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Neuroprotective effect of Naringin against organophosphorus compound induced Alzheimer disease

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Abstract

Organophosphorus compounds (OPCs), such as Malathion, are widely used pesticides known to induce oxidative stress, contributing to neurodegenerative diseases like Alzheimer's disease. Naringin, a flavonoid with antioxidant and neuroprotective properties, was studied for its potential to protect against Malathion-induced neurotoxicity. In this study, in-silico docking analysis was performed to assess Naringin's effect on acetylcholinesterase (AChE) activity. In-vivo studies examined Naringin's impact on behavior changes examination and oxidative stress markers, such as superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA), in Malathion- exposed rats. Naringin was administered at low (80 mg/kg) and high (160 mg/kg) doses, while diazepam was used as a positive control. In-silico analysis revealed that Naringin strongly binds to AChE, suggesting its neuroprotective potential. In-vivo results demonstrated that Naringin improved cognitive function in behavioral tests, including the Novel Object Recognition (NOR) and Morris Water Maze (MWM). Additionally, Naringin treatment significantly increased SOD and CAT activities while reducing MDA levels, indicating reduced oxidative stress, particularly at the higher dose. Naringin also restored AChE activity in a dose-dependent manner. Diazepam showed neuroprotective effects, but Naringin, especially at the higher dose, exhibited comparable or superior efficacy. These findings suggest that Naringin's antioxidant and AChE inhibitory properties may protect against Malathion-induced neurotoxicity, making it a promising therapeutic candidate for neurodegenerative diseases.

Keywords: Naringin, Acetylcholinesterase (AChE) inhibition, Malathion, Neuroprotection, Oxidative stress, Organophosphorus compounds

1. Introduction

Organophosphorus compounds (OPCs), such as Malathion, have been extensively used in agriculture and pest control due to their potent insecticidal properties. Despite their widespread use, OPCs are known to pose significant risks to human health, particularly due to their neurotoxic effects (Abou-Donia, 2003). One of the most concerning aspects of OPC exposure is its potential link to the development of neurodegenerative diseases, such as Alzheimer's disease (AD). Alzheimer's disease, a progressive neurodegenerative disorder characterized by memory loss, cognitive decline, and behavioral disturbances, has been associated with environmental toxins, including OPCs. Malathion, an organophosphate insecticide, is particularly implicated in this regard due to its ability to induce oxidative stress, disrupt cholinergic signaling, and promote

neuroinflammation. The neurotoxic effects of Malathion are primarily mediated through the inhibition of acetylcholinesterase (AChE), an enzyme crucial for breaking down the neurotransmitter acetylcholine in the synaptic cleft. This inhibition leads to an accumulation of acetylcholine, resulting in prolonged cholinergic activity, neuronal dysfunction, and, ultimately, neurodegeneration (Fulton and Key, 2001; Marrero-Rosado et al., 2021; Segall et al., 2003).

Oxidative stress, which involves an imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defenses, is a major contributing factor in Malathion-induced neurotoxicity. ROS, such as superoxide radicals and hydrogen peroxide, can cause significant damage to cellular components, including lipids, proteins, and DNA, leading to cellular dysfunction and death. In the brain, oxidative damage is particularly harmful due to the high metabolic rate and relatively low antioxidant capacity of neuronal cells (Akhgari et al., 2003; Bashir et al., 2021). The accumulation of ROS in the brain has been linked to the pathogenesis of various neurodegenerative diseases, including Alzheimer's disease, where oxidative stress is thought to play a key role in the formation of amyloid-beta plaques and tau tangles, hallmark features of the disease. Moreover, oxidative stress can exacerbate neuroinflammation, further contributing to neuronal damage and cognitive decline. Given these mechanisms, mitigating oxidative stress and restoring AChE activity represent important therapeutic targets for preventing or slowing the progression of neurodegenerative diseases associated with OPC exposure (Aroniadou-Anderjaska et al., 2023; Bashir et al., 2021; Farkhondeh et al., 2020).

Naringin, a flavonoid abundantly found in citrus fruits such as grapefruits, has garnered considerable attention in recent years for its potent antioxidant and neuroprotective properties. Flavonoids, as a class of polyphenolic compounds, have been widely studied for their ability to scavenge free radicals, reduce oxidative damage, and modulate various cellular signaling pathways involved in inflammation and apoptosis. Naringin, in particular, has demonstrated significant neuroprotective effects in various models of neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, and ischemic stroke (Ben-Azu et al., 2019; Dashputre et al., 2023). These protective effects are largely attributed to Naringin's ability to enhance the activity of endogenous antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT), while reducing the levels of oxidative stress markers, such as malondialdehyde (MDA), a lipid peroxidation product. Additionally, Naringin has been shown to inhibit the activity of AChE, thereby helping to restore normal cholinergic signaling in the brain. This dual action of Naringin— its antioxidant and anti-cholinesterase effects—makes it a promising candidate for counteracting the neurotoxic effects of OPCs like Malathion (Balachandran et al., 2023; Golechha et al., 2011; Yu et al., 2022).

Due to multi-targeted and therapeutic effect of naringin, this study was designed to explore its protective effects against Malathion-induced neurotoxicity, both in silico and in vivo. The first objective of the study was to evaluate the binding affinity of Naringin to AChE through in-silico docking analysis (Kaur and Prakash, 2020; Maratha and Mahadevan, 2012; Oladapo et al., 2021). Molecular docking is a widely used computational technique to predict the interaction between small molecules and target proteins, providing valuable insights into the potential efficacy of drug candidates (Zahiruddin et al., 2020). By examining the binding interactions between Naringin and AChE, we sought to determine whether Naringin could effectively inhibit the enzyme, thereby reducing acetylcholine accumulation and ameliorating the cholinergic dysfunction associated with OPC exposure. In this context, a strong binding affinity would suggest that Naringin could act as an AChE inhibitor, offering neuroprotective benefits against Malathion-induced neurodegeneration.

Taking these facts int consideration, this study aimed to explore the neuroprotective effects of Naringin against Malathion-induced neurotoxicity through both in-silico and in-vivo approaches. By combining molecular docking analysis with behavioral, biochemical, and enzymatic

assessments, we sought to provide a comprehensive understanding of Naringin's therapeutic potential. The findings from this study could contribute to the development of novel strategies for preventing or treating neurodegenerative diseases associated with OPC exposure, such as Alzheimer's disease. Moreover, the outcome of the study explores the broader implications for the use of Naringin and other flavonoids as

neuroprotective agents in various models of neurodegeneration, highlighting the importance of dietary antioxidants in maintaining brain health and preventing cognitive decline.

2. Material and methods

2.1. Chemical and reagents

Malathion, Naringin (95% Pure, B. No. 0202) were purchased form Chemika Biochemika , 1% pentobarbital sodium, 10% formalin were purchased from Sisco Research Laboratories Pvt. Ltd. (SRL) – India. Laboratory animals feed was purchased form VRK Nutritional solution, Maharastra (Batch No. 009).

