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Original Article

# Neuroprotective Effects of Proso Millet (*Panicum miliaceum* L.) Free Phenolic Extract in an $A\beta_{1-42}$-Induced Alzheimer's Disease Mouse Model

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## ABSTRACT

**Background:** Alzheimer's Disease (AD) is a neurodegenerative condition linked with inflammation and oxidative stress of neurones. The conventional anti-inflammatory drugs showed little efficacy in reducing AD, thus new anti-AD drugs have become more important. Inhibitors of soluble Epoxide Hydrolase (sEH) have been recently reported to retain the memory function in diabetic rats, but their effectiveness in reducing memory loss in $A\beta_{1-42}$-induced AD model is not known. **Objectives:** The objective of the present study was to assess the sEH inhibition potential of proso millet free phenolics (*Panicum miliaceum* L., PM-FP) *in vitro* and to compare its effect with the standard sEH inhibitor 1-Trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) Urea (TPPU) in ameliorating AD. **Materials and Methods:** As AD is also linked with loss of the sense of smell, we assessed the ability of sEH inhibitors to retain the olfactory function (buried pellet food test) in AD mice as well as memory loss (Novel object recognition test, discrimination index). Further, we have also evaluated the effect of treatment on ROS production in brain. **Results:** Proso Millet seeds Free Phenolics extract (PM-FP) was found to be a potent inhibitor of human sEH and mouse sEH with $IC_{50}$ of 6.40 and 16.38 µg/mL respectively. AD was induced in mice by intra-cerebroventricular injection of $A\beta_{1-42}$ which resulted in olfactory and memory dysfunction. **Conclusion:** Proso millet and standard sEH inhibitors improved olfactory and memory function with a reduction in ROS generation in AD mice. The present study demonstrates that sEH inhibition by PM-FP is a promising approach to treat AD.

**Keywords:** Alzheimer's disease, Novel object recognition test, *Panicum miliaceum* L., Proso millet, Soluble epoxide hydrolase, TPPU, Oxidative stress.

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## INTRODUCTION

Alzheimer's Disease (AD) is a progressive neurodegenerative disease that affects/alters memory, personality, cognitive ability and other functions which eventually lead to death due to total brain failure. The clinical symptoms of AD are impairment in memory that interferes with daily life, difficulty in planning or solving problems, new problems with words in speaking or writing and difficulty understanding visual images and spatial relationships (Kumar *et al.*, 2015). AD has been recognized as the sixth leading cause of death overall and the fifth among adults over 65 years in the United States. In 2018, more than 122,000 people died from AD, an increase of 146% from the year 2000. The primary pathology of AD is the formation of senile plaques by Aβ and neurofibrillary tangles by Tau hyperphosphorylation (Sharma *et al.*, 2019). Although numerous theories have been proposed for the pathogenesis of AD, only 2 types of drugs have been approved by FDA for treatment, and these drugs only help in alleviating symptoms of the disease. Alzheimer's Disease (AD) is known to be a chronic inflammatory disease. Neuroinflammation is closely associated with oxidative stress in AD and regulates the crosstalk between the immune system and the central nervous system. Hence, it is critical to broaden the horizon in the search for new anti-inflammatory targets, preferably soluble epoxide hydrolase inhibitors which preserve memory in diabetic rats (Minaz *et al.*, 2018; Pardeshi *et al.*, 2019). Soluble Epoxide Hydrolase (sEH) enzyme, which is highly expressed in relatively high concentrations in mice and human brains, metabolises EETs and other Epoxyfatty Acids (EpFA) to Dihydroxyeicosatrienoic Acids (DHETs). Epoxyeicosatrienoic acids (EETs) are known to

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cause vasodilatation, reduce inflammation, diminish oxidative stress, and inhibit the pathological Endoplasmic Reticulum (ER) stress response (Sun *et al.*, 2021), while DHETs are known to eliminate, reduce or modify the beneficial effects of EETs. Recent research shows that genetic deletion of sEH ameliorates the symptoms of AD in mice. In recent research, sEH inhibition is shown to protect memory and cognition. Several studies highlight the widespread role of EETs and other EpFA in the function of the Central Nervous System (CNS). We thought that sEH Inhibitors (sEHI) would protect EETs in the brain and reduce the formation of Reactive Oxygen Species (ROS), neuroinflammation and neurodegeneration, which would result in a beneficial effect in AD (Griñán-Ferré *et al.*, 2020). Proso millet (*Panicum miliaceum*) and Barnyard millet (*Echinochloa frumentacea*) are members of the family Gramineae and are major millets cultivated in India, Africa, Japan and other Asian countries (Ramadoss and Sivalingam, 2020). Proso millet has been recently reported to have substantial health benefits and functional properties, such as antioxidant activity (Shen *et al.*, 2018). Our preliminary studies showed the sEH inhibitory activity of Proso millet. Therefore, in the present study, we investigated the effect of free phenolic extract of Proso Millet (PM-FP) in maintaining memory in $A\beta_{1-42}$ mice model of Alzheimer's disease.

## MATERIALS AND METHODS

### Chemicals and reagents

The 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea (TPPU) was gifted by Dr. Bruce D. Hammock, Professor, Department of Entomology and Nematology, University of California, Davis. 96 Briggs Hall, One Shields Ave, Davis, CA 95616. $A\beta_{1-42}$ was obtained from Juniper Life Sciences (AS-24224; Bengaluru, Karnataka). 2′,7′-dichlorofluorescein diacetate was obtained from Sigma-Aldrich (CAT Number 4091-99-0). The chemicals and reagents used were of analytical grade.

### Collection and authentication of seeds of Proso Millet (PM-FP)

Seeds of proso millet (*Panicum miliaceum* Linn. PM-FP) were obtained from the Vice-Chancellors Farm of the University of Agricultural Sciences, Mandya, Karnataka, India and authenticated by Professor V. Biligiriranga, Department of Botany, JSS College of Arts, Commerce and Science, Mysore, Karnataka, India.

### Extraction of free phenolics

Free phenolic acids were extracted using the method of Ayumi *et al.* (1999). 500 g of powder was extracted with 70% ethanol (4×500 mL, 1 hr each), the supernatants were obtained by centrifugation and concentrated, and the pH was adjusted in the range of 2-3 with 4 M HCl. Phenolic acids were obtained by ethyl acetate extraction (5×200 mL) and the collected extracts were dried with anhydrous disodium sulfate, filtered and concentrated (Subba Rao and Muralikrishna, 2002). Proso Millet hydroalcoholic extract (PM-FP) was used as treatment drug at high dose (200 mg/kg) (Shen *et al.*, 2018). The standard drug TPPU (1 mg/kg) (Ramadoss and Sivalingam, 2020) and vehicle (0.3 mL of 0.5% CMC) were given orally using an oral gavage syringe.

