Original Article
DOI: https://doi.org/10.70749/ijbr.v3i8.2162

INDUS JOURNAL OF BIOSCIENCE RESEARCH
https://ijbr.com.pk
ISSN: 2960-2793/ 2960-2807

Computational and Experimental Evidence for the Neuroprotective Role of 4-Methyl-2-(5-phenyl-6-thioxo-1,3,5-thiadiazinan-3-yl)pentanoic Acid via NRF2/TLR4 Signaling in Aging Mice

Saleha Gul[1,2], Gul Nabi Khan[2], Shahid Ali Shah[4], Rasool Khan[3], Haleema Ali[3], Umer Sadique[5], Farrah Zaidi[1]*

[1] Institute of Zoological Sciences, University of Peshawar, Pakistan
[2] Department of Zoology, Islamia College University, Peshawar, Pakistan
[3] Institute of Chemical Sciences, University of Peshawar, Pakistan
[4] Department of Biology, University of Haripur, Haripur, Khyber Pakhtunkhwa, Pakistan
[5] College of Veterinary Sciences, Faculty of Animal Husbandry and Veterinary Sciences, University of Agriculture, Peshawar, Pakistan

ARTICLE INFO

Keywords: D-Gal, Pentanoic Acid, Mice, TLR4, Nrf-2, Astrocytes, Microglia.

Correspondence to: Farrah Zaidi,
Institute of Zoological Sciences, University of Peshawar, Pakistan

Email: zaidi_farrah@uop.edu.pk

Declaration
Authors' Contribution
All authors equally contributed to the study and approved the final manuscript

Conflict of Interest: No conflict of interest.

Funding: No funding received by the authors.

Article History
Received: 01-05-2025 Revised: 12-07-2025
Accepted: 21-07-2025 Published: 03-08-2025

ABSTRACT

Background: Aging is an inevitable biological process and a significant risk factor for numerous neurodegenerative and psychiatric diseases. Aim: The current study offers the first complete assessment of the neuroprotective effects of a novel pentanoic acid derivative (PAD), 4-Methyl-2-(5-Phenyl-Thioxo-1,3,5-Thiadiazinan-3-yl), in a D-galactose (D-Gal)-induced model of brain aging in male mice. **Material and methods**: Thirty males were assigned randomly to six groups (n = 10); a normal control, a D-Gal group (100 mg/kg), and a D-Gal + PAD group (25 mg/kg). D-galactose was given intraperitoneally once a day for 56 days whereas PAD was started at day 29 for 28 days. **Results:** Western blotting showed that chronic exposure to D-galactose induced microglial and astrocytic activation, increased TLR4 and NF-κB signaling, decreased Nrf2 expression, and increased PARP-1 activity in the mouse brain. An administration of PAD inhibited these changes by reducing glial activation, the level of TLR4, and increasing Nrf2, and reducing the activity of PARP-1. In Silico molecular docking studies were conducted for further mechanistic validation, which demonstrated that PAD forms stronger stable hydrogen bonds with specific residues within the KEAP1 binding pocket that may disrupt the KEAP1–NRF2 complex and initiate NRF2 signaling. Furthermore, PAD showed high binding affinity toward the TLR4 active site, indicating inhibition of TLR4 mediated inflammatory signaling. **Conclusion:** Taken together, these results demonstrate that this new PAD compound demonstrated neuroprotective effects through dual modulation of NRF2 and TLR4 pathways. The PAD compound represents a promising drug candidate for age-associated neurodegenerative diseases due to its potential to act as both a NRF2 activator and TLR4 inhibitor. Further pharmacologic and mechanistic investigation will be required to substantiate its drug-like features and therapeutic efficacy

INTRODUCTION

Aging is a nearly inevitable and an increasingly progressive biological event that continues to be the major risk factor for many neurodegenerative and psychiatric conditions (e.g. Alzheimer's disease (AD), Parkinson's disease (PD), and related dementias [1]. Through the ages, cumulative molecular, cellular, and structural changes in the brain including mitochondrial dysfunction, decline of proteostasis, genomic instability, and changes in glial-neuronal interaction emerged that lay the foundation for cognitive decline and increased risk for neurodegeneration [2]. One of the main mechanistic themes in brain aging is oxidative stress. The brain accumulates reactive oxygen and nitrogen species (ROS/RNS) in aging, while the endogenous antioxidant defense capacity decreases. The resulting state of redox imbalance can lead to neuronal damage, synaptic erosion, and activation of glial inflammatory pathways [3].

The D-galactose (D-gal) induced aging mouse model has become one of the most widely used experimental systems for aging research in recent years [4, 5]. Numerous studies have shown that long-term D-gal administration produces physiological and biochemical alterations that closely resemble natural aging in animals [6, 7]. In particular, D-gal–induced brain aging in mice mimics many features observed in human aging, such as heightened oxidative stress, neuronal damage, inflammation, and apoptosis [3, 8, 9].