2.2. In-silico docking analysis

2.2.1. Selection and Preparation of Protein

For selection of protein for molecular docking analysis, scientific literature was retrieved for determination of reported protein identification code for neuroprotective activity. The 3D crystal structure of acetylcholinesterase (PDB ID) was obtained from the Protein Data Bank (PDB ID: 6ZWE_ https://www.rcsb.org/structure/6ZWE). The protein specification were found as Method: X-RAY DIFFRACTION, Resolution: 3.00 Å, R-Value Free: 0.242, R-Value Work: 0.198 and R- Value Observed: 0.199. during preparation of the protein, each aspect of the protein was removed form water molecules and hydrogen atoms were added. The protein was optimized by assigning charges using Autodock Tools and non-polar hydrogens were merged (Gnanaraj et al., 2022).

2.2.2. Ligand Preparation

3D structure of the structure of Naringin was downloaded from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). The ligand was acquired in SDF format that was converted in to PDB format using discovery studio visualizer (2021). The ligand was prepared by adding charges, optimizing the geometry, and converting it into the PDBQT format using AutoDock Tools (Salar et al., 2023).

2.2.3. Molecular Docking Studies

AutoDock Vina was employed for the molecular docking process. The grid box was centered on the active site of AChE, and parameters were set. The docking simulation predicted the binding affinity of Naringin with AChE. The Command Prompt and precondition were used to process the docking analysis. The docking results were analyzed using Discovery Studio Visualizer to observe key interactions such as hydrogen bonds, π-alkyl stacking, and van der Waals forces within the active site. During analysis, heatatom from the protein was removed and specified the protein and

ligand interaction. Hydrogen bonding confirmer was achieved on the processed protein and 3D and 2D structure of the interaction was recorded (Gautam, 2022; Gautam et al., 2021; Khan et al., 2022; Misra et al., 2023).

2.3. In-vivo Studies

For in-vivo study, 42 male Wistar rats (120 ± 10 g), 5 to 7 weeks old were used in this examination. The animals were procured from the Central Animal Facility at the All India Institute of Medical Sciences (AIIMS), New Delhi with the approval no. 48/Animal/BS/CAF/22-23 and CPCSEA No. 1204/PO/RE/S/08/CPCSEA. They were housed under controlled environmental conditions with a temperature of 20 ± 2˚C and humidity of 50 ± 10%. The study was approved by the ethical committee of the Animal House, Kharvel Subharti College of Pharmacy (KSCP), Meerut. All procedures followed ethical guidelines outlined by the National Institutes of Health (NIH) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) for the care and use of laboratory animals (Gnanaraj et al., 2022).

2.3.1. Experimental model and treatment schedule

In the study, male Wistar rats were divided into four groups to evaluate the neuroprotective effects of naringin against malathion-induced neurodegeneration and oxidative stress. Group A, serving as the control, was provided with a regular diet and distilled water for 21 days. Group B was administered malathion (50% concentration, 0.02 mg/ml in ethanol) orally for 21 days to induce neurodegeneration. Group C received malathion as well as a low dose of naringin (80 mg/kg in methanol) orally for 21 days to investigate the neuroprotective effects of the lower dose. Group D was given malathion along with a high dose of naringin (160 mg/kg in methanol) orally over the same period to assess the effects of the higher dose of naringin on neuroprotection. Group E received Diazepam (7.5 mg/kg) as positive control.

The control group (Group A) was established to validate the success of the model creation via comparing to the treatment models. Group B served to induce memory impairment in rats through malathion exposure. Groups C and D were included to examine the protective action of low and high doses of naringin on malathion-induced neurodegeneration. Malathion treatment was administered to Groups B, C, D and E continuously for three weeks. Following this, Groups C and D were administered their respective doses of naringin for an additional week to determine the impact on memory restoration. Behavioral tests were then conducted to assess cognitive function

and evaluate naringin's neuroprotective efficacy against the malathion-induced cognitive decline. The body weight of all the animals was measured before and posttreatment. The brain tissues were collected for the assessment of oxidative markers such as malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), and acetylcholinesterase as per the standard protocol (Ekici et al., 2014; Gahlot and Yadav, 2021; Gaurav et al., 2022).

2.3.2. New object recognition

The New Object Recognition (NOR) test was designed to evaluate memory and cognitive function in rats as part of this study. The test was divided into two phases: the training phase and the testing phase. During the training phase, rats were placed in a test box with their backs facing two identical objects positioned at one end. They were allowed five minutes to explore these objects freely. After an hour, one of the two identical objects were replaced with a novel object, while the other remained unchanged. In the testing phase, the rats were returned to the test box and again given five minutes to explore. The recognition index was calculated to assess memory performance, using the formula (TN-TF)/(Tn+Tf), where Tn represents the time spent exploring the new object and Tf denotes the time spent on the familiar object. This index provided a quantitative measure of the rats' ability to recognize and differentiate between the novel and familiar objects, helping to evaluate memory retention and cognitive function (Gnanaraj et al., 2022).

2.3.3. Morris Water Maze

The Morris Water Maze (MWM) test was employed to assess the spatial learning and memory of the rodents in this study. The test involved five consecutive days of training during which each rat was placed in a circular pool filled with opaque water. A hidden platform was submerged just below the water surface, and the rodents were required to locate it using spatial cues. The escape latency, defined as the time taken to find the platform within 90 seconds, was recorded. If a rat found the platform in under 90 seconds, it was allowed to stay on it for 30 seconds. In cases where the platform was not located within the time limit, the rats were gently placed on the platform for 30 seconds to improve memory acquisition. On the sixth day, the platform was removed to evaluate memory retention. Parameters including the number of platform crossings, time spent in the target quadrant, and distance traveled in search of the platform were measured over a 90-second trial. These outcomes provided key insights into the rats' ability to remember the platform's location, assessing both short- and long-term spatial memory (Lee et al., 2016) and (Gnanaraj et al., 2022).

2.3.4. In-vivo anti-oxidant activity

The antioxidant potential of naringin on the brain against malathion induced oxidative stress was assessed by isolating the cortex and hippocampus regions from brain. The extracted brain tissues were meticulously homogenized in PBS buffer (pH 7.4). The tissue homogenate was subjected to centrifugation at 3000 g for 15

minutes at 4°C. The supernatant obtained after centrifugation was carefully separated for further analysis. The levels of catalase (CAT), superoxide dismutase (SOD), and malondialdehyde (MDA) in the supernatant were determined using an ELISA reader as per the reference protocol (Gaurav et al., 2022).

2.3.5. Acetylcholinesterase Inhibition activity

The acetylcholinesterase (AChE) activity assay was conducted using the following method with some modification (Lee et al., 2016). The brain tissue was quickly extracted from rat and homogenized in a sodium phosphate buffer solution. For the assay, 33 µL of the supernatant was combined with 470 µL of phosphate buffer (pH 8) and 167 µL of 5,5′-dithiobis(2-nitrobenzoic acid) (3 mM), and the mixture was incubated at 37°C for 5 minutes. Subsequently, 280 µL of acetylthiocholine iodide (1 mM) was added, and the reaction was allowed to proceed for another 5 minutes at 37°C. AChE activity was then quantified by measuring absorbance at 412 nm with a spectrophotometer (Gahlot and Yadav, 2021).

2.3.6. Histopathological studies

For the histopathological studies, brain hippocampi tissue samples were collected from all experimental groups and fixed in 10% formalin. These samples were then embedded in paraffin and sectioned at 5 µm thickness. Sections were stained with hematoxylin and eosin (H&E) to evaluate general neuronal architecture and cellular damage. Thereafter, each section were evaluated under compound microscope for examination of histological changes among different treated group (Gahlot and Yadav, 2021).