### *In vitro* screening of sEH inhibition activity

*In vitro* sEH inhibition activity was determined by using a fluorescent substrate (cyano(6-methoxy-naphthalen-2-yl) methyl trans-[(3-phenyloxiran-2-yl) methyl] carbonate; CMNPC) (Jones *et al.*, 2005). The test was carried out using recombinant human or mouse sEH proteins. The enzymes were pre-incubated with the plant extracts (final concentration 0.5-125 µg/mL) at 37°C for 5 min in 100 mM sodium phosphate buffer (200 µL, pH 7.4) containing 0.1 mg/mL of BSA and 1% of DMSO. The reaction was initiated by addition of the substrate, CMNPC (final [S]=5 µM) and the activity was measured by the formation of the fluorescent 6-methoxynaphthaldehyde product (λex=330 nm, λem=465 nm) every 30 sec for 10 min at 37°C with a SpectraMax M2 (Molecular Devices) spectrophotometer. $IC_{50}$ were determined by regression from the linear part of the curve of the activity in the presence of the inhibitor. All the tests were done in triplicate. Herbal extract were tested in the sEH inhibition assay and the one exhibiting high activity against mouse and human sEH was chosen for further *in vivo* investigation.

### Animals

The animal experiments were performed as per the guidelines of Committee for the Control and Supervision of Experiments on Animals (CCSEA). The experimental protocol was approved by institutional animal ethical committee, JSS College of Pharmacy, JSS Academy of Higher Education and Research, Mysuru, Karnataka, India (Protocol Approval IAEC NO: JSSAHER/CPT/IAEC/076/2021) and efforts were made to reduce the pain during the experiment. Female $C_{57}$BL/6 mice (2-3 months old) were obtained from a CCSEA registered animal supplier. The mice were maintained at the quarantine center and fed with sufficient food, water *ad libitum* access and maintained under normal room temperature (23°C±2°C) conditions, 12-hr light/dark cycle was duly also maintained. As the previous reports show the female gender as a risk factor for AD, female black mice were selected for the study.

### Investigation of PM-FP against $A\beta_{1-42}$ induced alzheimer's disease in mice

The animals were allowed to acclimatize for 7 days and then grouped into five groups as follows: Normal control, Sham control, Amyloid-β1-42 ($A\beta_{1-42}$, disease control), Standard (TPPU, 1 mg/kg) and Treatment (PM-FP, 200 mg/kg). Each group consisted of 14 animals and the changes in behaviour were noted initially. $A\beta_{1-42}$ peptide was injected on day 1 and induction period of 7

days was given. The behaviour of the animals was recorded again after the induction period. Once AD was induced, all animals were administered with test drug orally for 21 days. $A\beta_{1-42}$ stock (1 mM) was prepared in DMSO and diluted tenfold (100 µM) in PBS to prepare the $A\beta_{1-42}$ monomer solution. The oligomers were formed by incubating the monomer solution for 5 days at 37°C. $A\beta_{1-42}$ was administered intracerebroventricularly (i.c.v) using a stereotaxic instrument (Stoelting, USA) and a 28-gauge stainless-steel needle of 3.0 mm length (Hamilton). Mice were anaesthetised with an intraperitoneal (i.p) injection of xylazine (20 mg/kg) and ketamine (80 mg/kg) cocktail and then placed on the stereotaxic apparatus. $A\beta_{1-42}$ (5.0 µL) was injected slowly into the right lateral ventricle with the following coordinates from bregma: anteroposterior=-0.9 mm, mediolateral=1.3 mm, and dorsoventral=-2.0 mm. The same coordinates were used for injecting PBS (5.0 µL) to the sham mice. The mice were carefully monitored to avoid any drop in temperature. The i.c.v. route of administration of the peptide is advantageous as it quickly spreads across the brain (Souza *et al.*, 2016).

## *In vivo* tests to evaluate memory

The following *in vivo* tests were conducted to evaluate the protective effects of extracts and chemicals in $A\beta_{1-42}$ induced Alzheimer's disease.

## Novel Object Recognition Test (NORT)

The novel object recognition test was conducted in a well-lit wooden box. The test was performed in three phases: habituation, training and test. In habituation, all the animals were allowed to explore the empty box for 5 min and then returned to their respective cages. During the training phase, two identical objects (object A) were placed in the box, and the animal was placed in the box and allowed to explore the objects for 8-10 min, making sure the animal spends 15-20 sec with each object. After 24 hr of training day, animals were tested for their novel object recognition memory by replacing one of the objects with a novel object (object B); the time spent with the novel object was recorded. The discrimination index was calculated for object recognition (Hansen *et al.*, 2010).

## ROS Determination

ROS estimation was done by the DCFHDA method. The production of ROS in the cells was estimated by using 2,7-Dichlorodihydrofluorescein-Diacetate (DCFH-DA) reagent. DCFH-DA is converted to fluorescent DCF by the action of ROS, which is the basis of this method. The brain tissues (hippocampus) of the mice were dissected and homogenised in 0.9% sodium chloride solution. This homogenate was then centrifuged at 8000 rpm for 10 min at 10°C and the supernatant was separated. The supernatant (2 µL) was taken out and 198 µL PBS was added. Then, 100 µL and 100 µL of 15 µM DCFH-DA solution were added to a 96-well microplate. The level of ROS was measured at an excitation wavelength of 485 nm and an emission wavelength of 525 nm using a multimode microplate reader (Perkin Elmer) (Wu *et al.*, 2013).

## Histopathological examination

At the end of the treatment period, animals from each group were euthanized and brain tissue was harvested. The hippocampal region was carefully isolated, fixed in 10% neutral buffered formalin for 48 hr, and subsequently processed for paraffin embedding. Sections of 5 µm thickness were cut using a rotary microtome and stained with Haematoxylin and Eosin (H & E). The stained sections were examined under a light microscope at 400X magnification to assess the histological architecture of the hippocampus, with particular attention to nerve cells, glial cells, and blood vessels. Representative photomicrographs were captured for each treatment group.

## Measurement of body weight

Body weight was recorded for all experimental animals at three time points: before induction, after the induction period (Day 14), and at the end of the 21-day treatment period, using a calibrated digital weighing balance. Dosing volumes were adjusted at each recording to maintain weight-based consistency throughout the experiment.

## Blood sample collection and haematology

Blood samples were collected from the retro-orbital plexus under brief isoflurane anaesthesia at the end of the treatment period. Samples were placed into individual K3-EDTA-containing tubes and processed immediately. Haematological parameters including Red Blood Cell Count (RBC, $10^3$/µL), White Blood Cell count (WBC, $10^3$/µL), and Haemoglobin Concentration (HGB, g/dL) were determined using an automated haematology analyser (Agappe Diagnostics, India) following standard laboratory procedures.

