CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

journal homepage: http://chimie-biologie.ubm.ro/carpathian_journal/index.html

NEUROPROTECTIVE EFFECTS OF HESPERIDIN: IN-VITRO AND IN-SILICO EVALUATION OF ITS ANTIOXIDANT AND ENZYME INHIBITORY ACTIVITIES

Mimansa Kandhwal[1], Amarjot Kaur Grewal[1]✉, Manjinder Singh[1]✉, Varinder Singh[2], Thakur Gurjeet Singh[1]

[1]*Chitkara College of Pharmacy, Chitkara University, Punjab, India.*
[2]*Department of Pharmaceutical sciences and Technology, Maharaja Ranjit Singh Punjab Technical University, Bathinda, Punjab, India.*
✉*amarjot.kaur@chitkara.edu.in;* ✉*manjinder.singh@chitkara.edu.in;*

https://doi.org/10.34302/crpjfst/2024.16.4.15

Article history:
Received
October 23th, 2024
Accepted
November 30th, 2024

Keywords:
Acetylcholinesterase;
Hesperidin;
Monoamine oxidase;
Neuroprotection;
Flavonoid.

Abstract
Brain disorders are persistent medical conditions characterized by a gradual deterioration in neurological functioning. There is a worldwide increase in interest in phytomedicines for their beneficial health benefits and low adverse effects. Hesperidin (Hsp), a flavanone glycoside in citrus fruit peels, has many pharmacological characteristics. Nevertheless, there is a need for more comprehensive investigations that elucidate the underlying mechanism of action. The objective of this work is to assess the neuroprotective impact of Hsp using in-vitro tests for the inhibition of acetylcholinesterase (AChE), monoamine oxidase (MAO), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) (H2O2), followed by in-silico techniques such as molecular docking and molecular dynamics. The outcomes of the current investigation demonstrate significant inhibitory effects on AChE, MAO, DPPH, and H2O2, which may be attributed to the intended pharmacological actions of Hsp. In-silico studies showed strong interactions of Hsp with targeted proteins. Thus, Hsp has the potential to be formulated as a neuroprotective medication.

1. Introduction

Neurons have a vital role in promoting communication, hence ensuring the efficient functioning of the human brain (Morrison 1997). The finite lifespan of neurons is a significant factor in the pathogenesis of various brain disorders, including Alzheimer's Disease, Parkinson's disease, Epilepsy, Huntington's disease, and major Depression. This is primarily attributed to the progressive reduction in neuronal population, alterations in structural integrity, and compromised functional capacities, collectively known as neurodegeneration. In addition to this, the modified functioning of metabolic enzymes, which subsequently impact the release of neurotransmitters, may also play a role in the pathophysiology of many affective disorders like anxiety and phobias. A complete understanding of the exact causes of brain disorders is not comprehensively elucidated; however, they are commonly correlated with abnormal production of reactive oxygen species, and disturbed activity of enzymes involved in the metabolism of Neurotransmitters.

The development and progression of brain diseases are associated with oxidative stress (Hayashi et al., 2012). The aforementioned situation occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the ability of the human body to mitigate the resulting damage. Reactive

oxygen species (ROS), including superoxide radicals, hydrogen peroxide, and hydroxyl radicals, are naturally occurring byproducts (Hoidal, 2001) from several biological processes, including metabolic activities. Usually, the human body has natural mechanisms to combat reactive oxygen species (ROS) and reduce damage. However, when the level of oxidative stress exceeds the ability of these protective systems, it may damage the structure and function of neurons (Rao et al. 2011; Popa et al. 2013). Reactive oxygen species (ROS) have been found to harm the brain, leading to the incorrect folding and buildup of specific proteins namely alpha-synuclein in Parkinson's disease, amyloid-beta in Alzheimer's disease, and huntingtin in Huntington's disease (Sun and Chen 1998; Abdelhamid et al. 2023; Liu et al. 2023). The formation of these misfolded proteins produces harmful clusters, disrupting cellular functions and facilitate the death of neuronal cells. ROS have the potential to cause adverse impacts on mitochondrial DNA and proteins, leading to compromised energy generation and an augmented ROS production (Li et al., 2022, Rehman et al., 2022). Antioxidant drugs have the potential to act as adjuvant drugs to benefit in brain disorders caused by ROS.

In addition to oxidative stress, the dysregulation of enzymes implicated in neurotransmitter metabolism also leads to disruptions in brain functionality, primarily linked to conditions such as Alzheimer's disease (AD) (Bai et al., 2022), major depression, and mood disorders (Bhatt et al., 2020). Alzheimer's disease (AD) is distinguished by the deterioration of cholinergic neurons, which are a specific kind of neuronal cell that plays a crucial role in facilitating communication within the central nervous system via the use of the neurotransmitter acetylcholine (ACh) (Bekdash, 2021). Acetylcholine (ACh) is mainly found in the hippocampal regions of the brain and is closely linked to memory and cognitive processes inside the human body. The enzyme acetylcholinesterase (AChE) is responsible for breaking down acetylcholine (ACh) in the synaptic cleft, which stops the passage of signals and impacts memory (Benzi and Moretti 1998). AD is characterized by increased activity of AChE, leading to the use of potent acetylcholinesterase inhibitors (tacrine and donepezil) as the primary therapy for AD (Marucci et al., 2021).

The course of depression symptoms is also influenced by changes in the levels of MAO-B in the brain (Klimek et al., 2003). The enzymatic catalyst known as monoamine oxidase (MAO) plays a crucial role in the modulation of neurotransmitters such as serotonin, norepinephrine, and dopamine in the cerebral region, (Shih et al., 1999). The primary role of MAO-B is the enzymatic breakdown of the monoamines which play a crucial role in regulating mood and motivation (Feinberg et al., 2016). Any change in the levels of monoamines contribute to manifestation of symptoms associated with depression and mood disorders. From extensive analysis of the literature, it has been determined that there is a need to develop drugs with neuroprotective properties to treat the brain disorders effectively. The significance of flavonoids in treating neurological diseases is increasing due to their diverse pharmacological actions (Ayaz et al., 2019, Kim et al., 2019). Hesperidin is a bioflavonoid predominantly found in Citrus fruits such as oranges, lemons, and grapefruits (Hajialyani et al., 2019). Its antioxidant and anti-inflammatory properties help protecting neural cells from oxidative stress and inflammation, which are often implicated in neurodegenerative diseases such as Alzheimer's and Parkinson's (Kim et al., 2019). Research suggests that hesperidin may improve cognitive function and memory, offering a promising complementary approach to traditional treatments for brain health (Roohbakhsh et al., 2014). Despite of diverse pharmacological actions of Hsp, studies revealing the mechanism of action associated with Hsp's neuroprotective action are scanty.