2.4. Statistical analysis

In this study, the data Data are expressed as Mean ± SD and analyzed using One-Way ANOVA, followed by Tukey's test. Significance level was determined at $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***). All the readings or experiments were measured or done in triplicate or multiplate (n=3/6) for robust evaluation of significant effect of the experimental data. Graph Pad Prism version-5 was used for the statistical analysis.

3. Results

3.1. In-silico docking analysis

In-silico molecular docking was conducted to determine the interaction between acetylcholinesterase (AChE) protein and the flavonoid as Naringin, analyzed through both 3D (A, B) and 2D (D) visualizations. This analysis was conducted at two different sites of the protein namely site 1 and site 2. In the 3D views (A, B), the protein-ligand binding site is shown with surface representation highlighting hydrogen bond acceptor (green) and donor (magenta) regions. Naringin is bound within the active site of AChE, interacting with key residues that contribute to the inhibition of the enzyme's activity, which is crucial for acetylcholine breakdown.

Naringin showed bonding patterns, including van der Waals forces, conventional hydrogen bonds, and pi-stacking interactions. During interpretation analysis, it was found that Naringin's hydroxyl groups form hydrogen bonds with residues like HIS-405, ALA-237, and THR-238, contributing to stable binding. Pi-alkyl and pi-pi T-shaped interactions between Naringin and aromatic amino acids (e.g., TRP-236) further stabilize the complex.

Biologically, these interactions suggest that Naringin inhibits AChE by showing significant interaction into its active site, potentially improving acetylcholine availability, which could counter neurodegenerative conditions like Alzheimer's disease by enhancing cholinergic signaling. The docking analysis reveals a promising role for Naringin as an AChE inhibitor, complementing its antioxidant properties.

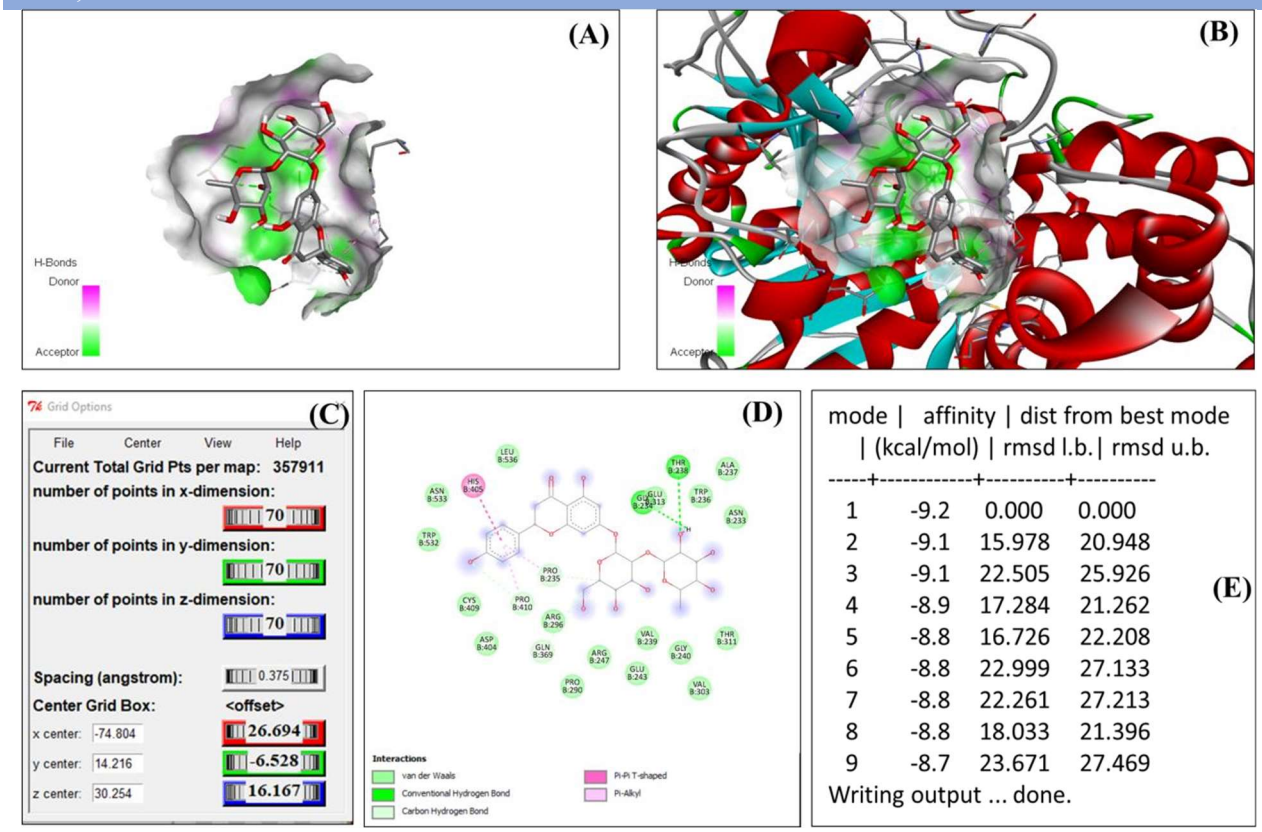


Figure 1: In-silico docking analysis of the acetylcholinesterase (AChE) protein and Naringin at site 1. The figure showed 2D ligand interaction with protein (Figure A), 3D interaction of the protein with ligand (Figure B), Grid boxing targets of the protein (Figure C), 2D interaction of the protein with ligand with conventional hydrogen bonding (Figure D) and the binding energy score of the ligand with protein in form of RMSD L.B and RMSD U.B.