## Estimation of endogenous antioxidants

Brain tissue collected at necropsy was stored at -20°C. Tissue homogenate was prepared at 8000 rpm in 10% w/v ice-cold Phosphate-Buffered Saline (PBS, pH 7.0) and centrifuged at 15,000 rpm for 10 min at 4°C. The resulting supernatant was used for antioxidant enzyme assays. Total protein was estimated by Bradford's method using Bovine Serum Albumin (BSA) as the standard, and all activities were normalised to milligrams of protein. Superoxide Dismutase (SOD) activity was estimated by the NBT photoreduction method. Superoxide radicals were generated by NADH oxidation with Phenazine Methosulfate (PMS). SOD in the sample competes with NBT for these radicals, reducing formazan formation. Absorbance was measured at 560 nm and one unit of SOD activity was defined as the amount of enzyme required to inhibit NBT reduction by 50% under the assay conditions. Catalase (CAT) activity was measured

spectrophotometrically by following the decomposition of hydrogen peroxide at 240 nm. The reaction mixture contained 20 µL of sample supernatant and 180 µL of freshly prepared $H_2O_2$ in PBS, and absorbance was recorded every 30 sec over 3 min at 30°C. Activity was expressed as µmol $H_2O_2$ consumed per minute per milligram of protein, using the molar extinction coefficient of 43.6 $M^{-1}cm^{-1}$. Glutathione (GSH) levels were quantified using Ellman's reagent (DTNB). Proteins were precipitated from the homogenate using TCA, and the cleared supernatant was reacted with DTNB at 25°C for 30 min. The resultant yellow-coloured TNB product was measured at 412 nm. GSH content was expressed as µmol/mg protein. Lipid Peroxidation (LPO) was assessed using the Thiobarbituric Acid Reactive Substances (TBARS) method. Brain homogenate was incubated with TCA, HCl, and TBA, then heated at 95°C for 15 min to generate the pink-coloured TBA-MDA adduct. Absorbance was recorded at 532 nm and LPO was expressed as nmol/mg protein using the molar extinction coefficient of $1.56 \times 10^5$ $M^{-1}cm^{-1}$.

### Estimation of NRF2 by ELISA

NRF2 levels in brain tissue lysates were measured using a competitive ELISA kit (Krishgen Biosystems, India). The NRF2 in the sample competes with biotin-labelled NRF2 for binding to the antibody pre-coated on the microplate wells. After incubation and washing, avidin-HRP conjugate was added followed by TMB substrate; colour development, inversely proportional to NRF2 concentration, was stopped and read at 450 nm. Results were expressed as pg/mL.

### Statistical analysis

All the experimental data were statistically analysed by using GraphPad Prism (version 9) and expressed as mean±SEM ($n$=14) and evaluated by using Two-way ANOVA followed by Tukey's Multiple Comparison test.

## RESULTS

### *In vitro* data of sEH inhibition activity

The herbal extracts were tested for the inhibition of sEH in human and mouse sEH. $IC_{50}$ values of all the samples are shown in Table 1. Of the extracts tested, PM-FP (Proso millet) showed the strongest inhibitory activity against human sEH ($IC_{50}$ 6.40 µg/mL) and mouse sEH ($IC_{50}$ 16.38 µg/mL).

### Effect of PM-FP on body weight

Body weight was recorded before induction, after induction, and after the 21-day treatment period (Figure 1A). At baseline, body weights were comparable across all five groups. Following ICV injection of $A\beta_{1-42}$, a modest but noticeable decline in body weight was observed in the disease control (Control) group compared to the normal and sham control groups, consistent with the nutritional and metabolic consequences of amyloid-induced neurodegeneration. After 21 days of treatment, the normal and sham control groups maintained their weight, while the Control group showed persistent weight loss. PM-FP (Treatment) and standard sEH inhibitor (Standard) groups-maintained body weight in a range comparable to the sham group, suggesting that treatment did not produce overt toxicity or weight-compromising side effects. No statistically significant differences were detected between groups at any of the three time points, indicating that the effects of PM-FP on the animals were pharmacologically selective rather than broadly systemic.

### Effect of PM-FP on haematological parameters

Haematological evaluation was carried out at the end of the treatment period to assess any potential haematotoxicity of PM-FP and to examine systemic effects of amyloid-induced AD on blood cell indices. RBC, WBC, and HGB levels were measured in five groups (Figures 1B-1D). Red Blood Cell (RBC) counts were significantly lower in the normal group compared to the sham, control, and treatment groups ($p$<0.01), while the remaining groups showed broadly comparable counts in the range of 7.0–7.3 × $10^3$/µL (Figure 1B). The Treatment group showed slightly higher RBC counts than the Control, though not to a statistically significant degree. White Blood Cell (WBC) counts were markedly elevated in the sham control group relative to the disease control ($p$<0.01), which likely reflects the surgical trauma and wound response following ICV injection of PBS. The disease control group showed WBC values close to those of the normal group. Interestingly, the Treatment group showed somewhat higher WBC compared to the Control ($p$<0.01), which may indicate enhanced immune activity; however, values remained within a physiologically acceptable range (Figure 1C). Haemoglobin (HGB) concentration was slightly lower in the normal group (approximately 10 g/dL) compared to all other groups, with the Treatment group showing the numerically highest HGB among all groups (approximately 11.6 g/dL; $p$<0.01 vs. normal). This is unlikely to be a pathological finding, and the standard, control, and treatment groups all maintained HGB values within the normal mouse reference range of 10.2–16.6 g/dL. Taken together, the haematological data indicate that PM-FP treatment did not cause haematotoxicity (Figure 1D).

**Table 1: *In vitro* data of sEH inhibition activity of herbal extracts against human and mouse sEH.**

| Sl. No. | Common Name | Scientific Name | Human sEH $IC_{50}$ (µg/mL) | Mouse sEH $IC_{50}$ (µg/mL) |
|---|---|---|---|---|
| 1. | Proso millet | *Panicum miliaceum* L. | 6.40 | 16.38 |