In the present investigation, in-vitro assays were conducted to assess the antioxidant characteristics and AChE and MAO-B inhibitory ability of Hsp. To confirm the

efficacy of the pharmacological investigations on the specific enzymes in achieving the intended therapeutic outcome, molecular docking experiments were conducted, followed by molecular dynamics studies. The primary objective of this study is to assess the neuroprotective properties of Hsp through the utilization of in-vitro assays measuring the inhibitory effects on acetylcholinesterase (AChE), monoamine oxidase (MAO), and 1,1-diphenyl-2-picrylhydrazyl (DPPH). Additionally, in-silico techniques such as molecular docking and molecular dynamics studies were employed to investigate the potential of Hsp as an adjuvant therapy for various brain disorders.

2. Material and Methods

Hesperidin was procured from Sigma Aldrich. All chemicals were of analytical grade.

2.1. *In vitro* AChE inhibitory activity

The inhibitory effect of the test extract on AChE activity was evaluated by the spectrophotometric method of Ellman et al. (Classics Ellman et al. 1961). Donepezil (10–80 µg/ml) was used as standard AChE inhibitor. The control, standard, and test samples contained the following: • Control = Phosphate buffer (0.1 M, 8 pH, 2.6 ml) + DTNB (0.01 M, 0.1 ml) + distilled water (0.1 ml) + AChE (0.1 U ml−1, 0.1 ml) + ATI (0.075 M, 0.1 ml). • Standard = Phosphate buffer (0.1 M, 8 pH, 2.6 ml) + DTNB (0.01 M, 0.1 ml) + Donepezil (0.1 ml) + AChE (0.1 U ml−1, 0.1 ml) + ATI (0.075 M, 0.1 ml). • Test = Phosphate buffer (0.1 M, 8 pH, 2.6 ml) + DTNB (0.01 M, 0.1 ml) + extract (0.l ml) + AChE (0.1 U ml−1, 0.1 ml) + ATI (0.075 M, 0.1 ml). All the readings were taken in triplicate. The percentage inhibition was calculated in comparison to control (test sample absent).

The percentage inhibition was calculated using the following equation:

$$\% \text{ Inhibition} = 1 - (\text{absorbance of test sample} / \text{absorbance of control}) \times 100. \quad (1)$$

2.3. *In vitro* MAO-B inhibitor activity

The fluorometric technique was used to conduct the in-vitro MAO-B inhibition test, whereby the percentages and IC50 values of Hsp was determined. This inhibition assay is based on the principle of revealing the hydrogen peroxide ions via a reaction called as horseradish peroxide coupled reaction by utilizing amplex red (10-acetyl-3,7-dihydroxyphenoxazine) reagent by the method of Can et al., 2017 (Can et al. 2017).

2.4. *In vitro* antioxidant property

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay (Blois 1958; Kaur et al. 2017) was used to evaluate the antioxidant activity of Hsp. The expression of DPPH radical scavenging activity was determined by calculating the percentage inhibition using the following equation:

$$\% \text{ Inhibition} = \{(\text{Absorbance of control} - \text{Absorbance of test sample}) / \text{Absorbance of control}\} \times 100 \quad (2)$$

2.5.Statistical Analysis

Acetylcholinesterase, MAO-B inhibitory, DPPH and H_2O_2 inhibitory assays were presented as mean±SD and statistically analysed by one-way ANOVA followed by Tukey's multiple comparison test.

2.6. *In silico* Studies

2.6.1. Molecular docking studies

The affinities of Hsp with acetylcholinesterase and MAO-B were investigated by molecular docking tests using the Biovia Discovery Studio application. The molecular docking of Hsp was performed using the crystalline structures of the acetylcholinesterase (AChE) and MAO-B (2V52 (1.6) proteins, which were obtained from the RCSB-protein database library (PDB) (https://www.rcsb.org/) (Sharma et al. 2023; Wu et al. 2003). The structures underwent pre-processing, preparation, and optimization using the "Macromolecule" module for later analysis.

The first phase in protein pre-processing was the removal of water molecules and heteroatoms, followed by the protonation technique using the 'Add Polar' function. Following this, the "Define and Edit Binding Site" tool was used to construct and generate a binding site encompassing the co-crystallized ligands. MarvinSketch and ChemDraw 16.0 software were used to construct Hesperidin's SMILES notations and molecular structure, respectively. The ligands were synthesized and underwent comprehensive minimizing utilization of the Small Molecules module. The technique used for the docking study was the "Dock Ligands (CDOCKER)" approach. Visual examination was used to analyze the molecular interactions between docked complexes of Hsp with AChE and MAO-B. The formation and establishment of a binding site encompassing the co-crystallized ligands depended on the docking scores of the amino acids found in AChE and MAO-B.

2.6.2. Molecular dynamic Simulations

The molecular dynamics (MD) studies were performed (with simulation for 20 nanoseconds) using the molecular dynamics component of Schrodinger's Desmond software. Additionally, the thermal equilibrium of hesperidin concerning time was assessed (Furlan et al., 2021; Bhatia et al., 2023; Li et al., 2023; Sharma et al., 2023; Mahajan et al., 2020; Nagu et al., 2021). The protein-ligand connection was strengthened by reducing or minimizing energy simulations, which allowed for the attainment of hesperidin's optimal structure via interactions over time. The study investigated the connection between the complex ligand and the target macromolecule during the whole 100 nanosecond simulations, using simulation-interaction analysis (Sharma et al. 2023). This methodology offers a valuable understanding of essential ligand-interacting relationships that may be used to ascertain a ligand's affinities with enhanced accuracy. The first construction of the component used the orthorhombic box-shaped TIP3P solvable method, whereas the ionic potential of the input system was altered by applying a 0.15 M salt solution. The modeling operation was conducted using the NPT consortium and a time interval of 1.0 fs. The Nose-Hoover Chain methodology provided a constant temperature of 310 K, while the Martyn-Tobias-Klein method was utilized to fix the barometric pressure at 1.01325 bar.