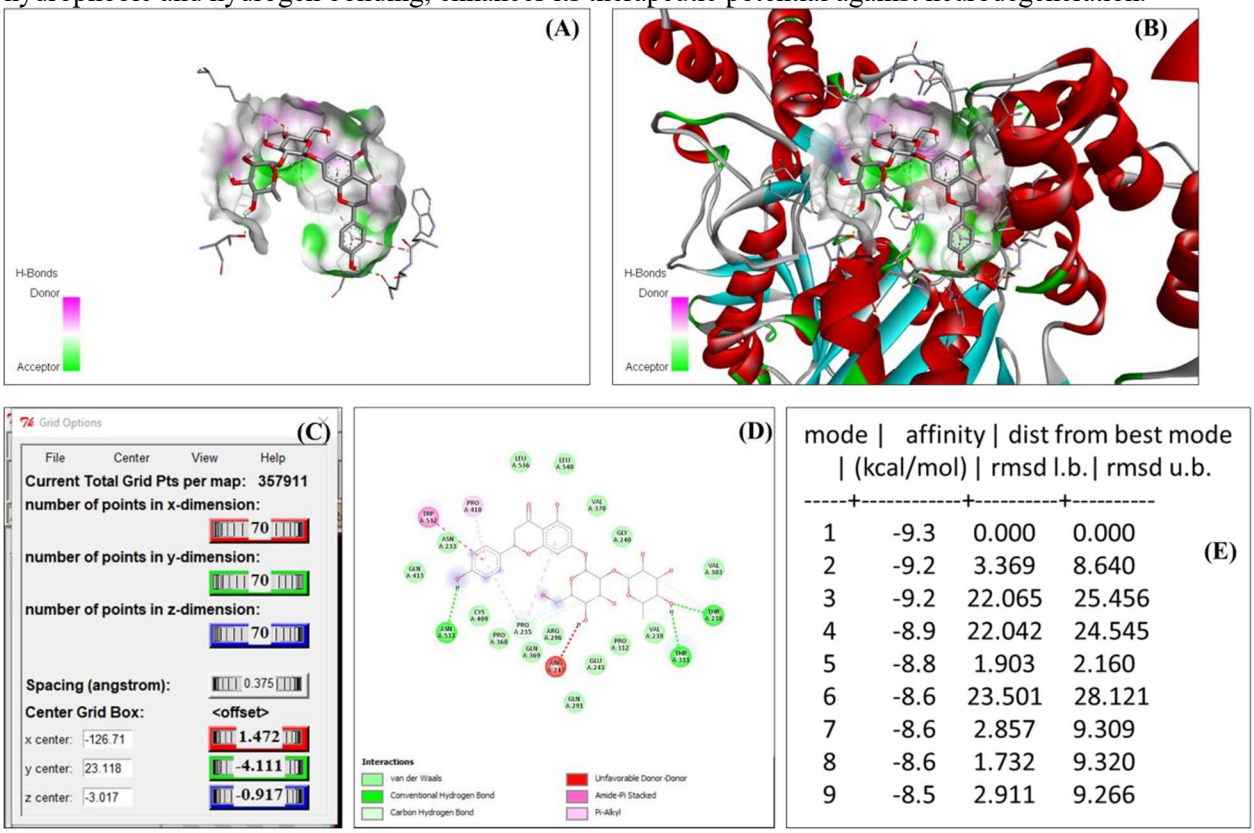
The in-silico docking analysis between acetylcholinesterase (AChE) and Naringin at site 2 was conducted and showed promising insights into their molecular interaction, specifically at binding site 2. The 2D interaction plot (Figure 2A) highlights the conventional hydrogen bonds formed between the ligand (Naringin) and key residues in AChE, enhancing the binding affinity and stability. The 3D visualization (Figure 2B) provides a comprehensive view of the interaction within the active site of the enzyme, demonstrating a close fit between Naringin and the hydrophobic core of the protein. Hydrogen bond donors and acceptors, shown in pink and green, respectively, indicate significant stabilization through multiple interactions. Figure 2C represents the grid parameters used to define the docking region, ensuring precision in positioning. The 2D interaction map (Figure 2D) shows that key residues such as HIS (His405), TRP (Trp236), and

PRO (Pro296) form critical interactions with Naringin. HIS engages in pi-pi stacking, enhancing the stability of the ligand within the binding pocket, while TRP supports van der Waals interactions, which are crucial for the hydrophobic environment. The hydrogen bonds, notably with PRO, further stabilize the ligand within the active site. The combination of these interactions results in a lower pocket energy bundle, signifying a strong, energetically favorable binding, crucial for AChE inhibition.

From a biological perspective, these interactions suggest that Naringin could effectively inhibit AChE by occupying the active site and interfering with its enzymatic activity. This mechanism aligns with therapeutic goals in neurodegenerative disorders like Alzheimer's disease, where AChE inhibition is a common strategy to boost acetylcholine levels and mitigate cognitive decline. The strong binding energy score, reflected in the RMSD values, suggests robust docking accuracy, confirming Naringin's potential as an acetylcholinesterase

inhibitor.

Moreover, the docking outcomes suggest that Naringin's multi-residue binding, particularly through hydrophobic and hydrogen bonding, enhances its therapeutic potential against neurodegeneration.



mode	affinity (kcal/mol)	dist from best mode rmsd l.b.	rmsd u.b.
1	-9.3	0.000	0.000
2	-9.2	3.369	8.640
3	-9.2	22.065	25.456
4	-8.9	22.042	24.545
5	-8.8	1.903	2.160
6	-8.6	23.501	28.121
7	-8.6	2.857	9.309
8	-8.6	1.732	9.320
9	-8.5	2.911	9.266

Figure 2: In-silico docking analysis of the acetylcholinesterase (AChE) protein and Naringin at site 2. The figure showed 2D ligand interaction with protein (Figure A), 3D interaction of the protein with ligand (Figure B), Grid boxing targets of the protein (Figure C), 2D interaction of the protein with ligand with conventional hydrogen bonding (Figure D) and the binding energy score of the ligand with protein in form of RMSD L.B and RMSD U.B.

3.2. In-vivo studies

3.2.1. New object recognition

The New Object Recognition (NOR) test revealed differences in memory and cognitive function across the treatment groups. Rats in Group A (normal control) exhibited a high recognition index, showing strong memory retention. Group B (malathion-treated group) displayed a significant reduction in the recognition index, indicating memory impairment likely caused by neurotoxic oxidative stress due to malathion exposure.

Group C (Malathion + Naringin Low Dose) demonstrated a moderate improvement in recognition index compared to Group B, suggesting that the lower dose of naringin partially mitigated memory deficits. However, the most notable improvement was observed in Group D (Malathion + Naringin

High Dose), where rats exhibited a near-normal recognition index, indicating a strong neuroprotective effect of the high dose of naringin in combating malathion-induced cognitive decline. Group E (diazepam-treated) also showed improved memory but was less effective than high-dose naringin. Moreover, this study indicates that naringin, particularly at a high dose, has a significant potential to improve memory and cognitive function in rats exposed to oxidative stress, likely through its antioxidant properties.

Table 1: Recognition Index observed among the different treated groups.

Group	Treatment	Recognition Index
Group A	Control	High
Group B	Malathion	Low
Group C	MNLD	Moderate
Group D	MNHD	High
Group E	Diazepam	Moderate-High

3.2.2. Morris Water Maze

The Morris Water Maze (MWM) test results demonstrated distinct differences in spatial learning and memory retention across the treatment groups. In Group A (normal control), the rats showed rapid learning with decreased escape latency over the five-day training period, and strong memory retention was evident on the probe day, as these rats spent significantly more time in the target quadrant and crossed the platform location frequently. In contrast, Group B (malathion-treated) showed a marked increase in escape latency during the training days, indicating impaired learning and memory acquisition, likely due to neurotoxic effects from malathion-induced oxidative stress. These rats exhibited poor performance on the probe day, with fewer platform crossings and less time spent in the target quadrant.

Group C (Malathion + Naringin Low Dose) showed a moderate improvement in performance compared to Group B. Although the escape latency remained elevated, the rats demonstrated better memory retention than the malathion group, spending more time in the target quadrant and

crossing the platform location more frequently. Group D (Malathion + Naringin High Dose) showed significant recovery, with escape latencies similar to those in the normal control group. On the probe day, these rats displayed strong memory retention, as indicated by increased time in the target quadrant and a higher number of platform crossings, comparable to Group A. These results suggest that naringin, particularly at a higher dose, effectively mitigated malathion-induced spatial memory impairments, likely due to its antioxidant and neuroprotective properties.