NRF2 (nuclear factor erythroid 2–related factor 2) is an important transcription factor with the ability to regulate

cellular antioxidant and cytoprotective mechanisms. Under basal conditions, NRF2 is attached to KEAP1 and destined for degradation. However, in the setting of oxidative stress, NRF2 translocates to the nucleus, binds antioxidant response elements (AREs) and promotes genes that support and maintain redox balance [10]. Aging, and decreased NRF2 activity, compromises oxidative defense, confounding mitochondrial function followed by chronic inflammation and cellular senescence [11]. Concurrently, neuroinflammation is one of the major contributors to the aging brain, with TLR4 (Toll-like receptor 4) serving as a central mediator. Expressed on microglia or astrocytes, TLR4 is activated by damage- or pathogen-associated molecular patterns (DAMP/PAMPs); this leads to activation of the MyD88/TRIF/NF-κB signaling pathways and further stimulation of pro-inflammatory cytokine release [12]. Chronic TLR4 activation promotes low-grade inflammation ("inflammaging"), and interferes with synaptic and neuronal integrity [12, 13]. Crosstalk between nuclear factor erythroid-2(p45) related factor 2 (NRF2) and Toll-like receptor 4 (TLR4)/nuclear factor-kappa B (NF-κB) signaling cascades enhances oxidative and inflammatory injury with suppressed NRF2 leading to increased inflammasome activity and continued TLR4 signals leading to prolonged suppression of NRF2 [14]. As such, strategies that inhibit TLR4 mediated inflammation but promote NRF2 activation may limit neurodegeneration associated with aging. The D-Gal-induced rodent model is frequently used to simulate brain aging, the administration of D-Gal leads to modeling oxidative stress, glial activation, and poor cognitive performance, thereby allowing the biological research of nutraceutical compounds associated with a neuroprotective effect [15].

In recent years, considerable attention has been directed toward the development of novel synthetic compounds designed to mimic the pharmacophoric characteristics of naturally occurring bioactive molecules. Such compounds are of particular interest due to their potential to exert multifaceted therapeutic effects, including antioxidant, anti-inflammatory, and neuroprotective actions. Among these, pentanoic acid derivatives (PADs) have emerged as promising candidates owing to their diverse biological activities. A recently synthesized derivative, 2-(4-methyl-2-(5-phenyl-6-thioxo-1,3,5-thiadiazinan-3-yl)pentanoic acid), has demonstrated notable pharmacological potential in preliminary studies [16]. Despite these findings, the neuroprotective capacity of PADs has not yet been elucidated. The present study, therefore, undertakes the first systematic evaluation of this PAD compound in a D-galactose (D-Gal)-induced aging mouse model. Specifically, we investigate whether PAD can modulate the NRF2/TLR4 signaling axis—by activating NRF2-mediated antioxidant defenses and inhibiting TLR4-driven inflammatory responses—to alleviate oxidative stress, neuroinflammation, and neurodegeneration. In addition, in silico molecular docking and pathway analyses were performed to further characterize the interaction of PAD with NRF2 and TLR4 targets, supporting its potential as a dual-action neuroprotective agent for the management of age-related neurological disorders.

MATERIAL & METHODS

Grouping of animals

Male albino mice, aged eight weeks and each weighing 30 ± 2 grams, were purchased from the Veterinary Research Institute in Peshawar. Mice were acclimatized for one week prior to the experiments and were housed under laboratory conditions. The temperature of the animal wards was maintained between 21°C and 25°C with a relative humidity of 45–55% and a 12-hour light/dark cycle, with animals provided standard laboratory chow and water ad Libitum. All experiments took place in the Pathology Laboratory, College of Veterinary Sciences, University of Agriculture, Peshawar, in accordance with the guidelines provided by the Research Ethical Board of the University of Peshawar, Pakistan. There were three experimental groups, each with ten mice, which were as follows:

- Group I (Control group): Mice in this group received normal saline (0.9%) intraperitoneally (i.p), which they received one time a day, for a period of eight weeks.
- Group II (D-galactose group): Mice received D-galactose (100 mg/kg body weight i.p) once a day for eight weeks, to induce aging-like changes in the mice.
- Group III (D-gal + PAD group): Mice in this group received D-galactose (100 mg/kg i.p.) one time per day for eight weeks w/ a novel pentanoic acid derived compound (PAD) for the period of four weeks (3 times/ week, 25 mg/kg).

Pentanoic acid derivative (2-(4-methyl-2-(5-phenyl-6-thioxo-1,3,5-thiadiazinan-3-yl))) used in this study is a compound that has recently been synthesized and donated by Dr. Rasool Khan at the Institute of Chemical Sciences, University of Peshawar. The synthesis and initial characterization of this compound was recently described by Haleema Ali et al. (2023).

Brain Tissue Harvesting and Homogenization:

At the conclusion of the experimental time points (as indicated above), all mice were humanely euthanized under isoflurane anesthesia. Brain tissues were quickly harvested, rinsed with ice-cold phosphate-buffered saline (PBS) to eliminate any excess blood and weighed. Next, the tissues were homogenized in T-PER™ Tissue Protein Extraction Reagent (Thermo Scientific, USA), containing protease and phosphatase inhibitors, to preserve the protein in the samples. Homogenates were centrifuged for 15 minutes at 12,000 rpm at 4°C, then the supernatants were collected for analysis specifically for Western blotting.

