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ORIGINAL ARTICLE

6-Hydroxy flavone rescue ethanol-induced apoptotic neurodegeneration via activation of p-Akt signaling pathway in the developing mice brain

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ABSTRACT

Objective: To investigate the potential use of 6-Hydroxyflavone (6-HF) activation of phosphorylated Akt (p-Akt) to reduce neurodegeneration induced by Ethanol in the developing mice brain of post-natal day 7 (PND-7) mice.

Methods: This experimental study was conducted from March to June 2023 at Neuro Molecular Medicines Research Center, in collaboration with Shaheed Benazir Bhutto Women University, Peshawar-Pakistan. Twenty postnatal day-7 (PND-7) mice were randomly divided into four groups: control, EthOH, EthOH + 6-HF, and 6-HF. Ethanol (5 mg/kg) was administered subcutaneously to the EthOH group. 6-HF was administered at a dose of 30 mg/kg to both the EthOH + 6-HF and 6-HF groups. Four hours after administration, all mice were sacrificed for brain tissue collection and Western blot analysis. Densitometric quantification of protein bands was performed using ImageJ software. Statistical analysis was done using one-way ANOVA followed by post-hoc Tukey's test, with $p \leq 0.05$ considered significant.

Results: Significant differences in p-Akt levels along with BAX, BCL-2, Cas-3 and PARP-1 protiens were observed in the brain homogenates of PND 7 mice in various groups. Post hoc tukey test revealed significant increase ($p<0.001$) in the p-Akt, BAX, BCL-2, Cas-3 and PARP-1 levels in EthOH group as compared to control group mice. However, significant increase ($p<0.05$) was observed in p-Akt level along with significant retrieval ($p<0.001$) in BAX, BCL-2, Cas-3 and PARP-1 expression level in EthOH+6-HF group as compared to EthOH group.

Conclusion: : Administration of 6-HF significantly improved ethanol induced neurodegeneration in brain of the PND-7 mice, potentially through modulation of the p-Akt signaling pathway.

Keywords: Ethanol (MeSH); 6-hydroxy flavone (MeSH); Neurodegeneration (MeSH); Nerve Degeneration (MeSH); p-Akt signaling pathway (Non-MeSH); Caspase-3 (MeSH).

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INTRODUCTION

Moderate alcohol consumption has been associated with protective effects against certain conditions, including cardiovascular diseases.[1] However, excessive alcohol intake can lead to significant harm, affecting vital organs and contributing to neurodegenerative disorders such as neuronal death, cognitive decline, behavioral disturbances, and memory impairment.[2] Ethanol poses an even greater risk to the developing brain, where concentrations as low as 0.5 g/L can induce apoptotic neurodegeneration.[3]

Prenatal exposure to ethanol is a well-established cause of fetal alcohol spectrum disorders (FASD), a leading contributor to global mortality and neurodevelopmental abnormalities.[4] FASD affects multiple critical brain regions-including the cerebellum, cerebral cortex, hippocampus, basal ganglia, amygdala, and thalamus-resulting in significant impairments in memory and learning.[5] To explore these effects further, experimental animal models have been used to study ethanol exposure during both prenatal and neonatal stages. Ethanol disrupts postnatal brain development by inducing apoptosis and neuronal death, leading to mental retardation and other adverse outcomes.[6] Various strategies have been explored to mitigate the harmful effects of ethanol during these vulnerable developmental periods.

Flavonoids are essential plant pigments, primarily responsible for the yellow coloration of flower petals, which helps attract pollinating insects. They play key roles in floral pigmentation, ultraviolet (UV) filtration, and symbiotic nitrogen fixation.[7] As biologically significant polyphenols, flavonoids are widely distributed in higher plants and are commonly present in the human diet. They exhibit a broad spectrum of pharmacological and biological activities, including antioxidant, anti-inflammatory, anti-allergic, and anticancer properties.[8] Certain flavonoids also possess antimicrobial properties against plant pathogens and may function as cell cycle regulators and chemical messengers. Dietary sources of flavonoids include onions, black and green tea, parsley, dark chocolate, blueberries, bananas, and citrus fruits.[9]