2.6.3. Drug likeliness and ADME studies

It has been demonstrated in periclinal studies of drug moieties discovered earlier that early estimation of ADME (absorption, distribution, metabolism, and excretion profile) in the discovery phase reduces drastically the fraction of pharmacokinetics-related failure in the clinical phases (Han et al., 2019). To predict the pharmacokinetic study Hsp the SWISS ADME, a web server tool was used. It provides the access to a pool of quick yet reliable predictive models for physicochemical characteristics, pharmacokinetics, and drug-likeness properties (Singh et al., 2017).

Using the swiss ADME programme, the pharmacokinetic characteristics of the Hsp was assessed (http://www.swissadme.ch). A wide molecular profile, including physicochemical characteristics, pharmacokinetics, solubility, lipophilicity, and drug likeliness were examined.

3. Results and Discusions

3.1. Pharmacological studies

3.1.1. In-vitro acetylcholinesterase and MAO-B inhibitory activity

Hsp was found to be strong AChE and MAO-B inhibitor (Table 1). The present study used donepezil and selegiline as the typical inhibitor of acetylcholinesterase and MAO-B, respectively.

3.1.2. In vitro Antioxidant Properties

Hsp showed strong inhibition of DPPH and H_2O_2 with comparable IC50 value to ascorbic acid (Table 1).

Table 1. The AChE inhibition, MAO-B inhibition and radical scavenging activities of Hesperidin and reference compounds.

Compounds	AChE Inhibitory activity IC_{50} values (Mean^n±SD, µg/ml)	MAO-B inhibitory activity IC_{50} values (Mean±SD, µg/ml)	Radical scavenging activity IC_{50} values (Mean±SD, µg/ml)	H_2O_2 Assay IC_{50} values (Mean±SD, µg/ml)
Hesperidin	17.18 ±0.93	28.49 ±1.72	8.92 ±0.91	11.28 ±0.61
Donepezil	19.27 ±1.41	-	-	-
Selegiline	-	26.29 ±1.72	-	-
Ascorbic Acid	-	-	7.63 ±0.83	9.11 ± 0.52

n=3. No statistical difference was observed in Hesperidin and test drugs in all activities.

Table 2. Docking score and key interactions of Hesperidin with AChE & MAO-B enzymes

Target Proteins	-CDOCKER Interaction Energy (kcal/mol)	Types of Interaction	Key Residues
AChE	-71.03	Conventional H-bond	Tyr121, Asp285, Ser286, Glu199, His440, Tyr70.
		Pi-Pi stacking	Trp84, Leu282
		Pi-donor H-bond	Tyr334
		Pi-alkyl	Trp84
		Van der Waals	Leu358, Phe290, Leu127,
MAO-B	-68.32	Conventional H-bond	Cys397, Arg42, Tyr435, Leu171
		Carbon H- bond	Gly57, Gln206, Ile264, Ala263, Thr426
		Pi-Cation	Arg42
		Pi-sigma	Gly13
		Pi-alkyl	Phe343, Tyr60

3.2. *In Silico* Studies

3.2.1. Molecular Docking

Figure 1 and 2 depicts hesperidin's molecular docking and protein interaction studies with AChE and MAO-B. Hsp exhibited interactions with both the peripheral anionic site (PAS, Trp279, Asp285, Ser286, Glu199, Tyr334) and catalytic anionic site (CAS, Trp84, Tyr70, His440) of AChE. The binding energies of hesperidin were -71.03 Kcal/mol, much higher than the binding energies of the standard medication donepezil, which were -51.89 Kcal/mol. Hesperidin exhibited interactions with the essential amino acid residues of the active site of MAO-B (Cys397, Arg42, Tyr435, Leu171, Gly57, Gln206, Ile264, Ala263, Thr426, Arg42, Gly13, Phe343, Tyr60) by several mechanisms such as hydrogen bonding, Pi-Pi stacking, Pi-Cation interactions, and Pi-alkyl interactions. Compared to the conventional medication selegiline, Hsp exhibited superior binding energies of -68.32 Kcal/mol, surpassing the latter's binding energy of -60.12 Kcal/mol. Table 2 presents

hesperidin's docking score and critical contacts with AChE and MAO-B enzymes. The interactions of potent test compound Hesperidin in the active gorge of AChE and MAO-B with the crucial residues are displayed in **Figure 1** and **Figure 2**.