Western Blotting:

The protein concentration from the brain homogenates was evaluated using the Bio-Rad Protein Assay Kit according to the manufacturer's guidelines. Equal amounts of total protein from each sample were used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine relative protein expression based on the molecular weight. The separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes using a semi-dry transfer apparatus. Blocking of membranes (5% skim milk in Tris-buffered

saline with 0.1% Tween-20 (TBST)) was performed at room temperature for one hour to limit background binding. Membranes were incubated at 4°C overnight with the following primary antibodies (from Santa Cruz Biotechnology, CA, USA, at a dilution of 1:2000): Anti-Iba-1 (SC-32725), Anti-GFAP (SC-33673), Anti-NF-κB (SC-8008), Anti-Nrf2 (SC-365949), Anti-TLR4 (SC-293072), Anti-PARP-1 (SC-8007) and β-actin (SC-47778) as internal loading control. After incubation, membranes were washed three times with TBST and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-mouse or anti-goat based on the species of primary antibody) diluted at 1:1000 for 1-2 hours at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) detection reagents (Bio-Rad, USA), and images were collected on X-ray film for quantification.

Molecular Docking Studies

Molecular Docking Investigations The structure of the 4-methyl-2-(5-phenyl-6-thioxo-1,3,5-thiadiazinan-3-yl) pentanoic acid ligand was designed using the KingDraw application, with the final structure generated and saved as a MOL file. The molecular file was generated in Structure Data File (SDF) format so that the docking software tools supported that format. For receptor preparation, the three-dimensional crystal structures for nuclear factor erythroid 2–related factor 2 (NRF2) and toll-like receptor 4 (TLR4) structure were obtained from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB). To maintain fidelity and reproducibility, a sequential approach of three programs utilizing four completely organic software programs was used for molecular docking simulations.

Discovery Studio (BIOVIA, Dassault Systèmes):

Discovery Studio was used to perform first processing for both the protein and ligand structures. The SDF ligand structure was converted to a PDB file structure for compatibility with the next phase of docking programs. Discovery Studio was used to clean up the receptor structure by removing water molecules, associated ligands (if any had been "soaked into" the protein), and other unnecessary heteroatoms, ultimately cleaning the protein structure for analysis during the docking process.

PyMOL Molecular Graphics System:

PyMOL was used for structural visualization and further cleaning of the receptor proteins. It enabled the removal of residual ligands or ions and provided a three-dimensional graphical representation of the ligand, allowing confirmation of its molecular geometry and spatial conformation prior to docking.

MGL Tools (AutoDock Tools):

The pore software suite was used to prepare protein and ligand files in PDBQT format for docking, which includes atomic coordinates, charge assignment, and torsional information. Dimensions of the grid boxes were optimized to include the active site predicted region of the target proteins.

AutoDock Vina:

Molecular docking simulations were performed using AutoDock Vina, which predicts the most favorable binding orientation and interaction energy of the ligand and target proteins (NRF2 and TLR4). The docking results revealed potential binding affinities, hydrogen bonding trends, and hydrophobic interactions utilized to speculate the compound's potential biological activity.

After docking, the individual protein–ligand complexes were visualized and analyzed in both PyMOL and Discovery Studio Visualizer in order to identify important interacting residues, hydrogen bonds, and conformational stability. The docking scores and binding poses were visualized and evaluated to begin to understand the documents interactions and likely binding affinity towards NRF2 and TLR4 as a starting point for structural understanding as it impacts potential determinants of modulator activity.

Statistical analysis:

The X-ray films developed for protein bands were subjected to densitometric quantification using the ImageJ software (National Institutes of Health, USA), a program well-established for the analysis of biomedical imaging data. The intensity of each band was normalized to the loaded control (β-actin), to account for potential variability in protein loading. Upon establishment of experiments, statistical comparisons between two groups were conducted using a Student's t-test to analyze differences between the means of two independent groups. Where an experiment consisted of more than 2 groups, the statistical comparison of interest was conducted for overall differences among the groups using one-way analysis of variance (ANOVA). Where ANOVA was statistically significant, the specific differences between group's means were identified using Bonferroni and Tukey's post-hoc statistics, controlling for multiple comparisons. Statistical analyses and graph/chart building were performed using GraphPad Prism (version 5.0). Data were shown as mean ± standard error of the mean (SEM). A p-value of ≤ 0.05 was considered statistically significant, whereas a 95% confidence level was used for all statistical tests to establish robustness and reliability.

RESULTS

PAD Reduced Microglial and Astrocytic Activation in the Brains of D-galactose–induced Aging Mice.

It has previously been shown that D-galactose (D-Gal) elicits neuroinflammation by activating the resident immune cells in the brain known as microglia and astrocytes (Xiong et al., 2023). In support of these findings, the present study found that chronic administration of D-Gal at a dose of 100 mg/kg for eight weeks significantly increased the expression levels of microglial and astrocytic markers in the brain homogenates of adult albino mice (Figure 1). Increased expression levels of these markers indicate glial activation that is indicative of neuroinflammatory mechanisms associated with aging. Interestingly, co-administration of the pentanoic acid derivative (PAD) significantly inhibited both microglial and astrocytic marker protein expression upregulation that was induced by D-Gal. Furthermore, the inhibition observed indicated that PAD is capable of downregulating selected neuroinflammatory processes that are initiated by D-Gal. The downregulation of Iba-1 (a microglial marker) and GFAP (an astrocytic marker) following treatment with PAD demonstrated that it can regulate neuroimmune activity and promote neural homeostasis.