Flavonoids possess a 15-carbon skeleton comprising a heterocyclic ring and two phenyl rings. Over 5,000 naturally occurring flavonoids have been identified from various plant sources. Based on their chemical structure, flavonoids are categorized into several

classes, including anthocyanidins, anthoxanthins, flavanones, flavanonols, and flavans.[10] One such compound, 6-Hydroxyflavone, is a flavone derived from the leaves of Barleria prionitis. Known for its medicinal properties, it has demonstrated potential as a therapeutic agent for various neurological disorders. It exhibits antioxidant, anti-diabetic, and anti-inflammatory effects and is considered a promising candidate for managing anxiety-like disorders and preventing bone loss.[11]

Alcohol consumption is known to impair behavior, memory, and cognitive function.[12] Despite multiple pharmacological efforts to counteract alcohol-induced neurotoxicity, there remains a critical need to explore novel therapeutic interventions. This study was planned to evaluate the neuroprotective potential of 6-Hydroxyflavone through the activation of p-Akt in reducing ethanol-induced neurodegeneration in the developing brains of postnatal day 7 (PND-7) mice.

METHODS

This experimental study was conducted between March and June 2023, at Neuro Molecular Medicines Research Center (NMMRC), Peshawar in collaboration with Department of Biochemistry, Shaheed Benazir Bhutto Women University, Peshawar, Pakistan. All procedures involving animal handling, housing, and grouping were approved by the Institutional Animal Ethical Committee, comprising subject matter experts, vide Ref#: 11/2022 dated: 10/01/2023. A total of ten healthy adult albino mice (both male and female), aged 7-8 weeks and weighing approximately 32 grams, were procured from the Veterinary Research Institute, Peshawar, Pakistan.

Mice were randomly divided into five pairs and acclimatized to laboratory conditions, with access to food and water ad libitum and maintained on a 12/12-hour light/dark cycle at a controlled temperature of 25 ± 1°C. Following reproduction, the pups were kept with their mothers until postnatal day 7 (PND-7). The sample size was determined using the resource equation method, resulting in 20 PND-7 mice. These pups were randomly allocated into four groups (n=5 per group): Control, Ethanol (EtOH), Ethanol + 6-Hydroxyflavone (EtOH + 6-HF), and 6-Hydroxyflavone (6-HF) groups. All groups were housed separately in labeled cages (Biobase, China) at the NMMRC, Peshawar. The EtOH group received a subcutaneous injection of ethanol at 5 g/kg. The EtOH + 6-HF and 6-HF groups were administered 6-HF at a dose of 30 mg/kg following ethanol administration in the respective group. Four hours post-treatment, all PND-7 mice were sacrificed for laboratory analysis.

All the PND-7 mice were sacrificed at the end of treatment time as per method reported previously.[13] Mice were decapitated and the whole brain was carefully extracted. Brain was homogenized and tissue supernatant was collected and stored at -20oC till further analysis. Bio-Rad protein assay solution was used to quantify the brain homogenates. The homogenates were fractionated using SDS-PAGE (Model: BIO-RAD Mini PROTEAN System Cat#1658050, USA) with 10% polyacrylamide gel. After transfer, the membranes were blocked with 5% skimmed milk, incubated overnight at 4°C with primary antibodies such as anti-Bcl-2 (SC-7382), anti-BAX (SC-7480), anti-Cas-3 (SC-7272), anti-PARP-1 (SC-8007), and anti-p-Akt (SC-514032) of Santa Cruz Biotech, CA, USA. Enhanced chemiluminescence detection reagent was used for visualization according to the manufacturer's instructions. ImageJ software was used to perform the densitometry analysis of the bands in arbitrary units (A.U.) relative to the untreated control.

The 3 dimensional structure of p-AKT was retrieved from protein data bank (PDB) (http://www.rcsb.org).with PDBID: 6HHG. The structure of the ligand 6-Hydroxyflavone (6HF) (PubChem CID: 72279) was retrieved from PubChem database (https://pubchem.ncbi.nlm.nih.gov/).