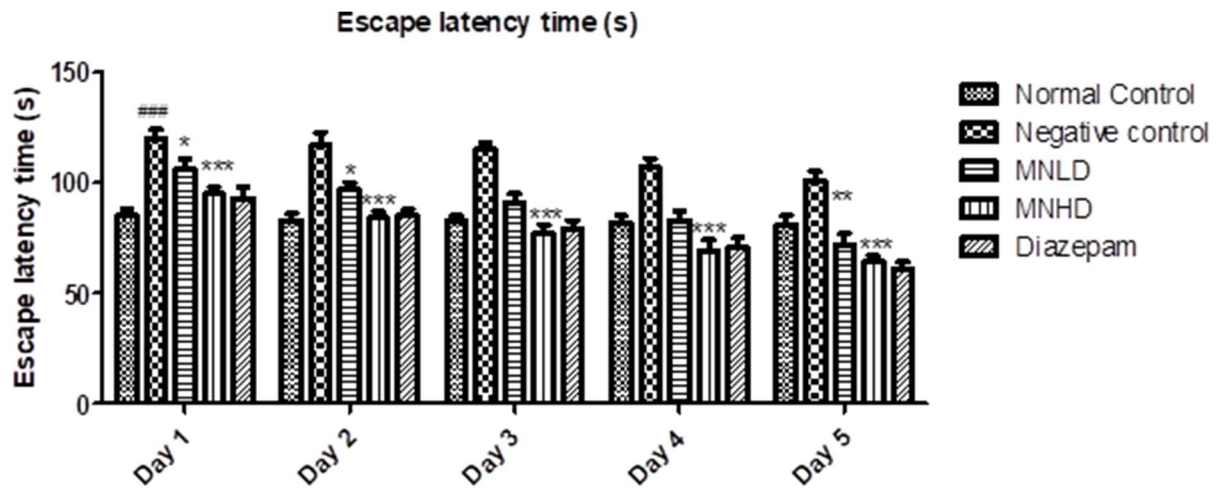


Figure 3: Estimation of escape latency time of animals treated in different group. Data are expressed as Mean ± SD and analyzed using One-Way ANOVA, followed by Tukey's test. Significance was determined at $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***).

In the study, the mean swimming distance and time spent in the target quadrant as parameters to assess the effect of Naringin in counteracting malathion-induced neurodegeneration. The negative control group (malathion-treated) shows significantly longer swimming distances and reduced time in the target quadrant, reflecting impaired spatial memory and cognitive decline due to neurodegeneration. Conversely, the Naringin-treated groups, particularly those receiving the high dose (MNHD), demonstrate markedly improved outcomes, with shorter swimming distances and increased time in the target quadrant. This improvement indicates that Naringin mitigates cognitive decline and neurodegeneration, particularly at higher doses. The MNLD group shows moderate improvement, suggesting dose-dependent neuroprotective effects. Diazepam, used as a positive control, also shows significant cognitive enhancement similar to the high-dose Naringin group.

Moreover, these results suggest that Naringin, especially in higher doses, exerts neuroprotective effects against malathion-induced neurodegeneration, improving cognitive function and spatial memory in rats. These outcomes highlight the therapeutic potential of Naringin in treating neurodegenerative disorders.

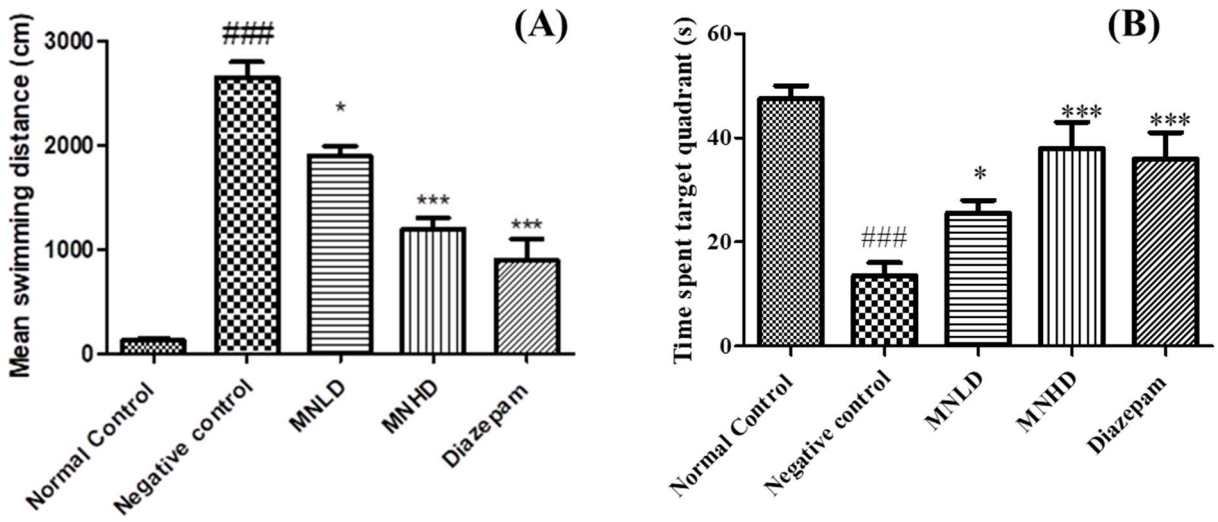


Figure 4: The figure depicts the neuroprotective effects of naringin on malathion-induced neurodegeneration by measuring mean swimming distance (Figure A) and time spent in the target quadrant (Figure B). Data are expressed as Mean ± SD and analyzed using One-Way ANOVA, followed by Tukey's test. Significance was determined at $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***). The highest significance levels are observed in treatments where naringin (both low and high doses) effectively reduced neurodegeneration, as indicated by shorter swimming distances and longer time spent in the target quadrant compared to the negative control group.

3.2.3. In-vivo anti-oxidant activity

The data on the antioxidant activity of naringin treatment, assessed across different groups, provides insight into its neuroprotective role against malathion-induced oxidative stress. Group A, the normal control group, maintained baseline levels of key antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH), reflecting normal physiological function without oxidative insult. Group B, which was exposed to malathion, showed significant depletion of these antioxidant enzymes, indicating heightened oxidative stress

and neurodegeneration. Malathion, an organophosphate, is known to generate reactive oxygen species (ROS) that overwhelm the antioxidant defense system, leading to lipid peroxidation, protein damage, and neuronal injury.

In Group C, rats treated with a low dose of naringin (80 mg/kg) showed partial restoration of antioxidant enzyme activity compared to the malathion group. This indicates that naringin at lower doses has some protective effect by scavenging free radicals and enhancing the brain's endogenous antioxidant capacity, although the restoration was not complete. Group D, treated with a higher dose of naringin (160 mg/kg), exhibited a more significant recovery of CAT, SOD, and GSH levels, suggesting a dose-dependent protective effect. Naringin at this dose was more effective in neutralizing ROS, reducing oxidative damage, and potentially mitigating malathion-induced neurodegeneration. This study highlights the antioxidant potential of naringin, with higher doses offering more substantial neuroprotection. It suggests that naringin's dose-dependent efficacy could be explored as a therapeutic strategy against oxidative stress-related neurodegenerative conditions.