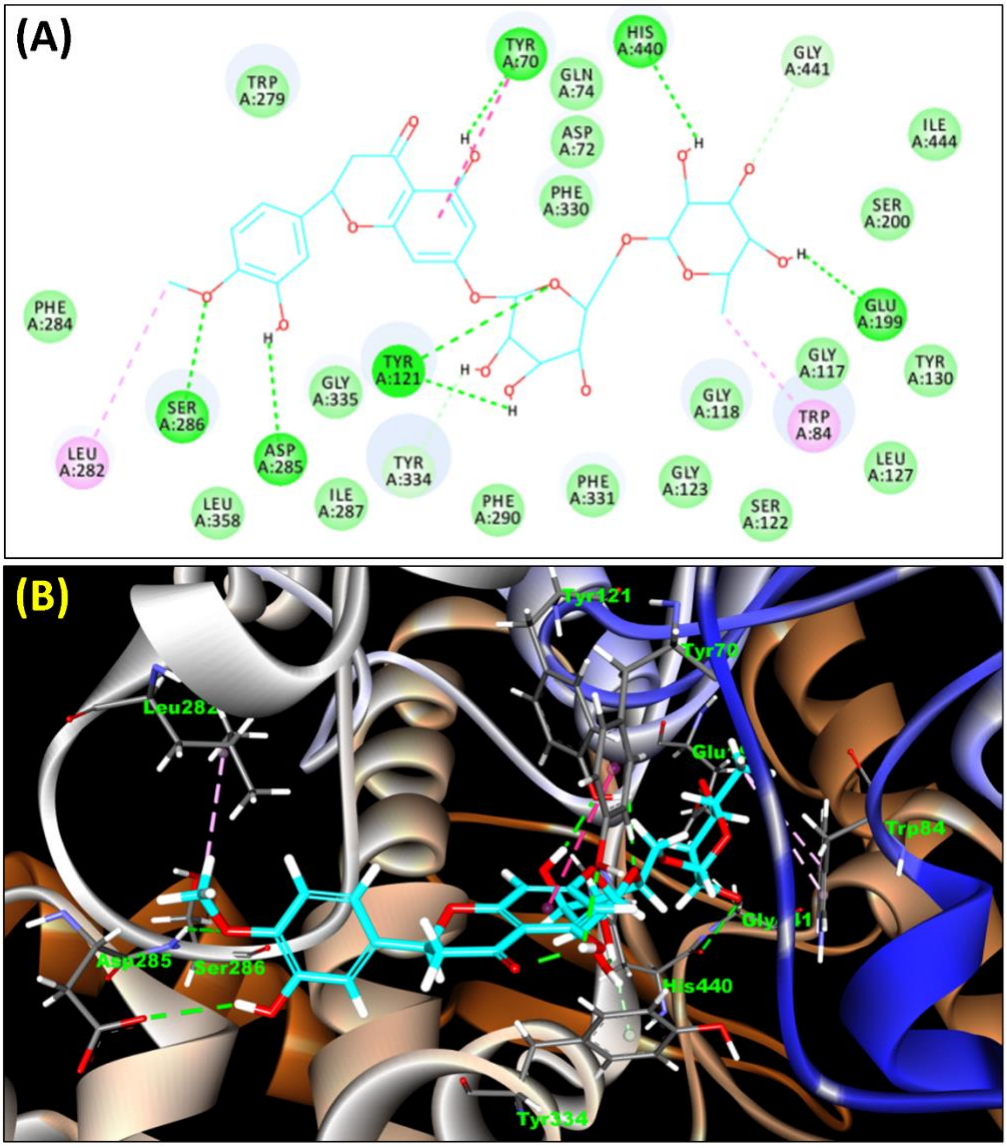

Figure 1. Interactions of Hesperidin within the binding site of AChE **(A)** 2D profile for different interactions of Hesperidin with AChE, and **(B)** 3D profile for binding of Hesperidin with TcAChE.

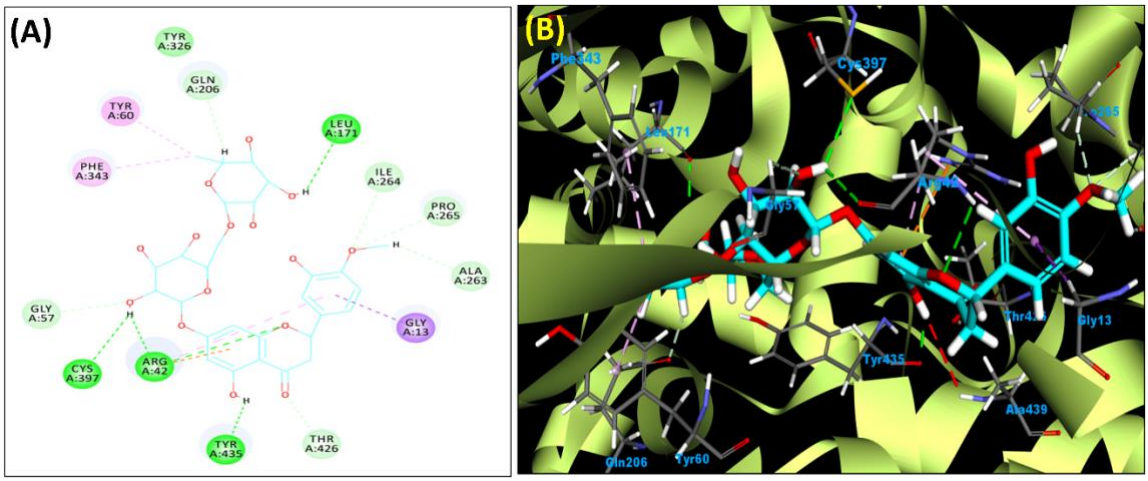

Figure 2. Interactions of Hesperidin within the binding site of MAO-B **(A)** 2D profile for different interactions of Hesperidin with MAO-B, and **(B)** 3D profile for binding of Hesperidin with MAO-B

3.2.2. Molecular dynamics simulations.

A molecular dynamics (MD) simulation was conducted on Hsp to investigate its thermodynamic stability, the dynamic behavior of the ligand-protein complex, and the influence of ligand association with the activated domain of Hsp on conformational changes (Furlan et al., 2021; Bhatia et al., 2023). The MD analysis maintained significant relationships while also revealing new linkages. The examination of the protein-ligand complex formed by AChE and Hsp demonstrated the presence of hydrophobic contacts with Trp279 and Tyr121, as well as polar and charged bonding interactions with His440 and Glu199, respectively (Figure 3A). The plot depicting the protein-ligand relationship (Figure 3B) comprehensively depicts these interactions. After conducting molecular dynamics (MD) simulations, the root mean square deviation (RMSD) of Hsp concerning the protein was calculated and is shown in Figure 3C.

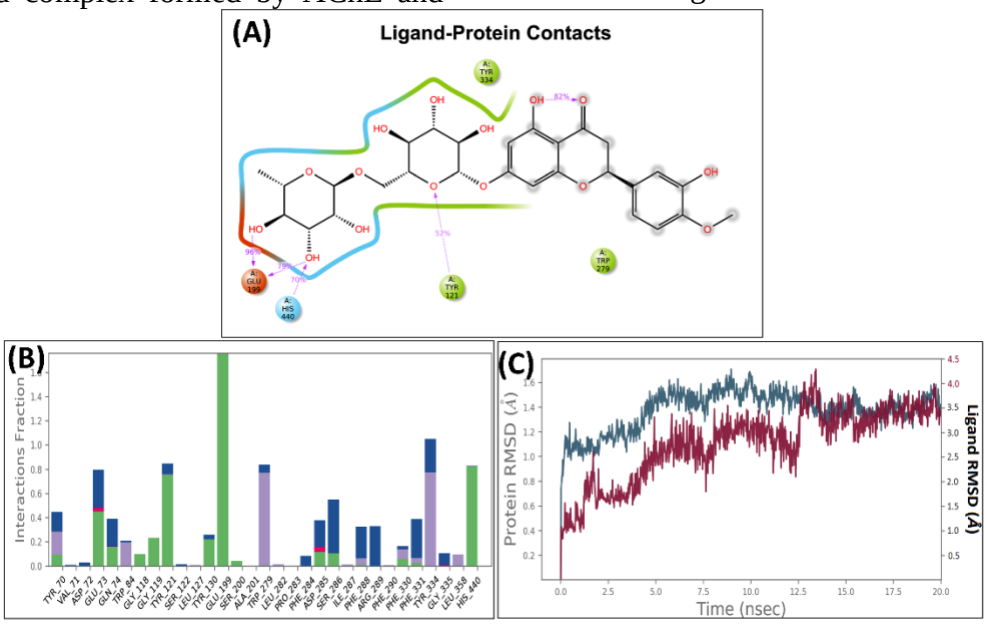

Figure 3. Post-MD interactions of Hesperidin with AChE; **(A)** Protein interactions fractions with the ligand (Hesperidin) plot throughout the simulation; **(B)** Protein-ligand contacts plot of compound Hesperidin with AChE; (C) RMSD trajectory plot for compound Hesperidin.