Overall, these findings provide further evidence to suggest that PAD may exert a neuroprotective and anti-inflammatory effect in the aging mouse brain by inhibiting excessive glial cell activation. Also, the reduction in activation of both microglia and astrocytes strengthens PAD's candidacy as a therapeutic target for alleviating D-Gal–induced neuroinflammation and neurodegeneration associated with aging (See figure 1).

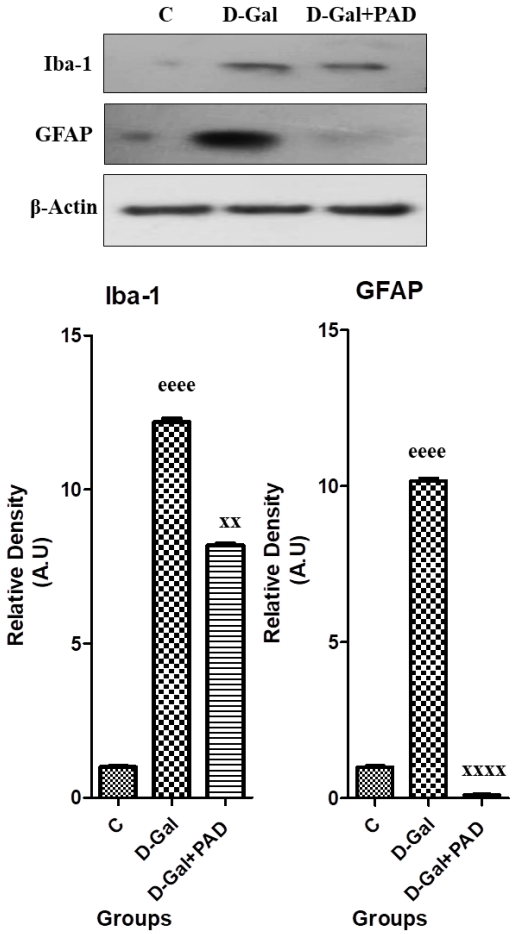

Figure 1. Influence of PAD on the protein expression of Iba-1 and GFAP in the brains of D-Gal-induced aging mice. Representative Immunoblots indicative of Iba-1, GFAP and β-actin in brain homogenates from experimental groups. Band intensities were measured using the ImageJ program and expressed in arbitrary units (A.U.). Histograms represent the means ± SEM of the measurements. Comparisons were made between the control and D-Gal groups (e) and D-Gal vs D-Gal + PAD (x). Statistical significance: ee $p \leq 0.0001$; xx $p \leq 0.01$; *xxxx $p \leq 0.0001$. Data indicate that PAD treatment significantly decreased the D-Gal-induced increase in Iba-1 and GFAP expression.

PAD Suppressed NF-κB and PARP-1 Expression in the Brains of D-Galactose–Induced Aging Mice.

Studies have shown that D-galactose (D-Gal) enhances neuroinflammatory and neurodegenerative activities by upregulating important proinflammatory mediators such as the nuclear factor kappa B (NF-κB) and poly (ADP-ribose) polymerase-1 (PARP-1), in the brains of aging mice (Wang et al., 2023). Similarly, in this study, the expression of NF-κB and PARP-1 proteins had a pronounced increase in brain homogenates from adult albino mice after chronic exposure to D-Gal (Figure 2). Higher levels of NF-κB and PARP-1 protein reflect the activation of inflammatory and apoptotic signaling pathways caused by oxidative stress, resulting in neuronal damage and cell death. PAD treatment, a derivative of pentanoic acid, significantly downregulated the D-Gal-induced overexpression of NF-κB and PARP-1. The decrease in NF-κB activation suggests a unique aspect of PAD in inhibiting the transcription of various cytokines and solving neuroinflammation. The downregulation of PARP-1 can also suggest a pharmacological role of PAD in preventing excessive DNA damage and subsequent neuron apoptosis. Overall, these data show that PAD has a strong anti-neuroinflammatory and neuroprotective impact through preventing the activation of NF-κB- and PARP-1-driven signaling pathways. This dual inhibition of signaling pathways highlights the therapeutic potential of PAD to mitigate D-Gal-induced oxidative stress, neurodegeneration, and neuroinflammation in the aging mouse brain as depicted in Figure 2.