Molecular docking of 6HF was performed with p-AKT to locate appropriate binding location Automated dockings were performed to locate the appropriate binding orientations using AutoDock4.2.[14] AutoGrid program was used to generate grid around experimentally defined binding cavity of p-AKT. Resulting docked complexes were clustered on the basis of energy with RMSD tolerance of 1.0 Å. HEX server (http://hex.loria.fr/). was used for Cross docking experiments. Detailed molecular interaction analysis of docked p-AKT-6HF complexes were performed using Chimera Discovery Studio.[15]

The original X-ray films from the Western blot analysis were scanned, and densitometric analysis of protein bands was conducted using ImageJ software. The integral optical density (IOD) of the protein bands was expressed in arbitrary units (A.Us) as mean ± standard error of the mean (SEM). Statistical significance was assessed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. A p-value of less than 0.05 ($p < 0.05$) was considered statistically significant.

RESULTS

6-HF activated p-Akt to reduce neurodegeneration against ethanol in PND-7 mice brain: Ethanol is the most common cause of inhibition of p-Akt protein activation. In the current study, significant differences ($p<0.001$) were observed in p-Akt protein expression in various experimental groups. Post hoc-tukey test revealed that ethanol significantly decreased ($p<0.001$) p-Akt protein in the brain homogenates of the EthOH group PND-7 mice. 6-HF administration, however significantly restored ($p<0.05$) p-Akt level in EthOH + 6-HF group mice as compared to EthOH group mice (Figure 1).

6-HF demonstrated successful amelioration of ethanol-induced apoptotic neurodegeneration in PND-7 mice: One way ANOVA showed significant differences in the expression levels of various apoptotic markers in the brain homogenates of PND-7 mice brain. Post-hoc tukey analysis revealed that ethanol caused significant increase ($p<0.001$) in the BAX, Caspase-3 and PARP-1 protein expression along with significant

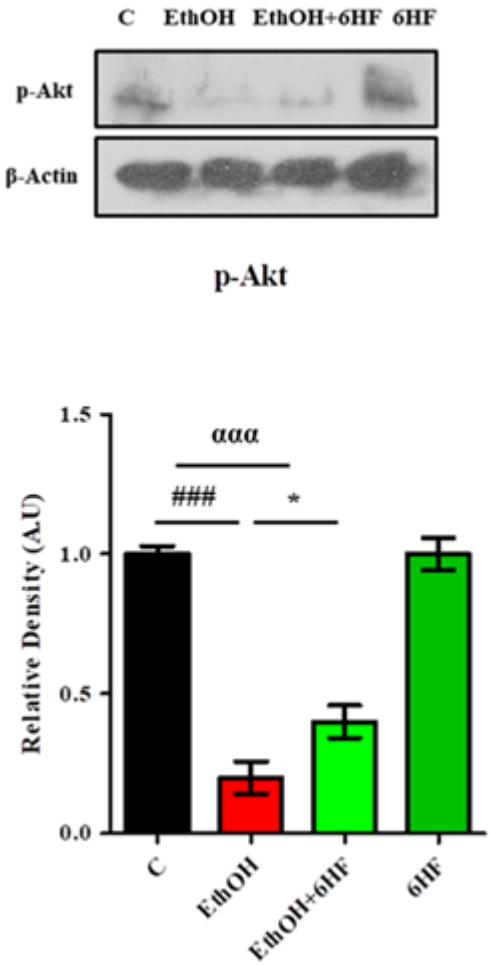

Figure 1: 6-HF demonstrated substantial amelioration in the expression level of p-Akt protein in the PND-7 mice brains of the EthOH+6-HF group. Shown are the Western blot results of p-Akt expression level, along with their bar-chart representation in various experimental mice groups respectively. β-Actin was used as a loading control'The results were determined using Image J software and bar-chart indicate mean in A.U ± SEM. Significance of one way ANOVA is expressed as α, significance of control vs EthOH is expressed as #, while significance of EthOH vs EthOH+6-HF is expressedas*. Significance: ***, ###p<0.001.