The similar pattern has been observed in the complex of MAO-B and Hesperidin which showed strong hydrophobic interactions with Ile198, Ala263, Tyr398 and Tyr435 along with charged interactions with active amino acid Arg42 (Figure 4A). The interactions are potted in the protein–ligand contacts plot (**Figure 4B**). The RMSD graph of the Hesperidin-MAO-B complex was displayed versus time (20ns) after MD simulation investigations (Figure 4C).

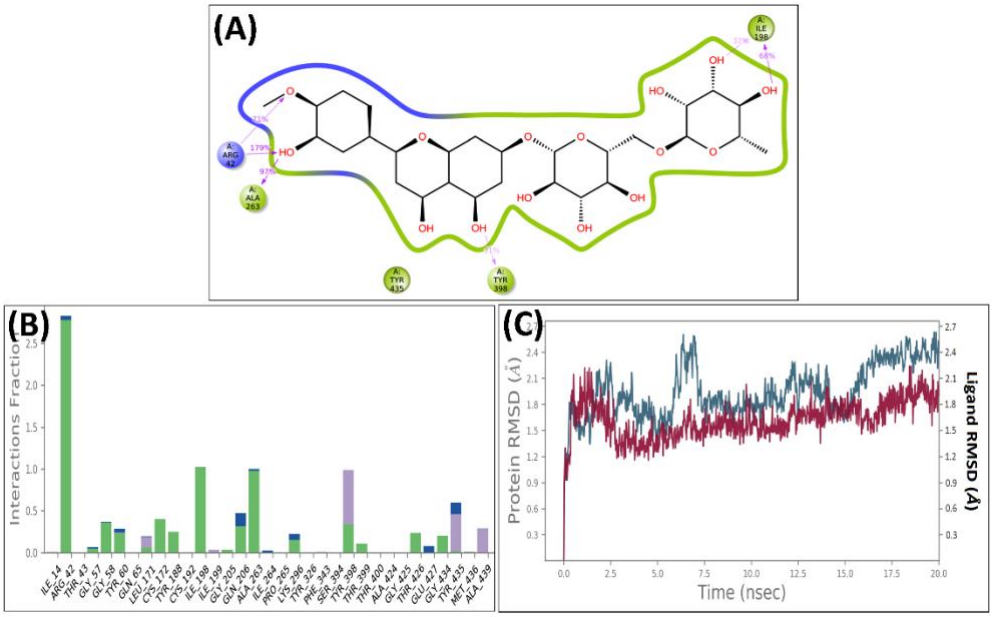

Figure 4. Post-MD interactions of Hesperidin with MAO-B; **(A)** Protein interactions fractions with the ligand (Hesperidin) plot throughout the simulation; **(B)** Protein-ligand contacts plot of compound Hesperidin with MAO-B; (C) RMSD trajectory plot for compound Hesperidin.

The root mean square deviation (RMSD) plots of Hsp with AChE and MAO-B demonstrated the stability of the complexes, with little variations (ranging from 1.2 to 2.5) seen within a 20 nanosecond time interval. The results obtained from the simulation demonstrate the inhibitory capacity of Hsp against AChE and MAO-B. The Hsp -MAO-B complex exhibited enhanced stability, as shown by its minor or negligible pulsation within the specified time frame. Conversely, the Hsp -AChE complex exhibited oscillation within the first 20 units of the binding cavity, after which it reached a stable conformation. During the simulated time necessary to elicit the therapeutic response, the complexes formed between Hsp and both target proteins exhibited remarkable stability inside the active site.

3.2.3. Drug Likeliness and ADME Studies

The pharmacokinetic studies ensure the anticipated pharmacological profile of targeted molecules. In order to identify new drugs, it is necessary to inquire absorption, distribution, metabolism, and excretion (ADME) profile at progressively earlier stages of the discovery process. In that situation, *in silico* models are appropriate substitutes for experimentation (Doogue et al., 2013). Our results (Table 3) demonstrated that Hsp has low GI absorption and has no blood brain barrier permeation. Furthermore, it also violates the Lipinski's rules. Therefore, there are challenges with Hsp molecule to develop it as a drug.

Table 3. The predicted Pharmacokinetic (ADME) profile of Hesperidin and Hesperetin molecules

Drug	MW	HBA	HBD	TPSA	Consensus Log P	Silicos-IT LogSw	GI absorption	BBB permeant	Lipinski #violations
Hesperidin	610.56	15	8	234.29	-0.72	-0.58	Low	No	3

3.3.Discussion

Brain disorders refer to a range of illnesses characterized by the progressive decline or death of neurons, particularly in the central nervous system, particularly the brain. These disorders often have a chronic trajectory and usually result in a gradual decline in cognitive, motor, emotional, and sensory functions. The potential development of various brain disorders can be attributed to the disruption of the cholinergic system, excessive activity of metabolizing enzymes such as monoamine oxidase (MAO) and acetylcholinesterase (AChE), and the presence of reactive oxygen species (Lawrence et al., 1995; Feinberg et al., 2016; Duncan et al., 2012; Liu et al., 2015; Pohanka, 2014; Jenner, 2003). Thus drugs targeting oxidative stress, AChE and MAO-B could be beneficial in treating brain disorders.