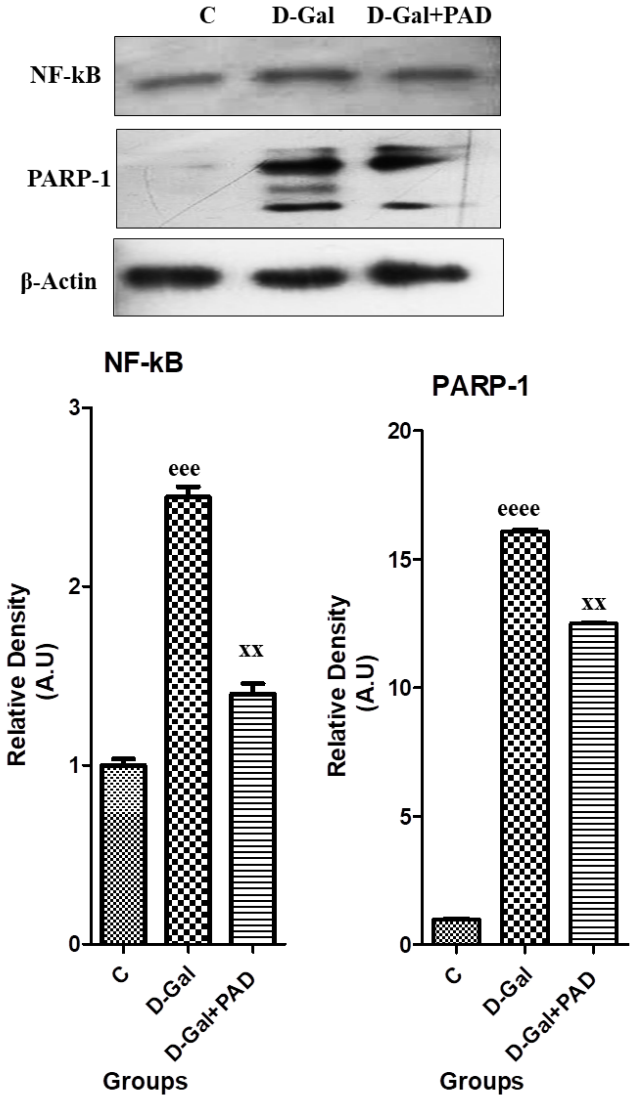

Figure 2. Effect of PAD on NF-κB and PARP-1 protein expression in the brains of D-Gal–induced aging mice. Representative Immunoblots showing the expression

levels of NF-κB, PARP-1, and β-actin in brain homogenates from the experimental groups. Protein band intensities were quantified using ImageJ software and expressed as arbitrary units (A.U.). Data are presented as the mean ± SEM. Statistical comparisons were made between the control and D-Gal groups (denoted by "e") and between the D-Gal and D-Gal + PAD groups (denoted by "x"). Statistical significance was indicated as follows: ^{eeee} p ≤ 0.0001; ^{xx}p≤ 0.01; ^{xxxx}p≤ 0.0001.

PAD Modulated Nrf2 and TLR4 Protein Expression in the Brains of D-Galactose–Induced Aging Mice

Previous studies have reported that chronic exposure to D-galactose (D-Gal) impairs antioxidant defense mechanisms and enhances neuroinflammation by downregulating nuclear factor erythroid 2–related factor 2 (Nrf2) and upregulating toll-like receptor 4 (TLR4) expressions in the brain (Saif Ullah et al., 2024). In agreement with these findings, the present study demonstrated that D-Gal administration markedly suppressed Nrf2 expression while increasing TLR4 levels in the brain homogenates of adult albino mice, as shown in Figure 3. The reduction in Nrf2 suggests a weakened antioxidant response, while elevated TLR4 expression indicates activation of pro-inflammatory signaling pathways associated with aging and oxidative stress. Interestingly, treatment with the pentanoic acid derivative (PAD) significantly reversed these D-Gal–induced alterations. PAD administration enhanced the expression of Nrf2, the master regulator of cellular redox homeostasis, indicating activation of the antioxidant defense system. Concurrently, PAD treatment downregulated TLR4 expression, implying a suppression of the innate immune signaling cascade that contributes to neuroinflammation. The observed modulation of Nrf2 and TLR4 suggests that PAD exerts dual protective effects it strengthens the brain's antioxidant capacity while simultaneously inhibiting inflammatory signaling pathways. Collectively, these results highlight PAD's potential as a neuroprotective agent capable of mitigating D-Gal–induced oxidative stress and inflammation by restoring the balance between Nrf2-mediated antioxidant responses and TLR4-dependent inflammatory signaling, as illustrated in Figure 3.