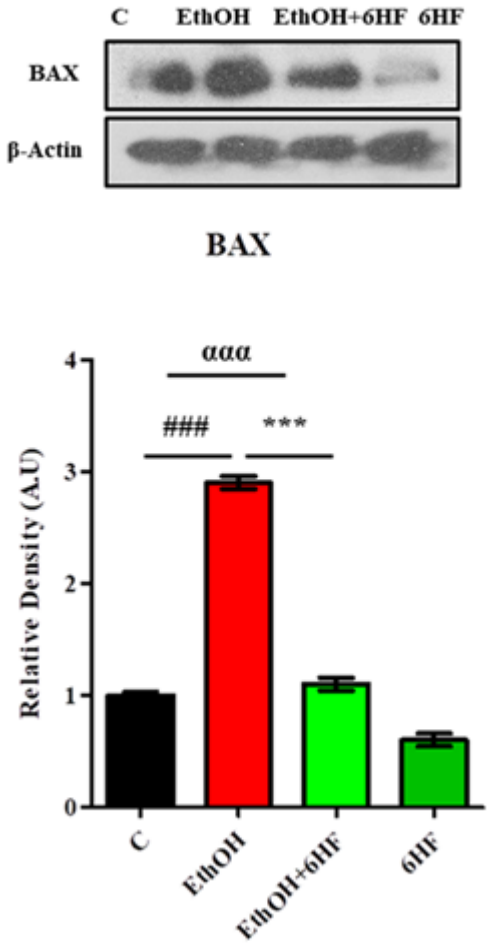

Figure 2: 6-HF successfully ameliorated the expression level of BAX protein in the PND-7 mice brains of the EthOH+6-HF group. Shown are the Western blot results of BAX expression level, along with their bar-chart representation in various experimental mice groups respectively. β-Actin was used as a loading control. The results were determined using Image J software and bar-chart indicate mean in A.U±SEM. Significance of one way ANOVA is expressed as α, significance of control vs EthOH is expressed as #, while significance of EthOH vs EthOH+6-HF is expressed as*. Significance: ***, ###p<0.001.

decrease ($p<0.001$) in the BCL-2 expression levels in EthOH group mice brain. However, 6-HF administration to the Ethanol group mice successfully retrieved the damage by significantly rectified ($p<0.001$) the BAX (Figure 2), Bcl-2 (Figure 3), Caspase-3 (Figure 4) and PARP-1 protein expression (Figure 5) in the brain homogenates of EthOH +6-HF group PND-7 mice as compared to the EthOH group mice.

Molecular docking: To understand the binding interaction of 6HF with p-AKT detailed interaction analysis was performed to analyze the critical residues of p-AKT involved in binding with 6HF. Trp80 of p-AKT made pi-staking interactions with 6-hydroxyphenyl group of 6HF. While Asn53 made hydrogen bond with 6-hydroxy moiety of 6HF. Leu210, Leu264, Lys268, Val270 and Asp292 of p-AKT were involved in hydrophobic interaction with 6-hydroxyphenyl and benzopyrone group of 6HF to keep well within the binding cavity (Figure 6).

DISCUSSION

This study evaluated the neuroprotective role of 6-HF in ethanol-induced neurodegeneration in PND-7 mice, focusing on p-Akt activation. Ethanol exposure significantly elevated p-Akt, BAX, BCL-2, Caspase-3, and PARP-1 levels ($p<0.001$), indicating increased apoptotic activity. However, co-administration of 6-HF significantly