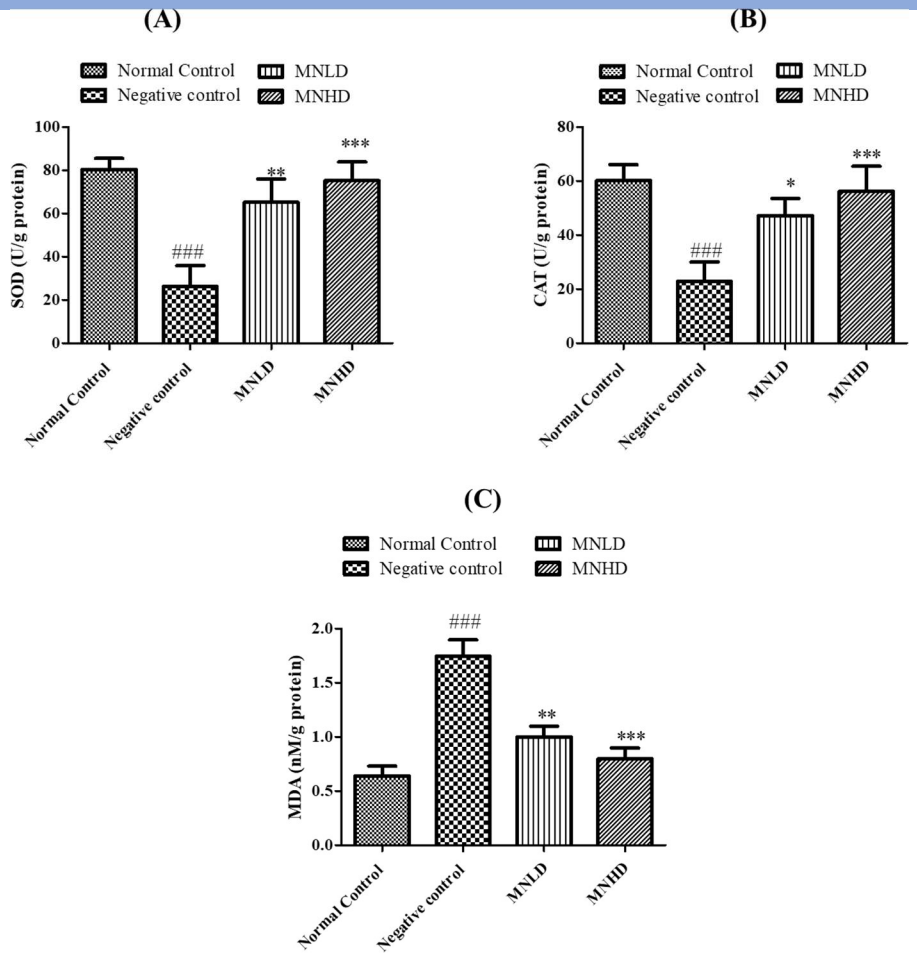


Figure 5: The figure shows the antioxidant activity (Figure A-SOD, Figure B-CAT and Figure C- MDA) of naringin in mitigating malathion-induced oxidative stress. The high malathion group exhibits elevated oxidative stress markers, while both the low (MNLD) and high (MNHD) doses of naringin significantly decrease these levels, demonstrating their antioxidant effect. Notably, the high dose (MNHD) of naringin shows a greater reduction in oxidative damage compared to the low dose. This suggests that naringin at higher concentrations effectively counters oxidative stress by enhancing the antioxidant defense system, which could protect against neurodegenerative damage. During analysis, the data was represented as Mean ± SD, using One Way ANOVA followed by Tukey test. the significance level was determined at $p<0.05$ while significance summary represented as least significant (*$p<0.05$), moderate significant (**$p<0.01$) and Highly significant (***$p<0.001$).

3.2.4. Acetylcholinesterase Inhibition activity

The Acetylcholinesterase (AChE) inhibition activity of naringin in various treatment groups subjected to malathion-induced neurodegeneration was evaluated and results Negative Control (malathion-treated) group exhibited a significant increase in AChE activity, indicating neurodegeneration caused by malathion as compared to control group. The MNLD (Malathion + Naringin Low Dose) group demonstrated a reduction in AChE activity compared to the Negative Control, signifying the neuroprotective effects of low-dose naringin. Similarly, the MNHD (Malathion + Naringin High Dose) group further reduced AChE activity, suggesting a dose- dependent neuroprotective response. The Diazepam-treated group also showed decreased AChE activity, confirming its effectiveness as a neuroprotective agent. The results indicate that naringin, particularly at a higher dose, effectively inhibits AChE activity, reducing neurodegeneration and oxidative stress induced by malathion.

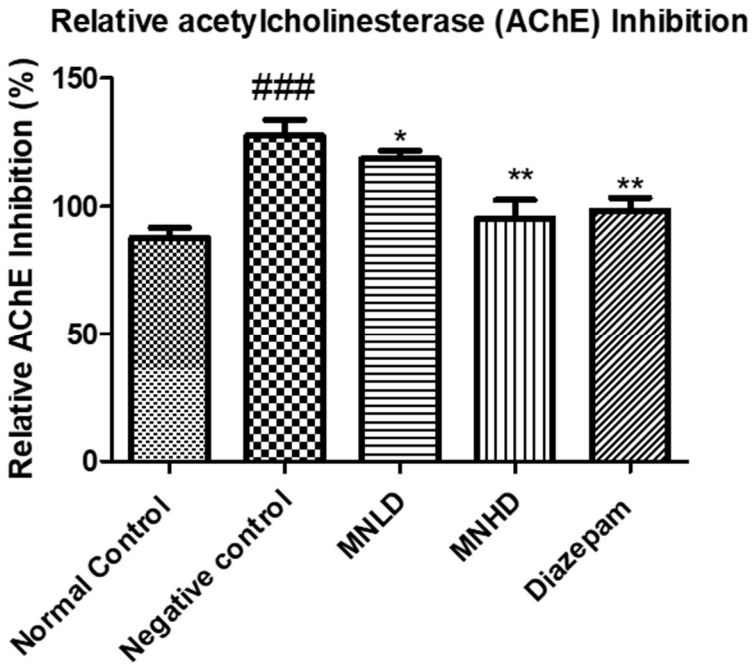


Figure 6: The figure illustrates Acetylcholinesterase (AChE) inhibition in rats exposed to malathion-induced neurodegeneration. Naringin, administered at both low and high doses, reduced AChE activity significantly, particularly in the high-dose group. This reduction indicates the neuroprotective effect of naringin against malathion's cholinergic dysfunction, suggesting its potential therapeutic application in neurodegenerative diseases. During analysis, the data was

represented as Mean ± SD, using One Way ANOVA followed by Tukey test. the significance level was determined at $p<0.05$ while significance summary represented as least significant ($*p<0.05$), moderate significant ($**p<0.01$) and Highly significant ($***p<0.001$).