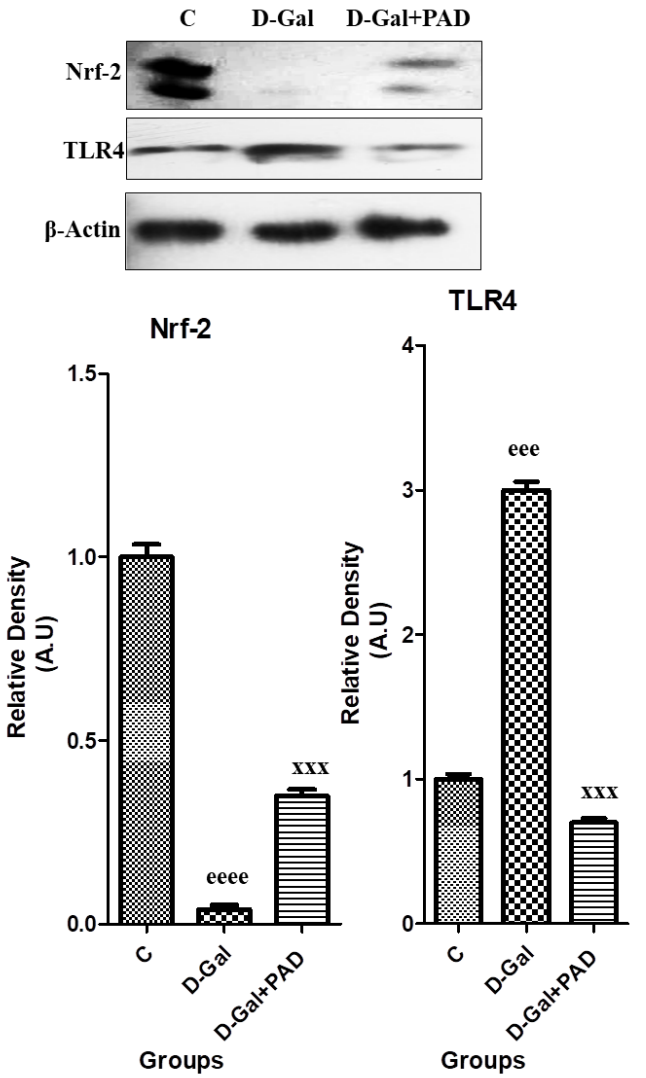

Figure 3. Effect of PAD on Nrf2 and TLR4 protein expression in the brains of D-Gal–induced aging mice. Representative Immunoblots illustrating the expression levels of Nrf2, TLR4, and β-actin in brain homogenates from the experimental groups. Densitometric analysis of the protein bands was performed using ImageJ software, and values were expressed in arbitrary units (A.U.). Data are presented as the mean ± SEM. Statistical comparisons were made between the control and D-Gal groups (denoted by "e"), and between the D-Gal and D-Gal + PAD groups (denoted by "x"). Levels of statistical significance were as follows: ^{eeee} p ≤ 0.0001; ^{xx} p ≤ 0.01; ^{xxxx} p ≤ 0.0001.

Molecular Docking Results and Interaction Analysis

Following molecular docking simulations, the binding interactions between 4-methyl-2-(5-phenyl-6-thioxo-1,3,5-thiadiazinan-3-yl) pentanoic acid and the target proteins KEAP1 (a regulatory protein of NRF2) and TLR4 were examined using PyMOL visualization software. The docking analysis provided insights into the potential molecular mechanisms through which the compound may modulate oxidative stress and inflammatory pathways. The docking results for KEAP1 demonstrated a binding affinity of –4.6 kcal/mol, indicating a moderate yet stable interaction between the ligand and the receptor. Structural visualization showed that the ligand was well accommodated within the NRF2-binding pocket of KEAP1. Hydrogen bonding interactions, depicted as yellow dotted

lines in the 3D model, were observed between the ligand and amino acid residues GLY305 and ILE474, with a bond distance of approximately 2.6 Å. These interactions suggest that the compound forms stable hydrogen bonds with crucial residues in the KEAP1 active site, potentially disrupting the KEAP1–NRF2 interaction and promoting NRF2 activation as shown in the figure 4A.

Similarly, docking of the compound with TLR4 revealed a higher binding affinity of –6.2 kcal/mol, reflecting a stronger and more stable interaction. Visualization in PyMOL confirmed that the ligand fitted precisely within the TLR4 active site. Hydrogen bond formation was detected with residues HIS458 and ASN433 at an average bond length of 2.4 Å. These close interactions indicate that the compound may effectively occupy the TLR4 binding pocket, thereby inhibiting receptor activation and attenuating downstream TLR4-mediated inflammatory signaling.

Overall, the docking outcomes suggest that the pentanoic acid derivative exhibits dual molecular interactions: it may enhance NRF2 activation by interfering with KEAP1 binding, while concurrently suppressing TLR4 signaling, thus highlighting its potential as a neuroprotective and anti-inflammatory agent as shown in the figure 4B.

Fig. 4A

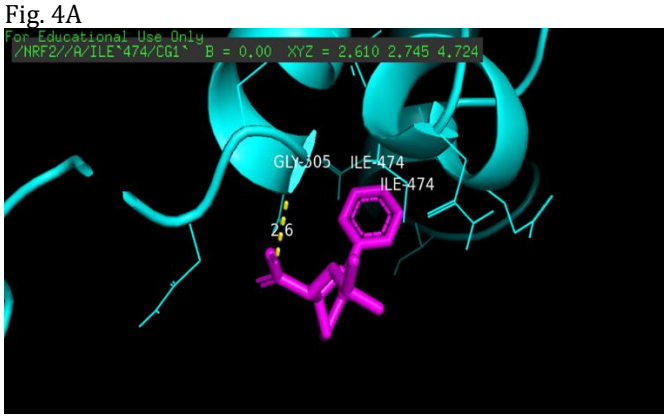

Fig. 4B

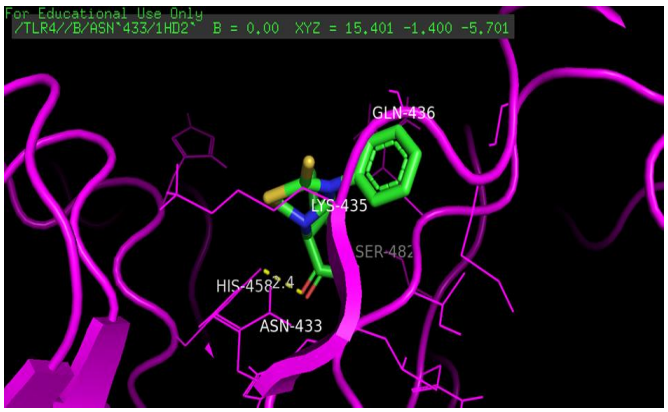

Figure 4. PAD activated Nrf-2 and inhibited TLR4 proteins. Shown are the interactions of PAD and Nrf-2 (A) and TLR4 (B) proteins.