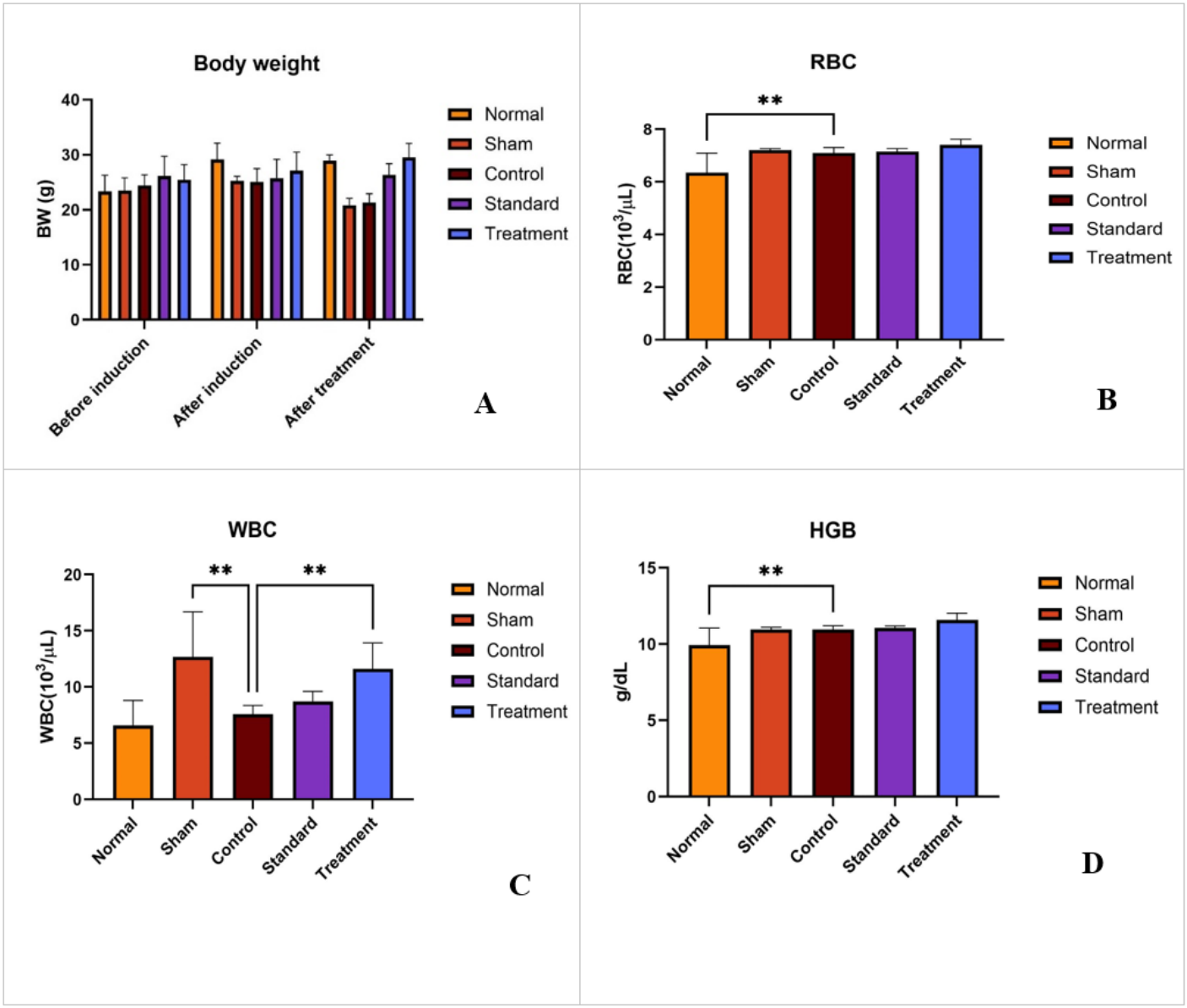


**Figure 1:** Effect of PM-FP on body weight (g) and haematological parameters. A: Effect of PM-FP on body weight (g) across experimental groups at three time points, values are expressed as Mean±SEM, *n*=14; B: Effect of PM-FP on Red Blood Cells (RBC) counts ($10^3$/µL) across experimental groups, values are expressed as Mean±SEM, *n*=14; ***p*<0.01 vs. Control; C: Effect of PM-FP on White Blood Cell (WBC) counts ($10^3$/µL) across experimental groups, values are expressed as Mean±SEM, *n*=14; ***p*<0.01; D: Effect of PM-FP on Haemoglobin (HGB) concentration (g/dL) across experimental groups, values are expressed as Mean±SEM, *n*=14; ***p*<0.01. Data for the Normal, Sham, Control, Standard, and Treatment (PM-FP 200 mg/kg) groups are presented.

## Effect of PM-FP on endogenous antioxidant enzymes and lipid peroxidation

Oxidative stress is a central feature of Aβ-induced neurodegeneration. We, therefore measured three key antioxidant enzymes, i.e., SOD, CAT, and GSH-along with the oxidative stress marker LPO, in brain tissue from all experimental groups (Figures 2A-2D). Superoxide Dismutase (SOD) activity was significantly reduced in the disease control group compared to the normal and sham control groups ($p<0.001$). This depletion is consistent with the well-documented failure of antioxidant defenses under the oxidative load imposed by amyloid beta. Both the standard sEH inhibitor-treated group and the PM-FP Treatment group showed significantly elevated SOD activity compared to the Control ($p<0.05$ and $p<0.001$, respectively). The Treatment group SOD levels were notably closer to the normal group than to the disease control, reflecting meaningful restoration of antioxidant capacity (Figure 2A). Catalase (CAT) activity showed a similar pattern: the disease control group exhibited significantly lower CAT activity compared to the normal group ($p<0.001$), while both the Standard and Treatment groups showed significant

