. In

addition, the ROS inducers greatly affected the intracellular Ca<sup>2+</sup> levels, antioxidant enzyme activity, BDNF/Trkβ signaling, AChE activity, and hallmarks of AD. However, treatment with





**Fig. 8. Effect of CF90 and CFW on mRNA level related to cognitive function in  $\text{AlCl}_3$ -induced zebrafish larvae.** The concentration of  $\text{AlCl}_3$  was 25  $\mu\text{M}$ . Data are presented as mean  $\pm$  SE ( $n = 3$ ) and analyzed by one-way analysis of variance followed by Dunnett test. \* $p < 0.05$  vs. untreated normal zebrafish larvae; # $p < 0.05$  vs.  $\text{AlCl}_3$ -treated zebrafish larvae. CF, *Capsosiphon fulvescens*; Normal, untreated CF extracts; CF90, CF subcritical water extract at 90°C and 3 MPa pressure; CFW, CF water extract;  $\text{AlCl}_3$ , aluminum chloride.

CF90 and CFW extracts in  $\text{H}_2\text{O}_2$ -induced PC12 cells or  $\text{AlCl}_3$ -induced zebrafish larvae effectively regulated the excessive ROS production, intracellular  $\text{Ca}^{2+}$  levels, and mRNA expression of SOD, GPx, GSK3 $\beta$ ,  $\beta$ -amyloid, tau, AChE, BDNF, and Trk $\beta$ . Thus, the current study suggested that CF90 and CFW extracts may improve the impairment of cognitive function and synaptic plasticity by regulating ROS generation in neurodegenerative diseases.

Oxidative stress is closely associated with the progression of several neurodegenerative diseases and aging (Singh et al., 2019). Excessive neuronal ROS generation causes lipid peroxidation, DNA repair dysfunction, and loss of the antioxidant defense system (Rodríguez-Fuentes et al., 2015). Several studies have reported that excessive ROS generation in neurons due to external stimuli induces an imbalance in antioxidant homeostasis, stimulates intracellular  $\text{Ca}^{2+}$  content, and leads to cell death or apoptosis (Buccellato et al., 2021; Gordeeva et al., 2003; Rico et al., 2007). In addition, neurotoxicity of  $\text{H}_2\text{O}_2$  and  $\text{AlCl}_3$  in various experimental models has been reported to cause oxidative brain damage, trigger apoptosis, and ultimately cause irreversible damage to neurons (Justin-Thenmozhi et al., 2018; Nampoothiri et al., 2015). Therefore, the inducers were considered pathological factors for neuronal development and neurodegenerative diseases, such as AD. In our study, treatment of cultured PC12 neuronal cells with  $\text{H}_2\text{O}_2$  showed the induction of cell death in a dose-dependent manner, and significantly amplified intracellular ROS production and  $\text{Ca}^{2+}$  content than in normal group. In addition, exposure of zebrafish larvae to  $\text{AlCl}_3$  significantly altered ROS

production as well as mRNA expression of SOD and GPx genes. However, CF90 and CFW extracts restored the imbalance between oxidative stress and  $\text{Ca}^{2+}$  content. Our results collectively suggested that CF90 and CFW extracts can ameliorate neuronal damage by modulating oxidative imbalance in  $\text{H}_2\text{O}_2$ -induced PC12 neuronal cells and  $\text{AlCl}_3$ -induced zebrafish larvae.

Oxidative stress in neurodegenerative process is stimulated by the accumulation of A $\beta$  plaques and hyperphosphorylated tau tangles, leading to signs of cognitive impairment, such as AD (Liu et al., 2015). Therefore, the imbalance between A $\beta$  production and clearance may serve as an important mediator of synaptic loss that promotes NFT and senile infectious disease formation (Kern & Behl, 2009). Hence, oxidative stress is regarded as a critical factor in AD progression. Justin-Thenmozhi et al. (2016) had reported that  $\text{AlCl}_3$  consumption in rats not only induces A $\beta$  accumulation by increasing oxidative stress, but also increases AChE activity in the hippocampal and cortical regions of the brain, leading to the progression of memory deficits in rats (Justin-Thenmozhi et al., 2016). Cao et al. (2016) had further suggested that  $\text{AlCl}_3$  can induce neuroinflammation, resulting in learning and memory impairments in rats. BDNF and Trk $\beta$  play critical roles as major neurotrophic factors in synaptic plasticity and cognitive function (Cao et al., 2016). Our study showed that excessive ROS generation due to exposure to  $\text{AlCl}_3$  in zebrafish larvae significantly increased or decreased the mRNA expression of A $\beta$ , tau, GSK3 $\beta$ , AChE, BDNF, and Trk $\beta$  genes. However, administration of CF90 and CFW effectively controlled the expression of mRNA genes associated with AD or cognitive

function through restoration of normal antioxidant enzyme activity.

In summary, our results revealed that H<sub>2</sub>O<sub>2</sub> or AlCl<sub>3</sub> could enhance excessive ROS generation in PC12 neuronal cells and zebrafish, resulting in severe cognitive impairment and loss of synaptic plasticity. In contrast, CF90 and CFW extracts effectively improved neurodegeneration by normalizing antioxidant enzyme activity, inhibiting AChE activity, downregulating A $\beta$ , GSK3 $\beta$ , and tau gene expression, and upregulating BDNF/Trk $\beta$  signals.

### Competing interests

No potential conflict of interest relevant to this article was reported.

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### Availability of data and materials

Upon reasonable request, the datasets of this study can be available from the corresponding author.

### Ethics approval and consent to participate

This article does not require IRB/IACUC approval because there are no human and animal participants.

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