DISCUSSION

The present study demonstrates that the novel pentanoic acid derivative (PAD) exerts multifaceted neuroprotective effects in a D-galactose (D-Gal)-induced brain-aging model in adult albino mice. Notably, PAD suppressed glial activation (microglia and astrocytes), inhibited key pro-inflammatory and pro-apoptotic mediators (NF-κB and PARP-1), and modulated the redox-inflammatory axis by activating Nrf2 while reducing TLR4 expression. Furthermore, molecular docking results suggest PAD may directly engage the regulatory proteins KEAP1 and TLR4, providing structural mechanistic support for its biochemical actions.

Chronic D-Gal treatment significantly elevated Iba-1 and GFAP protein levels, indicating microglial and astrocytic activation hallmarks of neuroinflammation in aging and age-related neurodegeneration. These findings align with previous reports showing that D-Gal triggers glial over-reactivity in rodent brain, thereby contributing to inflammaging and neural dysfunction [17, 18]. PAD co-administration markedly reduced Iba-1 and GFAP expression, suggesting its capacity to attenuate glial reactivity. By down-modulating both microglial and astrocytic activation, PAD likely disrupts the self-sustaining cycle of neuroinflammation that characterizes brain aging.

D-Gal exposure elicited substantial up-regulation of NF-κB and PARP-1 in the brain homogenates. NF-κB is a master transcription factor of inflammatory responses, while PARP-1 is linked with DNA damage, apoptosis and cellular energy depletion in stressed neural tissue [19]. Targeting these molecules is central to limiting neurodegenerative progression. Inhibiting NF-κB and PARP-1 via PAD treatment supports the notion that PAD not only suppresses glial activation but also interferes with intracellular inflammatory pathways and downstream apoptotic processes. This dual targeting gives the compound a strong anti-neuroinflammatory and neuroprotective profile.

Aging is characterized by a decline in Nrf2 activity and concurrent rise in TLR4 signaling, promoting oxidative stress, chronic inflammation and cellular senescence [3, 20]. In our model, D-Gal suppressed Nrf2 while elevating TLR4 protein levels, replicating aging-associated alterations in redox-inflammatory homeostasis. PAD reversed this pattern: it upregulated Nrf2, restoring antioxidant capacity, and downregulated TLR4, mitigating innate immune activation. The dual regulation of these pathways suggests PAD restores balance between oxidative defense and inflammatory signaling, thereby limiting age-related neurodegenerative changes.

Docking simulations indicated that PAD binds moderately to KEAP1 (≈ –4.6 kcal/mol) and more strongly to TLR4 (≈ –6.2 kcal/mol). Interaction with KEAP1 could relieve sequestration of Nrf2, facilitating its nuclear translocation and activation of antioxidant gene programmes. Simultaneously, binding to TLR4 may block receptor-mediated pro-inflammatory signaling, thereby reducing activation of NF-κB and glial cells. Together, these results provide a structural basis for the observed biochemical effects of PAD and support its potential as a dual-target therapeutic. Collectively, our findings suggest PAD is a promising neuroprotective agent in models of aging: it attenuates glial overactivation, suppresses inflammatory/apoptotic mediators, and enhances antioxidant defense via Nrf2 while inhibiting TLR4-driven inflammation. These actions may slow or prevent D-Gal-

induced aging-like brain changes and could extend to age-related neurodegenerative conditions. Future studies should address the behavioral endpoints, long-term outcomes, and dose-response relationships. Mechanistically, validating the docking predictions with mutagenesis or binding assays would strengthen the link between PAD–protein interactions and biological effects.

CONCLUSION

PAD modulates critical molecular pathways implicated in brain aging by suppressing neuroinflammation (glial activation, NF-κB, PARP-1), restoring antioxidant capacity (Nrf2), and inhibiting innate immune signaling (TLR4). Coupled with structural interaction data, these results highlight PAD as a compelling candidate for development in aging-associated neurodegenerative prevention or therapy.

Statements & Declarations

Funding
There is no funding for this work.

Competing interest
No competing interest was declared by the authors.

Author's contributions
Research Design: Saleha Gul, Farrah Zaidi, Umer Sadique, Shahid Ali Shah, and Gul Nabi Khan; Experimental Work: Saleha Gul, Shahid Ali Shah and Haleema Ali; Manuscript Writing: Saleha Gul, Shahid Ali Shah, and Gul Nabi Khan; Manuscript Review: Farrah Zaidi, Umer Sadique, Rasool Khan and Gul Nabi Khan.

Availability of data and materials
All data are included in this manuscript.

Ethics approval
This study was approved by the Research Ethics Board of the University of Peshawar, Pakistan (REB-05/06; Dated 24/06/2025)

Consent to participate
Not applicable.