3.2.5. Histopathological analysis

The histological section of the hippocampus from the normal control group displays typical neuronal architecture. The neurons appear healthy with clearly defined cellular boundaries and visible nuclei. There is an absence of neuronal shrinkage, vacuolization, or any other signs of degeneration, indicating the baseline, healthy condition of the hippocampal tissue. In the malathion-induced neurodegeneration (MNED) group, significant histopathological alterations are observed. Neurons show a high degree of shrinkage and irregularity in shape, indicating cellular stress and degeneration. Vacuolization and cytoplasmic swelling are prominent, suggesting cellular edema. The presence of pyknotic nuclei (dark-stained, shrunken nuclei) in many neurons reflects the onset of apoptosis, which is a characteristic feature of neurodegeneration. The overall structural integrity of the hippocampal tissue is compromised, with a notable loss of neuronal density. This supports the neurotoxic effects of malathion on hippocampal neurons. The hippocampal section from the MNLD group shows some protective effects compared to the MNED group. Although signs of degeneration are still present, they are less severe. Neurons exhibit a moderate level of vacuolization and cellular disorganization. While there is a reduction in the number of pyknotic nuclei compared to the MNED group, neuronal shrinkage and cytoplasmic edema are still evident. This suggests that Naringin at a low dose provides partial neuroprotection but is not entirely effective in reversing or preventing all degenerative changes induced by malathion. In the MNHD group, there is a marked improvement in neuronal integrity relative to both the MNED and MNLD groups. Neurons appear relatively intact, with fewer signs of shrinkage or vacuolization. The cellular

architecture is more preserved, and the incidence of pyknotic nuclei is significantly reduced. These observations indicate that a high dose of Naringin exerts substantial neuroprotective effects, mitigating malathion-induced neurodegeneration more effectively than the low dose. This suggests a dose-dependent response, where the higher concentration of Naringin provides enhanced protection against oxidative stress and apoptosis. The section from the Diazepam group shows an intermediate level of protection. While there is a reduction in cellular damage compared to the MNED group, Diazepam-treated neurons still exhibit mild shrinkage and vacuolization. Although Diazepam is known for its neuroprotective properties

through its anxiolytic and sedative effects, its direct role in counteracting malathion-induced neurodegeneration seems limited. Diazepam's effect is less pronounced than the high-dose Naringin, suggesting that while it may alleviate some symptoms of neurodegeneration, it may not address the underlying oxidative stress as effectively as Naringin.

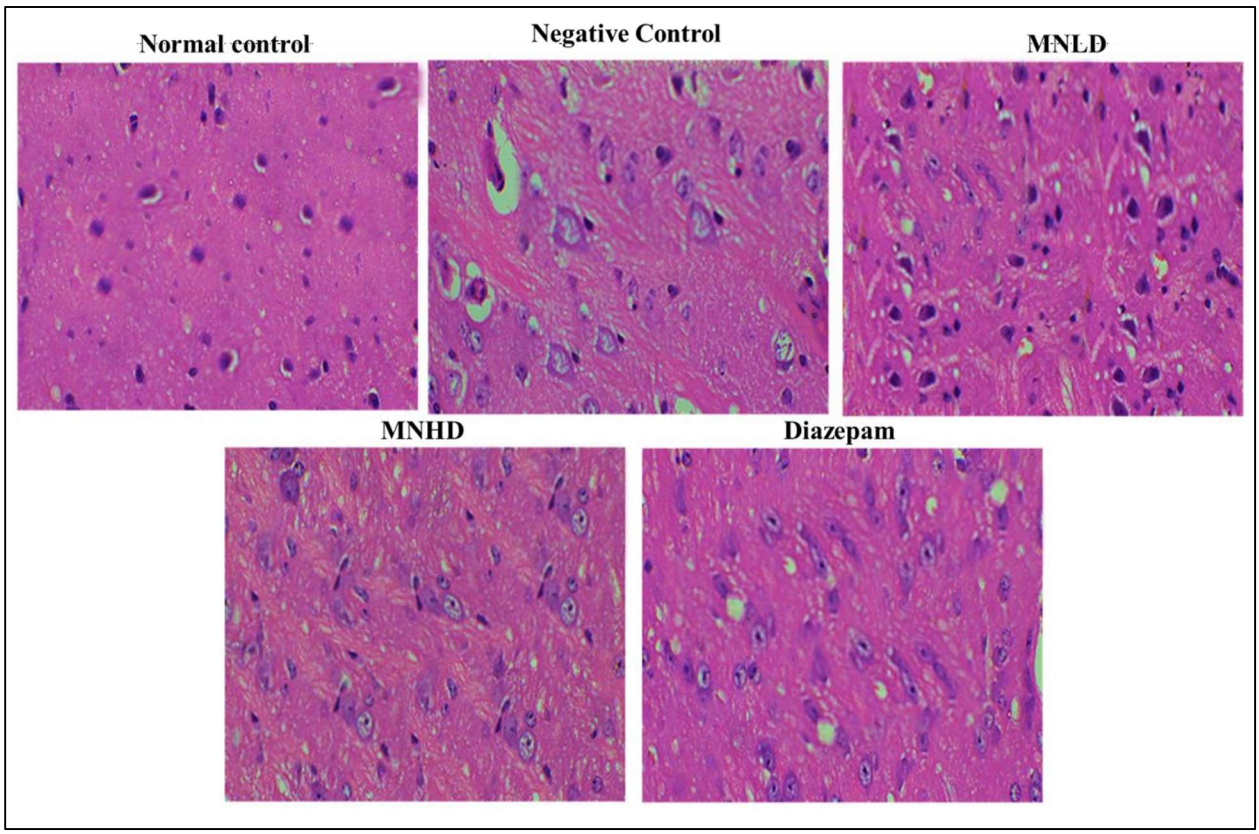


Figure 7: The figure shows histopathological sections of the hippocampus from an experimental study on malathion-induced neurodegeneration and the protective effects of Naringin at high (MNHD) and low doses (MNLD), along with a comparison to the Diazepam treatment group and a normal control group. This section is stained with hematoxylin and eosin (H&E) to highlight structural changes in the hippocampal tissue.

4. Discussion

Organophosphorus compounds (OPCs), such as Malathion, are widely used pesticides that have been implicated in neurodegenerative diseases like Alzheimer's disease due to their ability toinduce oxidative stress and associated complications. Naringin, a flavonoid with known

antioxidant and neuroprotective properties, was investigated for its potential protective effects against Malathion-induced neurotoxicity (Saadh, 2023; Salama et al., 2019).

In this study, in-silico docking analysis was conducted to determine the effect of naringin on acetylcholinesterase (AChE). The in-silico molecular docking analysis revealed a strong interaction between Naringin and acetylcholinesterase (AChE), focusing on two binding sites within the protein. Both 3D and 2D visualizations highlighted critical bonding patterns, such as hydrogen bonds, van der Waals forces, and pi-stacking interactions. Naringin formed stable hydrogen bonds with residues like HIS-405 and TRP-236, contributing to significant AChE inhibition (Gaurav, 2022; Gaurav et al., 2023). These interactions suggest that Naringin may enhance acetylcholine levels by blocking AChE activity, aligning with therapeutic strategies for neurodegenerative diseases like Alzheimer's. The docking analysis underscores Naringin's potential as a neuroprotective agent. Various studies has been published based on the in-silico docking studies conducted to explore the nephroprotective, nephroprotective and neuroprotective effect of phytochemicals, hence validating the molecular targets for illustrating the therapeutic pathway for treating acute and chronic ailments (Balachandran et al., 2023; Ben-Azu et al., 2019; Jahanshahi et al., 2021). In a study published by Sapna Salar et al 2024, reported the Network pharmacology and in-silico docking analyses highlighting the multi-targeted therapeutic effects of metabolites from *Nyctanthes arbor-tristis* in treating liver diseases. These metabolites appear to modulate the expression of crucial genes, including nitric oxide synthase (NOS), tumor necrosis factor-alpha (TNF-α), interleukins (ILs), and toll-like receptors (TLRs), which are involved in inflammatory pathways. Additionally, the regulation of serum aminotransferase levels suggests potential hepatoprotective effects. This comprehensive approach underscores the plant's efficacy in addressing liver pathology through diverse molecular mechanisms, paving the way for future therapeutic applications (Salar et al., 2023).