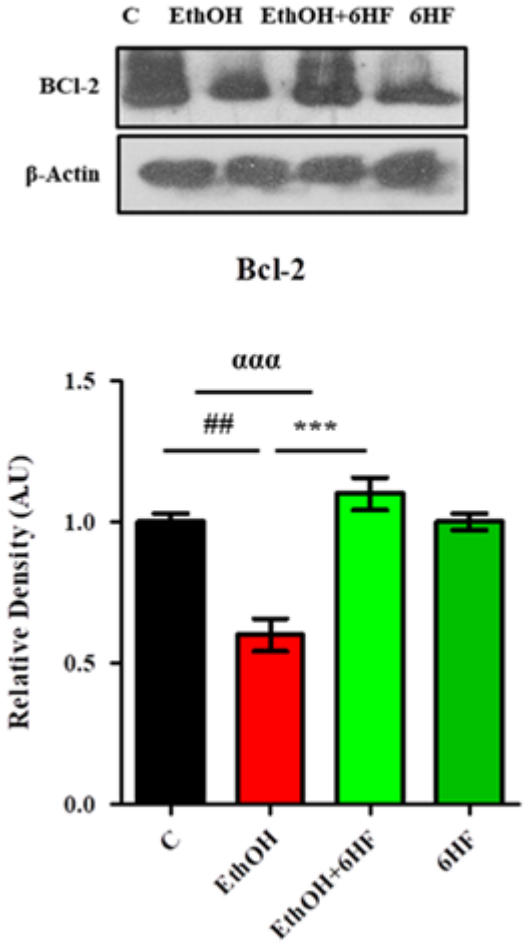

Figure 3: 6-HF significantly rectified the expression level of BCL-2 protein in the PND-7 mice brains of the EthOH+6-HF group. Shown are the Western blotresults of BCL-2 expression level, along with their bar-chart representation in various experimental mice groups respe-ctively. β-Actin was used as a loadingcontrol. The results were determined using Image J software and bar-chart indicate mean in A.U ± SEM. Significance of oneway ANOVA is expressed as α, significance of control vsEthOH is expressed as #, while significance of EthOH vs EthOH+6-HF is expressed as*. Significance: ***, ###p<0.001.

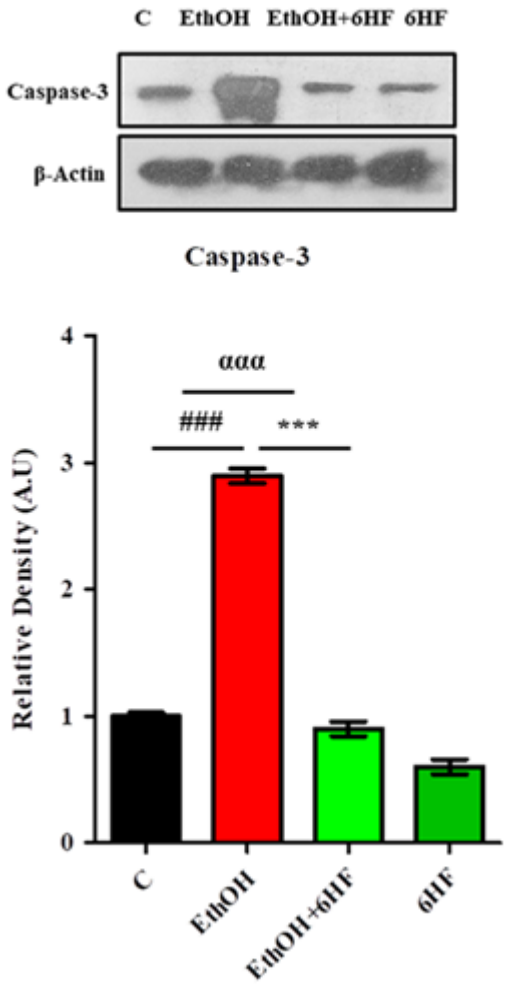

Figure 4: 6-HF demonstrated substantial amelioration in the expressionlevel of Caspase-3 protein in the PND-7 mice brains of the EthOH+6-HFgroup. Shown are the Western blot results of Caspase-3 expression level, along with their bar-chart representation in various experimental mice groups respectively. β-Actin was used as a loading control. The results were determined using Image J softwareand bar-chart indicate mean in A.U ± SEM. Significance of one way ANOVA is expressed as α, significance of control vs EthOH is expressed as #, while significance of EthOH vs EthOH+6-HF is expressed as*. Significance: ***, ###p<0.001.

enhanced p-Akt expression ($p<0.05$) and markedly reduced pro-apoptotic markers ($p<0.001$) compared to the ethanol group, suggesting that 6-HF mitigates ethanol-induced neuronal damage through p-Akt–mediated survival signaling.