Consent to publish
Not applicable.

REFERENCES

1. Brackhan, M., M. Arribas-Blazquez, and I. Lastres-Becker, *Aging, NRF2, and TAU: A Perfect Match for Neurodegeneration?* Antioxidants (Basel), 2023. **12**(8). https://doi.org/10.3390/antiox12081564
2. Schmidlin, C.J., et al., *Redox regulation by NRF2 in aging and disease.* Free Radic Biol Med, 2019. **134**: p. 702-707. https://doi.org/10.1016/j.freeradbiomed.2019.01.016
3. Davinelli, S., et al., *Dietary phytochemicals and neuro-inflammaging: from mechanistic insights to translational challenges.* Immun Ageing, 2016. **13**: p. 16. https://doi.org/10.1186/s12979-016-0070-3
4. Lei, H., et al., *Anti-aging effect of astragalosides and its mechanism of action.* Acta Pharmacol Sin, 2003. **24**(3): p. 230-4.
5. Shwe, T., et al., *Role of D-galactose-induced brain aging and its potential used for therapeutic interventions.* Exp Gerontol, 2018. **101**: p. 13-36. https://doi.org/10.1016/j.exger.2017.10.029
6. Zhang, Z.F., et al., *Purple sweet potato color attenuates oxidative stress and inflammatory response induced by d-galactose in mouse liver.* Food Chem Toxicol, 2009. **47**(2): p. 496-501. https://doi.org/10.1016/j.fct.2008.12.005
7. Kumar, A., A. Prakash, and S. Dogra, *Centella asiatica Attenuates D-Galactose-Induced Cognitive Impairment, Oxidative and Mitochondrial Dysfunction in Mice.* Int J Alzheimers Dis, 2011. **2011**: p. 347569. https://doi.org/10.4061/2011/347569
8. Ho, S.C., J.H. Liu, and R.Y. Wu, *Establishment of the mimetic aging effect in mice caused by D-galactose.* Biogerontology, 2003. **4**(1): p. 15-8. https://doi.org/10.1023/a:1022417102206
9. El Assar, M., J. Angulo, and L. Rodríguez-Mañas, *Oxidative stress and vascular inflammation in aging.* Free Radic Biol Med, 2013. **65**: p. 380-401. https://doi.org/10.1016/j.freeradbiomed.2013.07.003
10. Chu, C.T., et al., *Role of NRF2 in Pathogenesis of Alzheimer's Disease.* Antioxidants (Basel), 2024. **13**(12). https://doi.org/10.3390/antiox13121529
11. Zinovkin, R.A., N.D. Kondratenko, and L.A. Zinovkina, *Does Nrf2 Play a Role of a Master Regulator of Mammalian Aging?* Biochemistry (Mosc), 2022. **87**(12): p. 1465-1476. https://doi.org/10.1134/s0006297922120045
12. Kim, H.J., et al., *Toll-like receptor 4 (TLR4): new insight immune and aging.* Immun Ageing, 2023. **20**(1): p. 67. https://doi.org/10.1186/s12979-023-00383-3
13. Calvo-Rodriguez, M., et al., *Role of Toll Like Receptor 4 in Alzheimer's Disease.* Front Immunol, 2020. **11**: p. 1588. https://doi.org/10.3389/fimmu.2020.01588
14. Yang, J., L. Wise, and K.I. Fukuchi, *TLR4 Cross-Talk With NLRP3 Inflammasome and Complement Signaling Pathways in Alzheimer's Disease.* Front Immunol, 2020. **11**: p. 724. https://doi.org/10.3389/fimmu.2020.00724
15. Sadigh-Eteghad, S., et al., *Correction: D-galactose-induced brain ageing model: A systematic review and meta-analysis on cognitive outcomes and oxidative stress indices.* PLoS One, 2017. **12**(12): p. e0190328. https://doi.org/10.1371/journal.pone.0190328
16. Sadigh-Eteghad, S., et al., *D-galactose-induced brain ageing model: A systematic review and meta-analysis on cognitive outcomes and oxidative stress indices.* PLoS One, 2017. **12**(8): p. e0184122. https://doi.org/10.1371/journal.pone.0184122
17. Zhang, B., et al., *DL0410 Alleviates Memory Impairment in D-Galactose-Induced Aging Rats by Suppressing Neuroinflammation via the TLR4/MyD88/NF-κB Pathway.* Oxid Med Cell Longev, 2021. **2021**: p. 6521146. https://doi.org/10.1155/2021/6521146

18. Xiong, W., et al., *Nobiletin Mitigates D-Galactose-Induced Memory Impairment via Improving Hippocampal Neurogenesis in Mice.* Nutrients, 2023. **15**(9). https://doi.org/10.3390/nu15092228
19. Ahmad, S., et al., *Fisetin Rescues the Mice Brains Against D-Galactose-Induced Oxidative Stress, Neuroinflammation and Memory Impairment.* Front Pharmacol, 2021. **12**: p. 612078. https://doi.org/10.3389/fphar.2021.612078
20. Matsumaru, D. and H. Motohashi, *The KEAP1-NRF2 System in Healthy Aging and Longevity.* Antioxidants (Basel), 2021. **10**(12). https://doi.org/10.3390/antiox10121929