In this study, hesperidin, a plant flavonoid, was studied for its neuroprotective effect through in-vitro studies which were further validated using in-silico methods to explore its possible underlying mechanism of neuroprotection. The in-vitro experiments demonstrated that hesperidin exhibits favorable antioxidant properties, as evidenced by its strong DPPH and H_2O_2 inhibitory effects. Additionally, hesperidin has shown inhibitory activity against AChE and MAO-B enzymes, suggesting its potential therapeutic application in treating brain disorders. Further, in-silico molecular docking and molecular dynamics studies demonstrated that hesperidin interacted with crucial residues in the active areas of acetylcholinesterase and MAO-B enzymes. Molecular dynamics simulation research examines the functioning of biomolecular processes, including ligand binding, conformational changes generated by ligands or voltage, protein folding, and membrane transport. The results obtained from computational and laboratory experiments indicate that hesperidin has potential as a viable therapeutic strategy or supplementary treatment for managing brain disorders. The impact of oxidative stress on regulating enzyme activity, namely AChE and MAO-B, is well recognized. The AChE plays a crucial role in suppressing acetylcholine transmission at cholinergic synapses (Schallreuter et al., 2003). The rise in AChE activity may be attributed to many factors, including the loss of cholinergic neurons, the deposition of amyloid plaques, the formation of tau protein tangles, neuroinflammation, and the altered expression of acetylcholinesterase variations (Schallreuter et al., 2003; Uddin et al., 2020). ROS may oxidize the amino acid residues of AChE, leading to direct damage. The oxidative modification process can potentially disrupt the enzyme's active site, affecting its ability to bind to acetylcholine and facilitate effective breakdown. Excessive oxidative stress has capillary peroxidation, leading to the production of lipid peroxides that may cause damage to proteins, including AChE. The reduced function of enzymes may be indirectly attributed to the presence of damaged lipids and their breakdown products. According to Hasselmo (2006), acetylcholine is crucial in memory consolidation because it converts temporary memories into durable long-term memories. The regulation of acetylcholine levels inside the synaptic cleft by AChE plays a vital role in maintaining the proper functioning of neuronal circuits involved in memory formation. AChE does this by hydrolyzing excessive quantities of acetylcholine, thereby preserving the optimal equilibrium of this neurotransmitter (Lehmann and Fibiger 1979; Dańşman et al. 2022; Sharma et al. 2020). Therefore, changes in the functioning of AChE might result in impairments in memory, which is a

significant factor in the development of memory-related illnesses such as Alzheimer's. An inhibitory assay is conducted to evaluate the inhibitory activity hesperidin on AChE. The present investigation revealed that the IC_{50} value of hesperidin was comparatively lower than that of the donepezil. Consistent with previous research done by Singh et al. (2016), the present study suggests that hesperidin may possess efficacy in suppressing the activity of AChE, hence establishing its potential as a potent therapeutic agent for addressing diseases resulting from AChE dysregulation. Similarly, the activity of MAO-B is impacted by oxidative stress, a factor linked to the pathogenesis of several brain illnesses (Seif-El-Nasr et al., 2008). The enzymatic destruction of monoamine neurotransmitters, such as serotonin, dopamine, norepinephrine, and epinephrine, is attributed to monoamine oxidase, leading to the production of their respective metabolites (Tipton et al., 2004) which functions as a preventative strategy against the excessive activation of monoamine receptors. The maintenance of appropriate amounts of neurotransmitters inside synapses relies heavily on controlling monoamine breakdown by MAO-B. Thus, MAO-B inhibitors are often used in the treatment of several brain illnesses, including depression, epilepsy, and Parkinson's disorders (Jazvinġćak et al., 2023). In the present investigation, the IC_{50} value of hesperidin were determined to be 28.49 ±1.72 μg/ml, indicating its efficacy as a therapeutic agent for addressing illnesses associated with the modified activity of MAO-B.

Oxidative stress leads to a decrease in energy production and an increase in the synthesis of reactive oxygen species (ROS) (Federico et al. 2012; Niki et al. 2008; Tramutola et al. 2017; Barzilai et al. 2004). The process of neuronal death is expedited by oxidative stress since neurons possess an increased vulnerability to ROS due to their elevated metabolic rate, significant oxygen consumption, and restricted antioxidant defense systems (Niizuma et al., 2009). Flavonoids have the ability to act as scavengers of free radicals, hence assisting in the neutralization of these intrinsically unstable molecules via electron donation, leading to their eventual stability (Gupta et al., 2010). The antioxidant activities of hesperidin were assessed using the DPPH and H_2O_2 assays in this study. Numerous in vitro investigations have shown evidence that several plant flavonoids can mitigate the DPPH radical scavenging activity. Consistent with previous research, the present study found that hesperidin successfully scavenged DPPH radicals, with the extent of scavenging being dependent on the concentration of the radicals. In the H_2O_2 experiment, hesperidin demonstrated antioxidant characteristics. Therefore, it may be inferred that hesperidin has antioxidant capabilities, potentially providing neuroprotective benefits in the context of brain diseases.
In addition to inhibitory tests, the binding ability of hesperidin with AChE and MAO-B was assessed by molecular docking and molecular dynamic investigations. The investigations revealed that hesperidin displayed interactions with key amino acid residues located in the active region of the target proteins AChE and MAO-B. Consequently, hesperidin has shown the ability to block the receptor, castigating the symptoms associated with brain disorders. Further, Hsp molecule was further assessed for the determination of its drug-likeliness and ADME characteristics. The Lipinski's rule of five was used to evaluate the drug-like potential of the Hsp. Various parameters such as physicochemical properties, lipophilicity and solubility behaviors were predicted on the basis of topological polar surface area (TPSA), consensus log P, and ESOL LogS. Hsp showed optimal pharmacokinetic characteristics but did not comply with Lipinski's rules, indicating potential challenges in oral bioavailability, possibly due to sugar moieties hindering gastrointestinal absorption. Despite this, some drugs like erythromycin and rituximab also defy Lipinski's rules yet are clinically prescribed. Therefore, alternative administration routes may be explored to develop hesperidin as a viable drug candidate, considering its predicted ADME

properties and strong enzyme inhibition capabilities.

4. Conclusion and Future Perspectives

The present work confirmed by in-vitro and in-silico investigations that hesperidin has strong inhibitory effects on AChE and MAO-B, indicating its potential as a medication or adjunctive treatment for memory and mood disorders. In addition, it has strong antioxidant characteristics which reveal potential use in many neurological illnesses. Moreover, the computational investigations confirmed the in-vitro effects on the target proteins. Hesperidin had the excellent docking scores and substantial interactions with the target proteins AChE and MAO-B, as shown by the molecular docking experiments. Post-molecular dynamics simulations revealed the preservation of significant interactions with crucial amino acids in the active site. Therefore, it is necessary to conduct more in-vivo investigations on hesperidin to examine its pharmacological and mechanical properties.