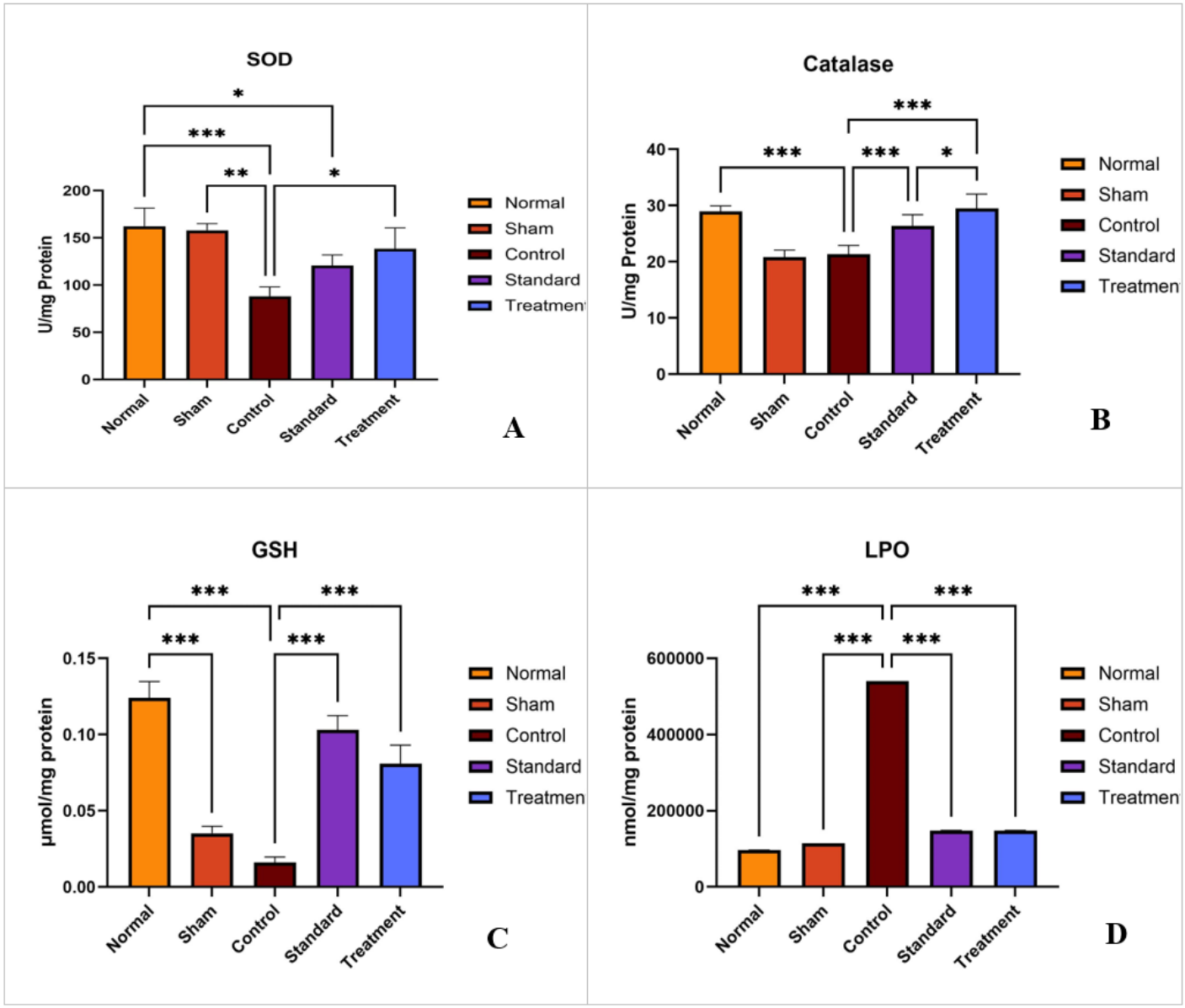


**Figure 2:** Effect of PM-FP on endogenous key antioxidant enzymes (SOD, CAT, and GSH) and oxidative stress marker Lipid Peroxidation (LPO). A: Effect of PM-FP on endogenous key antioxidant enzyme Superoxide Dismutase (SOD) activity (U/mg protein) across experimental groups, values are expressed as Mean±SEM, $n$=14; *$p$<0.05, **$p$<0.01, ***$p$<0.001; B: Effect of PM-FP on endogenous key antioxidant enzyme Catalase (CAT) activity (U/mg protein) across experimental groups, values are expressed as Mean±SEM, $n$=14; *$p$<0.05, ***$p$<0.001; C: Effect of PM-FP on endogenous key antioxidant enzyme Glutathione (GSH) levels (µmol/mg protein) across experimental groups, values are expressed as Mean±SEM, $n$=14; ***$p$<0.001; D: Effect of PM-FP on endogenous key antioxidant enzyme Lipid Peroxidation (LPO) levels (nmol/mg protein) across experimental groups, values are expressed as Mean±SEM, $n$=14; ***$p$<0.001. Data for the Normal, Sham, Control, Standard, and Treatment (PM-FP 200 mg/kg) groups are presented.

restoration of catalase activity ($p$<0.001 and $p$<0.05, respectively). The Treatment group achieved CAT activity comparable to that of the normal group, suggesting that PM-FP is effective at restoring this particular arm of the antioxidant defense system (Figure 2B). Glutathione (GSH) levels were dramatically depleted in the disease control group compared to the normal group ($p$<0.001), corroborating the severe oxidative stress state induced by $A\beta_{1-42}$. Both the Standard and Treatment groups showed significant recovery of GSH levels relative to the disease control ($p$<0.001 for both). While neither group fully reached normal levels, the degree of recovery was substantial, and the Treatment group's GSH levels approached those of the Standard group. The sham control group also showed reduced GSH relative to normal, which may reflect residual surgical stress from ICV PBS injection (Figure 2C). Lipid Peroxidation (LPO) in the disease control group was approximately five-fold higher than in the normal group ($p$<0.001), reflecting extensive oxidative membrane damage triggered by amyloid beta. Both Standard and Treatment groups showed significantly reduced LPO levels compared to the disease control ($p$<0.001 for both). Remarkably, the LPO values in the Standard and Treatment groups were close to the sham control, indicating near-complete suppression of amyloid-induced lipid

peroxidation. This finding strongly supports the antioxidant mechanism of action of PM-FP in the AD brain (Figure 2D).

## Effect of PM-FP on olfactory function: Buried pellet food test

The buried pellet food test was performed to assess the olfactory function. Sham control group did not show any significant variation in the time taken to unbury the buried pellet from day 0 to day 28 (8.83±0.307 vs 13.17±1.013 s). Disease control animals showed a significant increase in the variation in the time taken to find the pellet starting from day 7 (8.5±0.428 vs 34±1.570 s) as compared to the sham control. On day 7, all treated groups showed a significant increase in the time taken to unbury the pellet when compared to sham control (10.833±0.792). On day 14, there was a significant decrease in time taken to unbury the pellet when compared to disease control (36.16±1.108 vs 24.5±0.562, 22.66±1.855, 27.16±0.749, 24.83±1.579). On day 14, the PM-FP 200 mg/kg group showed a significantly decreased time to unbury the pellet when compared to disease control (24.83±1.579 vs 36.16±1.108). On day 28, all treated groups showed a significant decrease in time taken to unbury the pellet when compared to disease control (34±1.570 vs 19.66±0.333, 19.833±1.301, 23.83±1.222, 22.33±0.84). On day 14, the PM-FP 200 mg/kg group showed a significantly decreased time to unbury the pellet when compared to disease control (24.83±1.579 vs 36.16±1.108) (Figure 3A).

## Novel Object Recognition Test (NORT)

### *Time spent near novel object*

There were no significant changes in the time spent near novel object in sham control from day 0 to day 28 (4.8±0.7 vs 4.1±0.6). On day 7 and day 28, there was a significant decrease in the time spent near the novel object in disease control (1.4±0.7, 1.5±0.5) as compared to normal control. On day 7, all treated groups showed a significant decrease in time spent near the novel object when compared to sham control (1.4±0.7, 1.0±0.3, 1.6±0.4, 1.5±1.1 vs 2.4±0.3). On day 28, there was a significant increase in time spent near the novel object when compared to disease control (1.5±0.5, 3.4±0.5, 3.1±0.7, 3.0±0.41 vs 4.1±0.6). On day 28, TPPU and PM-FP 200 mg/kg treatment groups had a significant increase in time spent near novel object when compared to disease control (3.4±0.5, 3.1±0.7, 3.0±0.41 vs 1.5±0.5). All values are expressed as Mean±SEM, $n$=14.

## Discrimination Index

The discrimination index of sham control animals varied non-significantly from day 0 to day 28 (29.75±3.4, 20.06±0.9, 40.60±5.4). The discrimination index of disease control decreased significantly from day 7 to day 28 (10.35±1.5, 5.77±1.9). On day 7, all treated groups showed a non-significant decrease in discrimination index when compared to disease control (15.43±2.7, 11.26±2.5, 21.15±1.9 vs 10.35±1.5). On day 28, there was a significant increase in discrimination index when compared to disease control (57.71±3.9, 50.24±5.8, 39.63±3.6 vs 5.77±1.9). On day 28, the TPPU and PM-FP 200 mg/kg (39.63±3.6) treatment groups had a significantly increased discrimination index when compared to disease control (57.71±3.9, 39.63±3.6 vs 5.77±1.9). All values are expressed as percentage (%), $n$=14.