Gaurav et al., 2022 reported the Network pharmacological analysis of metabolites found in *Boerhaavia diffusa* and *Tinospora cordifolia* highlights the diverse physiological roles of polyphenols in addressing kidney dysfunction. By regulating various genes associated with inflammation, oxidative stress, and the renin-angiotensin system (RAS), polyphenols demonstrate potential as effective therapeutic agents. This comprehensive understanding supports the use of natural compounds in developing treatment regimens for kidney-related issues, suggesting that

approaches like herbal medicine can play a significant role in managing kidney health. Such insights pave the way for integrating these natural compounds into conventional therapies (Gaurav et al., 2022). Rat et al., 2021 conducted a study focused on the in-silico discovery of phytochemicals from *Withania somnifera* and their effectiveness against human soluble COMT. Using techniques like virtual screening, molecular docking, and molecular dynamics simulation, nine phytochemicals, including withaferin A and withanolide B, demonstrated strong binding efficiency compared to the drugs Opicapone and Entacapone. These findings suggest that these phytochemicals may serve as bioenhancers in L-DOPA therapy for Parkinson’s disease (PD). However, further experimental validation is required to confirm their potential as adjuvants in PD treatment (Rath et al., 2021).

The New Object Recognition (NOR) test demonstrated clear differences in memory across treatment groups. Rats exposed to malathion (Group B) showed significant memory impairment compared to the control (Group A). Naringin treatment at a low dose (Group C) improved recognition index, indicating partial memory recovery. However, the high dose (Group D) exhibited the most robust effect, nearly restoring memory function to normal levels, highlighting Naringin’s neuroprotective potential. Diazepam (Group E) improved memory but was less effective than high-dose Naringin. These results suggest that Naringin mitigates malathion-induced cognitive deficits through its antioxidant properties (Lee et al., 2016). Lee et al., 2016 explored the effects of Aronia melanocarpa berry extract on scopolamine-induced memory deficits in mice, using the Morris water maze and passive avoidance tests. The extract significantly alleviated memory impairment by reducing acetylcholinesterase (AChE) activity and enhancing brain-derived neurotrophic factor (BDNF) and phosphorylated CREB (p-CREB) expression in the hippocampus. The active compound, cyanidin-3-O-galactoside, contributed to these cognitive benefits. Overall, A. melanocarpa extract showed potential as a memory enhancer by improving synaptic signaling and inhibiting AChE, highlighting its therapeutic promise in neurodegenerative conditions (Lee et al., 2016).

Oxidative stress plays a pivotal role in neurodegeneration and epilepsy, as it leads to cellular damage through the overproduction of reactive oxygen species (ROS). In the context of malathion- induced neurotoxicity, ROS

disrupt antioxidant defenses, reducing key enzymes like superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH). Naringin treatment

demonstrates neuroprotective effects by restoring these antioxidants, with higher doses (160 mg/kg) offering more substantial protection. This dose-dependent restoration suggests that Naringin effectively scavenges free radicals and reduces oxidative damage, making it a potential therapeutic agent against neurodegeneration and epilepsy associated with oxidative stress.

Łukawski et al., 2023 reported in his study that in the PTX-induced seizure model, diazepam and phenobarbital significantly reduced lipid peroxidation (LPO) and nitric oxide levels in the frontal cortex, as well as nitric oxide increases in the hippocampus and midbrain. This highlights the antioxidant effects of certain antiepileptic drugs, like valproate, which prevents glutathione reduction and inhibits lipid peroxidation. Given the elevation of free radicals in seizure models, antioxidant therapies such as melatonin, selenium, vitamin E may serve as effective adjuncts in drug-resistant epilepsy, helping to reduce oxidative stress and improve seizure control (Łukawski and Czuczwar, 2023).

Moreover, the histopathological examination indicate that malathion causes significant neurodegenerative changes in the hippocampus, as evidenced by neuronal shrinkage, vacuolization, and increased apoptosis. These results are consistent with the known neurotoxic effects of organophosphates like malathion, which induce oxidative stress and disrupt normal cellular function. Naringin, a flavonoid with potent antioxidant properties, appears to offer a protective effect against malathion-induced neurotoxicity. The dose-dependent response observed suggests that higher doses of Naringin provide more substantial protection, likely by scavenging free radicals and enhancing antioxidant defense mechanisms. Naringin's efficacy at the high dose underscores its potential therapeutic utility in combating oxidative stress-related neuronal damage. Diazepam's limited neuroprotective effect, in contrast, highlights its primary action as a symptomatic treatment rather than a direct neuroprotective agent. While Diazepam can alleviate some behavioral symptoms associated with neurodegeneration, it may not mitigate the oxidative damage and apoptosis caused by malathion exposure to the same extent as Naringin. These findings suggest that Naringin, especially at higher doses, could be a promising candidate for the treatment of organophosphate-induced neurodegeneration. Future studies focusing on the molecular mechanisms underlying Naringin's neuroprotective effects could provide further insights into its potential as a therapeutic agent in neurodegenerative disorders.

Viswanatha et al., 2016 evaluated the anti-epileptic properties of methanolic leaf extract of *Punica granatum* (MLPG) in Swiss albino mice. MLPG showed significant dose-dependent protection against seizures induced by maximal electroshock (MES) and pentylenetetrazole (PTZ), while also increasing brain GABA levels. The results suggest that MLPG's anticonvulsant effects may be attributed to its influence on GABAergic pathways, with minimal impact on locomotor activity. This highlights MLPG's potential as a promising natural therapeutic for epilepsy (Viswanatha et al., 2016). Fisseha et al., 2022 evaluated anticonvulsant properties of *Biophytum umbraculum Welw. Syn* in hydroalcoholic and solvent fractions. The highest dose of 400 mg/kg showed significant seizure protection in both maximal electroshock and pentylenetetrazol tests. The butanol and chloroform fractions also displayed potent anticonvulsant effects, with no observed toxicity. This suggests that *Biophytum umbraculum* could be a promising candidate for developing new antiepileptic drugs, with the 400 mg/kg dose demonstrating optimal efficacy. Flavonoids, phenols, and other phytochemicals in the extracts may contribute to its therapeutic potential. Further studies are needed to validate these findings (Fisseha et al., 2022).

5. Conclusion

This study concludes that Naringin, a flavonoid, offers significant neuroprotective benefits against Malathion-induced neurotoxicity. Through both in-silico and in-vivo analyses, Naringin was shown to effectively inhibit acetylcholinesterase (AChE), improve cognitive function, and enhance antioxidant enzyme levels (SOD, CAT) while reducing oxidative stress markers like MDA in a dose-dependent manner. The high dose of Naringin (160 mg/kg) demonstrated superior efficacy compared to the lower dose and even the positive control, diazepam. These results suggest that Naringin holds promise as a therapeutic agent for preventing neurodegeneration, particularly in conditions involving oxidative stress and AChE dysregulation.

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