5.References

Ayaz, M., Sadiq, A., Junaid, M., Ullah, F., Ovais, M., Ullah, I., Ahmed, J., Shahid, M. (2019) Flavonoids as prospective neuroprotectants and their therapeutic propensity in aging associated neurological disorders. *Frontiers in Aging Neuroscience* 26;11,155.

Bai, R., Guo, J., Ye, X.Y., Xie, Y., Xie, T. (2022) Oxidative stress: The core pathogenesis and mechanism of Alzheimer's disease. *Ageing Research Reviews*, 1;77, 101619.

Barzilai, A., Yamamoto, K.I. (2004) DNA damage responses to oxidative stress. *DNA repair* 1;3,1109-15.

Bekdash, R.A. (2021) The cholinergic system, the adrenergic system and the neuropathology of Alzheimer's disease. *International Journal of Molecular Sciences* 28;22,1273.

Benzi, G., Moretti, A. (1998) Is there a rationale for the use of acetylcholinesterase inhibitors in the therapy of Alzheimer's disease? *European Journal of Pharmacology* 3;346(1):1-3.

Bhatia. S., Singh, M., Sharma, P., Mujwar, S., Singh, V., Mishra, K.K., Singh, T.G., Singh, T., Ahmad, S.F. (2023) Scaffold morphing and *in silico* design of potential BACE-1 (β-secretase) inhibitors: A hope for a newer dawn in anti-alzheimer therapeutics. *Molecules* 28;16, 6032.

Bhatt, S., Nagappa, A.N., Patil, C.R. (2020) Role of oxidative stress in depression. *Drug discovery today* 1;25,1270-6.

Blois, M.S. (1958) Antioxidant determinations by the use of a stable free radical. *Nature* 26;181,1199-200.

Can, N.O., Osmaniye, D., Levent, S., Saglık, B.N., Inci, B., Ilgın, S., Ozkay, Y., Kaplancıklı, Z.A. (2017) Synthesis of new hydrazone derivatives for MAO enzymes inhibitory activity. *Molecules* 22,1381.

Classics, E.G.L., Courtney, K.D., Andres, V., Featherstone, R.M. (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology* 7;2,88-95.

Danışman, B., Akçay, G., Gökçek, S.Ç., Kantar, D., Aslan, M., Derin, N. (2022) The role of acetylcholine on the effects of different doses of sulfite in learning and memory. *Neurochemical Research* 47;11,3331-43.

Doogue, M.P., Polasek, T.M., 2013. The ABCD of clinical pharmacokinetics. *Therapeutic advances in drug safety* 4;1,5-7.

Duncan, J., Johnson, S., Ou, X.M. (2012) Monoamine oxidases in major depressive disorder and alcoholism. *Drug Discoveries and Therapeutics* 30;6,112-22.

Federico, A., Cardaioli, E., Da, P.P., Formichi, P., Gallus, G.N., Radi, E. (2012) Mitochondria, oxidative stress and neurodegeneration. *Journal of Neurological Sciences* 15;322,254-62.

Furlan, V., Bren, U. (2021) Insight into inhibitory mechanism of pde4d by dietary polyphenols using molecular dynamics simulations and free energy calculations. *Biomolecules* 11;3,479-485.

Gupta, V.K., Rachna, K., Munish, G., Monika, G. (2010) Recent updates on free radicals scavenging flavonoids: An overview. *Asian Journal of Plant Sciences* 9;3,108-17.

Hajialyani, M., Hosein, F.M., Echeverría, J., Nabavi, S.M., Uriarte, E., Sobarzo, S.E. (2019) Hesperidin as a neuroprotective agent: A review of animal and clinical evidence. *Molecules* 12;24,648.

Han, Y., Zhang, J., Hu, C.Q., Zhang, X., Ma, B. and Zhang, P., (2019) *In silico* ADME and toxicity prediction of ceftazidime and its impurities. *Frontiers in pharmacology 10*, 434.

Hasselmo, M.E. (2006) The role of acetylcholine in learning and memory. *Current Opinion in Neurobiology* 1;16,710-5.

Hayashi, M., Miyata, R., Tanuma, N. (2012) Oxidative stress in developmental brain disorders. Neurodegenerative Diseases 1,278-90.

Hoidal, J.R. (2001) Reactive oxygen species and cell signaling. *American Journal of Respiratory Cell and Molecular Biology* 1;25,661-3.

Jenner, P. (2003) Oxidative stress in Parkinson's disease. *Annals of Neuroloy* 53;S3,S26-38.

Kaur, R., Singh, V., Shri, R. (2017) Anti-amnesic effects of Ganoderma species: A possible cholinergic and antioxidant mechanism. *Biomedicine and Pharmacotherapy* 1;92,1055-61.

Kim, J., Wie, M.B., Ahn, M., Tanaka, A., Matsuda, H., Shin, T. (2019) Benefits of hesperidin in central nervous system disorders: A review. *Anatomy & Cell Biology* 1;52,369-77.

Kim, J., Wie, M.B., Ahn, M., Tanaka, A., Matsuda, H., Shin, T. (2019) Benefits of hesperidin in central nervous system disorders: A review. *Anatomy & cell biology*. 52;4,369-77.

Klimek, V., Roberson, G., Stockmeier, C.A., Ordway, G.A. (2003) Serotonin transporter and MAO-B levels in monoamine nuclei of the human brainstem are normal in major depression. *Journal of Psychiatric Research* 37;5,387-97.

Lawrence, A.D., Sahakian, B.J. (1995) Alzheimer disease, attention, and the cholinergic system. *Alzheimer Disease & Associated Disorders* 1;9,37-49.

Lehmann, J., Fibiger, H.C. (1979) Acetylcholinesterase and the cholinergic neuron. *Life Sciences* 25;23,1939-47.

Li, A., Zheng, N., Ding, X. (2022) Mitochondrial abnormalities: A hub in metabolic syndrome-related cardiac dysfunction caused by oxidative stress. *Heart Failure Reviews* 27;4,1387-94.