## Reactive Oxygen Species (ROS) estimation

Sham control had an ROS level of 100 and disease control had an increased level of 180.1 relative absorbance units (at 570 nm). Standard TPPU and PM-FP (200 mg/kg) showed a significant decrease in ROS levels (125.64 and 124.19, respectively) compared to disease control (Figure 3B).

## Effect of PM-FP on nuclear factor erythroid 2-related factor (NRF2) levels

NRF2 is the master transcriptional regulator of antioxidant gene expression, and its downregulation has been consistently linked with oxidative neurodegeneration in AD. Brain NRF2 levels were measured by ELISA across all experimental groups. The normal and sham control groups showed comparably high NRF2 concentrations (~1700 pg/mL), reflecting robust basal antioxidant signaling. The disease control group had markedly lower NRF2 levels (~790 pg/mL), a significant decline compared to both the normal ($p$<0.001) and sham ($p$<0.001) groups. This suppression of NRF2 in $A\beta_{1-42}$ injected mice aligns with the concurrent reductions in SOD, CAT, and GSH, suggesting that impaired NRF2 signalling is a key upstream driver of the antioxidant enzyme deficits observed in this model. Treatment with PM-FP partially but meaningfully restored NRF2 levels (~1100 pg/mL) compared to the disease control. The Standard-treated group showed a similar degree of recovery (~1030 pg/mL). While neither group returned NRF2 to the levels of the normal or sham controls within the 21-day treatment window, the upward trend indicates that PM-FP engages the Nrf2 antioxidant response pathway, likely through sEH inhibition and the resulting elevation of EETs in the brain (Figure 3C).

## Histopathological examination of hippocampus

Haematoxylin and Eosin (H & E) stained sections of the hippocampus were examined across the Normal, Sham, Control, Standard, and Treatment groups to evaluate the neuroprotective effect of PM-FP on the hippocampal cytoarchitecture (Figures 4A-4E). The normal and sham control groups exhibited well-preserved hippocampal architecture at 400X magnification, characterised by densely packed, morphologically intact nerve cells with prominent nuclei, a normal complement of supportive glial cells, and patent blood vessels with no evidence of cellular infiltration or degeneration (Figures 4A-4B). The vehicle-treated disease control group ($A\beta_{1-42}$ injected) showed marked histopathological alterations in the hippocampus, including neuronal shrinkage, pyknotic nuclei, loss of normal

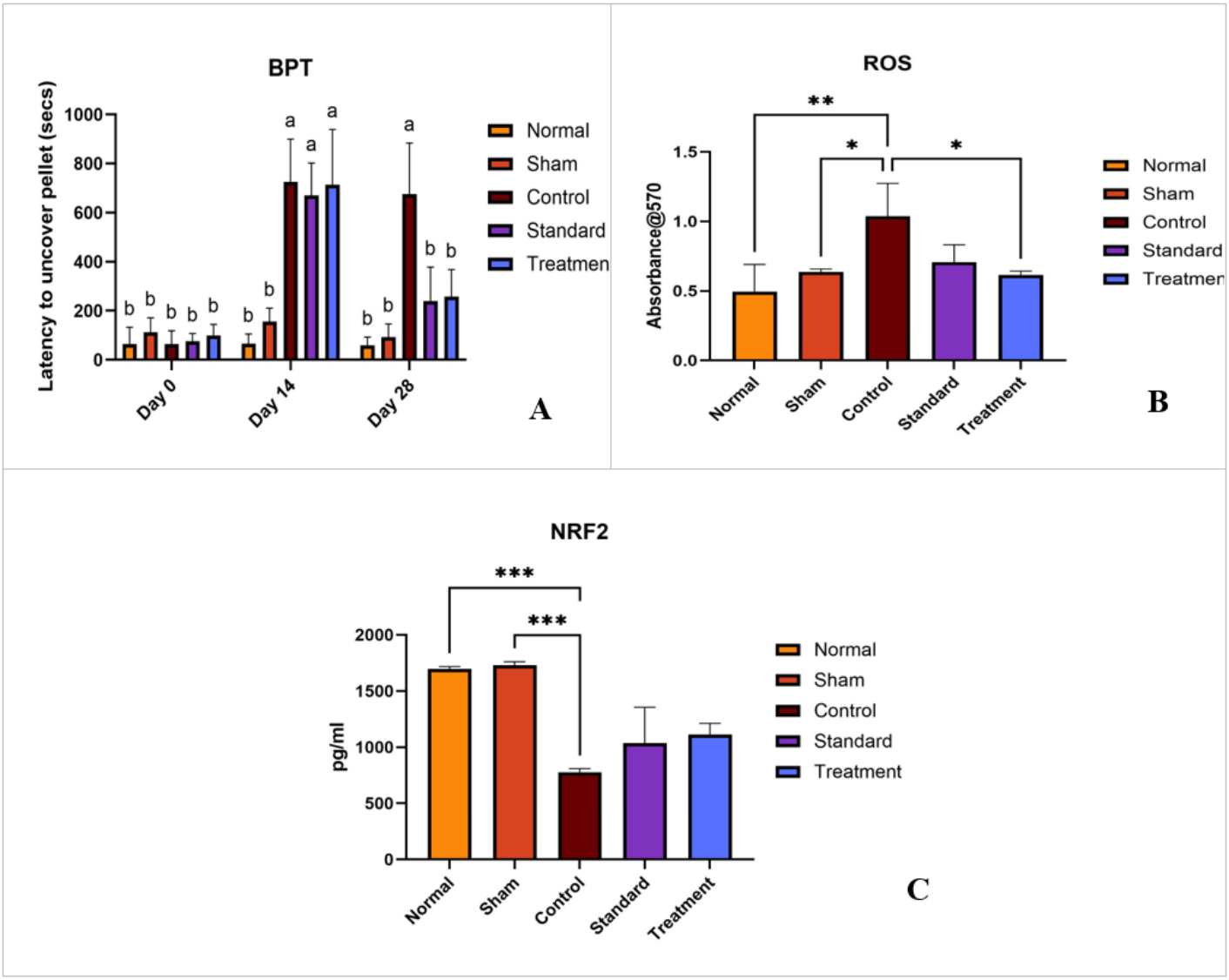


**Figure 3:** A: Effect of PM-FP on latency to uncover buried pellet (seconds) measured at Day 0, Day 14, and Day 28, values are expressed as Mean±SEM, *n*=14; [a]p<0.05 vs. sham control; [b]p<0.05 vs. disease control; B: Effect of PM-FP on ROS levels (absorbance at 570 nm) in brain hippocampus across experimental groups by the DCFH-DA method, values are expressed as Mean±SEM *n*=14; *$p$<0.05, **$p$<0.01; C: Effect of PM-FP on NRF2 concentration (pg/mL) in brain tissue across experimental groups, values are expressed as Mean±SEM, *n*=14; ***$p$<0.001. Data for the Normal, Sham, Control, Standard, and Treatment (PM-FP 200 mg/kg) groups are presented.

cytoarchitectural organisation, increased glial cell density, and congested blood vessels, consistent with $A\beta_{1-42}$ induced neurodegeneration (Figure 4C). These morphological changes in the disease control group corroborated the behavioural deficits observed in the olfactory and memory tests. Treatment with standard sEH inhibitor TPPU (1 mg/kg) resulted in notable restoration of the hippocampal architecture, with increased neuronal density, reduced pyknosis, and improved glial and vascular organisation compared to the disease control (Figure 4D). PM-FP at the high dose (200 mg/kg) produced a more pronounced restoration of hippocampal histology, approaching that of the sham control, with well-organised layers of neuronal cells, reduced degenerative changes, and normal-appearing glial cells and blood vessels (Figure 4E). These histopathological findings corroborate the neuroprotective effect of PM-FP as demonstrated by the behavioural and biochemical outcomes.