The use of animal models has become indispensable for investigating the pathological and clinical outcomes of various diseases, designing therapeutic interventions, and translating them from bench to bed.[16] The central nervous system is extremely sensitive to alcohol during development and the periods of vulnerability are temporally well defined. The time frames of vulnerability are different for different neuronal populations. Previous studies revealed that phosphorylated Akt play a major role against apoptosis.[17] Ethanol is a well-known agent to induce an increase in BAX proteins, BCL-2 protein inhibition and ultimately BAX/BCL-2 ratio upregulation in PND-7 pup's brain.[18] Bax-knockout mice showed ethanol-induced apoptosis in the developing brain through upregulation of BAX/BCL-2 ratio. A decrease in the BCL-2 level in cells leads to translocation of Bax to mitochondria, disruption of their membranes, release of cytochrome C and activation of caspase-3. All these events indicate that alcohol induces apoptosis in the developing brain via intrinsic (mitochondrial-mediated) apoptotic pathways.[19]

The findings of the current study align

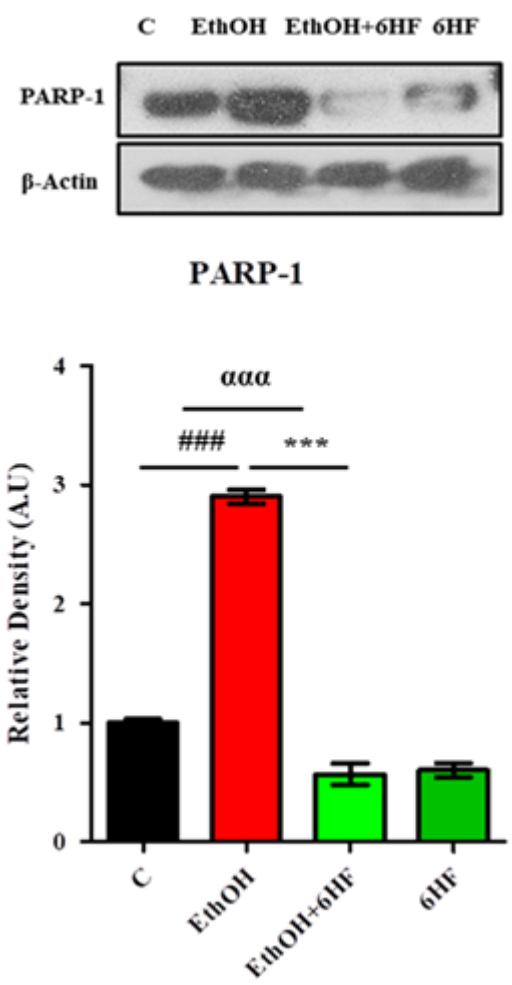

Figure 5: 6-HF successfully ameliorated the expression level of PARP-1 protein in the PND-7 mice brains of the EthOH+6-HF group. Shown are the Western blot results of PARP-1 expression level, along with their bar-chart representation in various experimental mice groups respectively. β-Actin was used as a loading control. The results were determined using Image J software and bar-chart indicate mean in A.U±SEM. Significance of one way ANOVA is expressed as α, significance of control vs EthOH is expressed as #, while significance of EthOH vs EthOH+6-HF is expressed as*. Significance: ***, ###p<0.001.