Li, Q., Zhang, H., Guan, S., Du, J., Zhang, Y., Wang, S. (2023) Molecular dynamics simulation of the inhibition mechanism of factor XIa by Milvexian-like macrocyclic inhibitors. *Computational and Theoretical Chemistry* 1225,114131.

Liu, J., Duan, W., Deng, Y., Zhang, Q., Li, R., Long, J., Ahmed, W., Gu, C., Qiu, Y., Cai, H., Hu, Y. (2023) New insights into molecular mechanisms underlying neurodegenerative disorders. *Journal of Integrative Neuroscience* 22;3,58.

Liu, T., Zhong, S., Liao, X., Chen, J., He, T., Lai, S., Jia, Y. (2015) A meta-analysis of oxidative stress markers in depression. *PloS one* 10;10,e0138904.

Mahajan, P., Kaushal, J. (2020). Epidemic trend of COVID-19 transmission in India during lockdown-1 phase. *Journal of Community Health* 45;6,1291-1300.

Marucci, G., Buccioni, M., Dal Ben, D., Lambertucci, C., Volpini, R., Amenta, F. (2021) Efficacy of acetylcholinesterase inhibitors in Alzheimer's disease. *Neuropharmacology*1;190,108352.

Morrison, J.H. (1997) Life and death of neurons in the aging brain. *Science* 278;5337,412-9.

Nagu, P., Parashar, A., Behl, T., Mehta, V. (2021) CNS implications of COVID-19: A comprehensive review. *Reviews in the Neurosciences* 32;2,219-234.

Niizuma, K., Endo, H., Chan, P.H. (2009) Oxidative stress and mitochondrial dysfunction as determinants of ischemic

neuronal death and survival. *Journal of Neurochemistry*109,133-8.

Niki, E. (2008) Lipid peroxidation products as oxidative stress biomarkers. *Biofactors* 34;2,171-80.

Pohanka, M. (2014) Alzheimer's disease and oxidative stress: A review. *Current Medicinal Chemistry* 21;3,356-64.

Rao, P.S., Kalva, S., Yerramilli, A., Mamidi, S. (2011) Free radicals and tissue damage: Role of antioxidants. *Free radicals and Antioxidants* 1;4,2-7.

Rehman, M.U., Sehar, N., Dar, N.J., Khan, A., Arafah, A., Rashid, S., Rashid, S.M., Ganaie, M.A. (2022) Mitochondrial dysfunctions, oxidative stress and neuroinflammation as therapeutic targets for neurodegenerative diseases: An update on current advances and impediments. *Neuroscience & Biobehavioral Reviews* 14:104961.

Roohbakhsh, A., Parhiz, H., Soltani, F., Rezaee, R., Iranshahi, M. (2014) Neuropharmacological properties and pharmacokinetics of the citrus flavonoids hesperidin and hesperetin—A mini-review. *Life sciences*. 15;113,1-6.

Schallreuter, K.U., Elwary, S.M., Gibbons, N.C., Rokos, H., Wood, J.M. (2004) Activation/deactivation of acetylcholinesterase by H_2O_2: More evidence for oxidative stress in vitiligo. *Biochemical and Biophysical Research Communications* 315;2,502-8.

Seif-El-Nasr, M., Atia, A.S., Abdelsalam, R.M. (2008) Effect of MAO-B inhibition against ischemia-induced oxidative stress in the rat brain. *Arzneimittelforschung* 58;4,160-7.

Sharma, P., Singh, M., Singh, V., Singh, T.G., Singh, T., Ahmad, S.F. (2023) Recent development of novel aminoethyl-substituted chalcones as potential drug candidates for the treatment of alzheimer's disease. *Molecules* 28;18,6579.

Sharma, P., Singh, V., Singh, M. (2023) N-methylpiperazinyl and piperdinylalkyl-O-chalcone derivatives as potential polyfunctional agents against Alzheimer's disease: Design, synthesis and biological evaluation. *Chemical Biology & Drug Design* 102;5,1155-75.

Sharma, V.K., Mehta, V., Singh, T.G. (2020) Alzheimer's disorder: epigenetic connection and associated risk factors. *Current Neuropharmacology* 18;8,740-53.

Shih, J.C., Chen, K., Ridd, M.J. (1999) Monoamine oxidase: from genes to behavior. *Annual Review of Neuroscience* 22;1,197-217.

Singh, V., Kahol, A., Singh, I.P., Saraf, I., Shri, R. (2016) Evaluation of anti-amnesic effect of extracts of selected Ocimum species using *in-vitro* and *in-vivo* models. *Journal of Ethnopharmacology* 4;193,490-9.

Singh, M., Kaur, M., Singh, N. and Silakari, O., (2017). Exploration of multi-target potential of chromen-4-one based compounds in Alzheimer's disease: Design, synthesis and biological evaluations. *Bioorganic & Medicinal Chemistry 25*;24,6273-6285

Sun, A.Y., Chen, Y.M. (1998) Oxidative stress and neurodegenerative disorders. *Journal of Biomedical Science* 5;6,401-14.

Tipton, K.F., Boyce, S., O'Sullivan, J., Davey, G.P., Healy, J. (2004) Monoamine oxidases: Certainties and uncertainties. *Current Medicinal Chemistry* 11;15,1965-82.

Tramutola, A., Lanzillotta, C., Perluigi, M., Butterfield, D.A. (2017) Oxidative stress, protein modification and Alzheimer disease. *Brain Research Bulletin* 1;133,88-96.

Uddin, M.S., Rahman, M.A., Kabir, M.T., Behl, T., Mathew, B., Perveen, A., Barreto, G.E., Bin-Jumah, M.N., Abdel-Daim, M.M., Ashraf, G.M. (2020) Multifarious roles of mTOR signaling in cognitive aging and cerebrovascular dysfunction of Alzheimer's disease. *Iubmb Life* 72;9,1843-55.

Wu, G., Robertson, D.H., Brooks III, C.L., Vieth, M. (2003) Detailed analysis of grid-based molecular docking: A case study of CDOCKER—A CHARMm-based MD docking algorithm. *Journal of computational chemistry* 24;13, 1549-1562.

Acknowledgment

The authors acknowledge and extend their appreciation to Chitkara University, Punjab, India for providing resources for completion of this research article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contribution

M.K.: Data Collection; AKG: Study concept and design; MJ: Data analysis and interpretation; VS: Writing original draft preparation; TGS: Conceptualization; KKM: Writing, review and editing.