## DISCUSSION

Alzheimer's disease is a very serious health issue. Although numerous studies have been conducted and various hypotheses have been tested to gain a better understanding of the pathophysiology of Alzheimer's disease, no promising and effective disease-modifying drugs have been found yet. While several drugs have gone for clinical trials, the findings are inconclusive. The causes for the failure of drugs in clinical trials are not well understood. Since natural compounds are known to possess notable antioxidant properties, in the present study free phenolic compounds from Proso millet seeds were isolated and

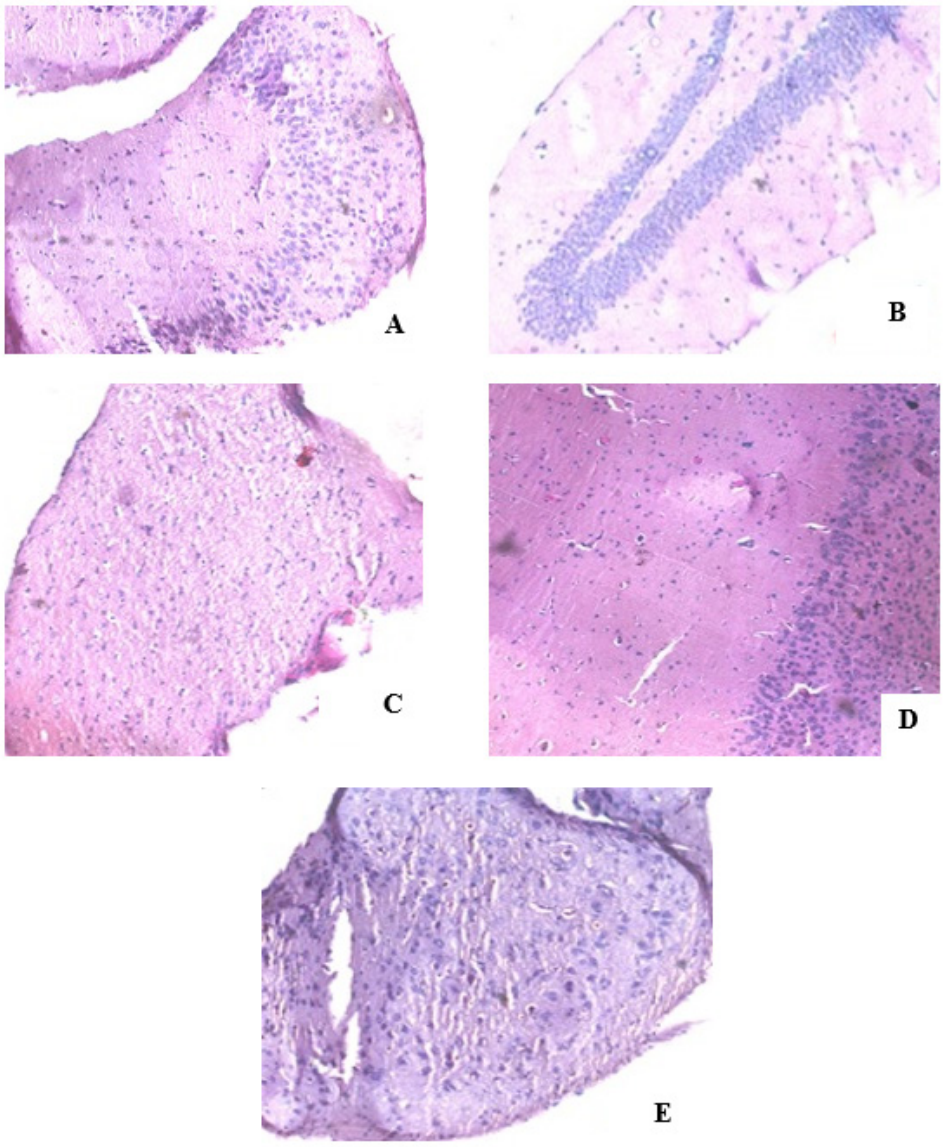


**Figure 4:** Histopathological examination of brain hippocampus (H & E stain, 400x). A: Photomicrograph showing the histological architecture of brain hippocampus in the Normal group; B: Photomicrograph showing the histological architecture of brain hippocampus in the Sham control group; C: Photomicrograph showing the histological architecture of brain hippocampus in the Vehicle (Disease Control, $A\beta_{1-42}$) group; D: Photomicrograph showing the histological architecture of brain hippocampus in the Standard (TPPU, 1 mg/kg) group; E: Photomicrograph showing the histological architecture of brain hippocampus in the Treatment (PM-FP, 200 mg/kg) group. Data for the Normal, Sham, Control, Standard, and Treatment (PM-FP 200 mg/kg) groups are presented

its effect on amyloid $\beta_{1-42}$ induced Alzheimer's disease in mice was examined. Soluble EH (sEH) is one of the important targets for Alzheimer's disease recently. As sEH inhibition was found to alleviate AD-related complications, the potential of PM-FP to inhibit sEH was evaluated. PM-FP was found to have sEH inhibitory activity and hence was screened *in vivo* for its effect on an $A\beta_{1-42}$ mice model of Alzheimer's disease (Jones *et al.*, 2005). In this study, the most common non-transgenic animal model for AD was used to study antioxidant and anti-inflammatory effect of Proso millet. The mice were administered with unilateral injection of $A\beta_{1-42}$ after i.c.v surgery and behavior tests were conducted. The induction of cognitive dysfunction was observed in all the groups except sham control animals on day 7 after $A\beta_{1-42}$ administration, which showed successful induction of Alzheimer's disease in the animals before treatment (Son *et al.*, 2021). Observed that olfactory deficit is related to cognitive and memory impairment and could be the first pathological finding in the diagnosis of AD dementia, which is in line with the findings of the present study (Son *et al.*, 2021; Burns, 2000). Olfactory dysfunction was measured by subjecting the animals to the buried pellet test, where the disease-induced animals took longer time to unbury the pellet on day 7 than on day 0. The cognitive dysfunction was assessed by subjecting the animals to NORT, where disease-induced animals demonstrated a low preference index for the novel object on day 7 when compared to day 0. Chen *et al.* (2014) reported that the Aβ-treated mice had a significantly reduced preference