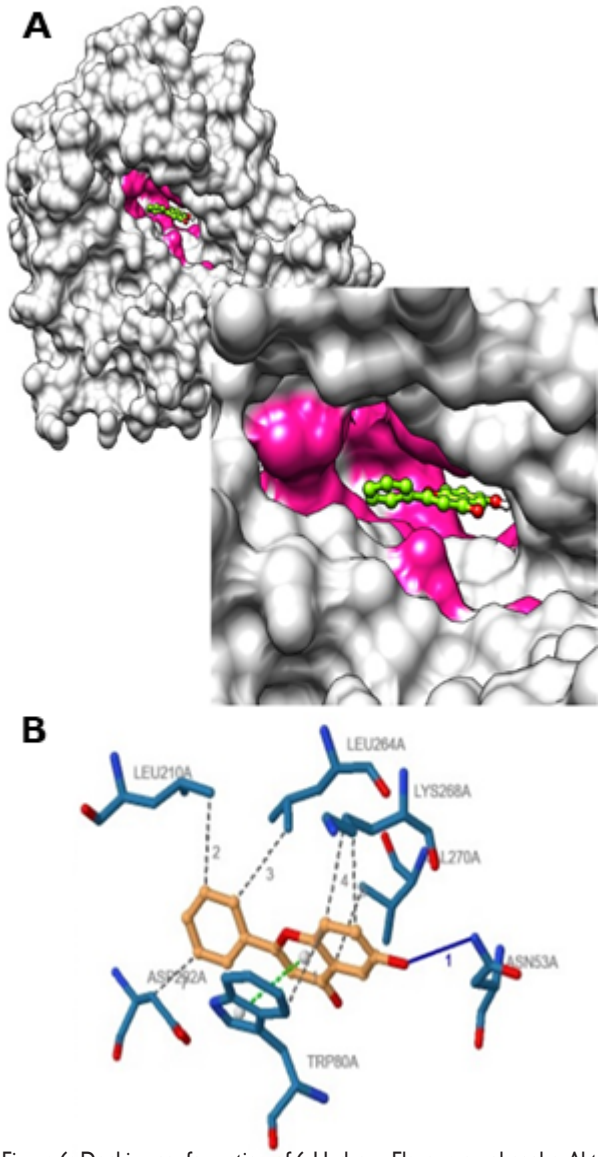

Figure 6: Docking conformation of 6-Hydroxy Flavone on phospho-Akt (A) 3D binding mode and interaction of 6-Hydroxy Flavone with p-Akt. Hydrophobic representation of p-Akt protein with bound 6-HF shown in zoom view. (B) 3D binding mode of p-Akt protein residues with 6-HF (shown in yellow) in stick model.

with previously published literature, which demonstrates that ethanol exposure leads to upregulation of BAX, suppression of BCL-2, and an elevated BAX/Bcl-2 ratio-indicating its neurotoxic effects on the immature brain.[20] Furthermore, studies show that taurine can restore BCL-2 levels and protect against apoptosis. [21] Given that ethanol-induced apoptosis is largely Bax-dependent, the observed restoration of BCL-2 in our study suggests a potential mechanism through which apoptosis was mitigated.

Caspase-3 is one of the important executioners of apoptosis.[22] The activation of caspase-3 plays an important part in the induction of apoptosis. Elegant studies reveal that inhibiting caspase-3 with an agent can reduce the deleterious effects of ethanol on the immature brain.[23] These authors have observed that by using Anthocyanins, melatonin and even glycine can exerts neuroprotection against ethanol-induced neurodegeneration via caspase-3 inhibition respectively.[24] Our western blot results reveal that a single injection of ethanol after four hours significantly induced an increase in the expression of capase-3 protein. The treatment with 6-HF slightly after the administration of ethanol significantly inhibited the caspase-3 protein expressions.

Ethanol has been shown to elevate the expression of PARP-1 protein,[25] and several studies have confirmed that ethanol intoxication induces DNA fragmentation in the brains of PND-7 mice.[26,27] Moreover, some natural

compounds have demonstrated neuroprotective effects against ethanol-induced DNA damage.[27] Consistent with these findings, our study observed a significant increase in PARP-1 protein expression following ethanol administration, while treatment with 6-HF effectively reduced PARP-1 levels in the brain homogenates of PND-7 mice.

CONCLUSION

This study demonstrated that 6-HF significantly attenuates ethanol-induced apoptotic neurodegeneration in the developing brain of PND-7 mice, likely through activation of the p-Akt signaling pathway. The treatment with 6-HF modulated key apoptotic markers including BAX, BCL-2, Caspase-3, and PARP-1, indicating its neuroprotective potential. These findings suggest that 6-HF may serve as a promising therapeutic candidate for mitigating ethanol-induced neurotoxicity. However, further in-depth studies are required to elucidate the precise molecular mechanisms and to explore its efficacy in other models of neurodevelopmental and neurodegenerative disorders.

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AUTHORS' CONTRIBUTION

Following authors have made substantial contributions to the manuscript as under:

MT: Acquisition, analysis and interpretation of data, drafting the manuscript, approval of the final version to be published

RJ & SAS: Conception and study design, acquisition, analysis and interpretation of data, critical review, approval of the final version to be published

NB: Acquisition, analysis and interpretation of data, critical review, approval of the final version to be published

HN: Analysis and interpretation of data, critical review, approval of the final version to be published

AJ: Acquisition, analysis and interpretation of data, drafting the manuscript, approval of the final version to be published

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

CONFLICT OF INTEREST

Authors declared no conflict of interest, whether financial or otherwise, that could influence the integrity, objectivity, or validity of their research work.

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DATA SHARING STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request