index (time spent exploring the new object) in comparison to the control mice after the induction period. In this study, TPPU (a synthetic sEH inhibitor) was used as the standard to assess the effect of sEH inhibition in the treatment of the condition. Notably, TPPU was found to be effective in restoring the Aβ-induced olfactory dysfunction, suggesting the therapeutic potential of sEH inhibition in improving the condition. Marowsky *et al.* (2009) reported the presence of presence of soluble epoxide hydrolase enzyme in the olfactory bulb of mouse. Lakkappa *et al.* (2018) reported the therapeutic potential of sEH inhibition in CNS. PM-FP at the high dose (200 mg/kg) was also effective against Aβ-induced olfactory dysfunction. Shen *et al.* (2018) reported that proso millet is highly associated with antioxidant activity in the brain. To our knowledge, we are the first to investigate the effects of sEH inhibition on olfaction. In the NORT study, animals with disease-induced olfactory dysfunction had less preference for the novel object as indicated by the time spent with the novel object in comparison to the sham control. Similar results were obtained by (Chen *et al.*, 2014). Standard TPPU-treated animals spent more time near the novel object when compared to disease control. Griñán-Ferré *et al.* (2020) found that TPPU-treated animals had an increased preference index. Animals treated with PM-FP (200 mg/kg) showed increased time near the novel object when compared to disease control. The discrimination index was significantly decreased in the disease control group as compared to the sham control group. The discrimination index of the animals was reversed by treatment with standard TPPU and PM-FP (compared to disease control) (Manoharan *et al.*, 2016). Reactive Oxygen Species (ROS) is very important in the normal functioning of the brain, but high levels of ROS can be detrimental by damaging DNA and proteins, resulting in oxidative stress. The role of ROS-induced oxidative damage has been extensively investigated in Alzheimer's disease and other neurodegenerative disorders. Similarly, we have observed increased levels of ROS in the disease control group compared to sham control in the present study. In the study by (Shi *et al.*, 2015) millets were reported to have effect on ROS levels. The standard TPPU and PM-FP (200 mg/kg) were found to be effective in reducing the Aβ-induced oxidative stress, as indicated by the reduction in ROS levels compared to disease control.

It has been observed that histopathological examination of the hippocampus provided morphological evidence corroborating the biochemical and behavioural findings of the present study. The disease control (vehicle-treated, $A\beta_{1-42}$ injected) group showed marked neuronal degeneration, pyknosis, and disorganisation of the hippocampal architecture, consistent with $A\beta_{1-42}$ mediated neurotoxicity. These morphological changes are well documented in Aβ-induced AD models and reflect the cascade of neuroinflammatory and oxidative events initiated by amyloid beta accumulation (Souza *et al.*, 2016). Treatment with TPPU resulted in appreciable restoration of the hippocampal cytoarchitecture, with better preservation of nerve cells, reduced glial activation, and improved vascular integrity, consistent with the known anti-neuroinflammatory effects of sEH inhibition (Sun *et al.*, 2012; Griñán-Ferré *et al.*, 2020). PM-FP at the high dose (200 mg/kg) produced a pronounced improvement in hippocampal histology compared to the disease control, showing near-normal hippocampal architecture. This morphological neuroprotection may be attributed to the antioxidant phenolic constituents of PM-FP, which likely act through sEH inhibition to sustain brain EET levels and attenuate neuroinflammation and oxidative damage (Shen *et al.*, 2018). The histopathological findings thus provide direct structural evidence for the neuroprotective potential of Proso millet free phenolics in $A\beta_{1-42}$ induced Alzheimer's disease.

## CONCLUSION

The present study demonstrates the beneficial effect of PM-FP in $A\beta_{1-42}$ mice model of Alzheimer's disease. The beneficial effect of Proso millet may be attributed to its sEH inhibitory activity, a new therapeutic target for AD. *In vitro* studies have shown sEH inhibitory activity of PM-FP, and *in vivo* studies have shown the beneficial effect of PM-FP in AD through improvement in olfactory function, memory, and reduction of oxidative stress. Histopathological examination of the hippocampus further confirmed the neuroprotective effect of PM-FP, with preservation of neuronal architecture compared to disease controls at the 200 mg/kg dose. This effect of PM-FP may be mediated through its sEH inhibitory activity, which elevates EETs in the brain, resulting in neuroprotective effect through antioxidant and anti-inflammatory mechanisms. But studies are underway to prove the sEH inhibitory activity *in vivo*.

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## ABBREVIATIONS

**AD:** Alzheimer's disease; **$A\beta_{1-42}$:** Amyloid $Beta_{1-42}$; **CCSEA:** Committee for the Control and Supervision of Experiments on Animals; **CNS:** Central Nervous System; **DHETs:** Dihydroxyeicosatrienoic acids; **EETs:** Epoxyeicosatrienoic acids; **EpFA:** Epoxyfatty acids; **ER:** Endoplasmic reticulum; **H & E:** Haematoxylin and Eosin; **IAEC:** Institutional animal ethical committee; **NORT:** Novel object recognition test; **PM-FP:** Proso millet free phenolics (*Panicum miliaceum* Linn.); **ROS:** Reactive Oxygen Species; **sEH:** Soluble epoxide hydrolase; **TPPU:** 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## AUTHOR CONTRIBUTIONS

**Baburayanakoppalu Manchegowda Jayanth:** Research concept and design, and collection and assembly of data. **Sathish Kumar Gunasekaran:** Data analysis, interpretation and writing the article. **Santhepete Nanjundaiah Manjula:** Critical revision and final approval of article. **Prashant Yuvaraj Mali:** Critical revision and final approval of the article.

## SUMMARY

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterised by inflammation, oxidative stress, and neuronal loss, for which effective disease-modifying therapies remain elusive. Soluble epoxide hydrolase (sEH) inhibition has emerged as a promising neuroprotective strategy by preserving epoxyeicosatrienoic acids (EETs) that reduce neuroinflammation and oxidative damage. In this study, the free phenolic extract of Proso millet (*Panicum miliaceum* L., PM-FP) was evaluated for its sEH inhibitory activity and neuroprotective potential in an $A\beta_{1-42}$-induced mouse model of AD. PM-FP exhibited potent inhibition of both human sEH ($IC_{50}$ 6.40 µg/mL) and mouse sEH ($IC_{50}$ 16.38 µg/mL) *in vitro*. *In vivo*, PM-FP (200 mg/kg) significantly improved olfactory function (buried pellet food test), recognition memory (NORT, discrimination index), antioxidant enzyme activities (SOD, CAT, GSH), NRF2 levels, and hippocampal cytoarchitecture, while reducing ROS and lipid peroxidation in AD mice, with effects comparable to standard sEH inhibitors TPPU and PTUPB. These findings demonstrate that sEH inhibition by PM-FP is a promising approach to treat AD.

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