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SCREENING OF HERBAL MOLECULES FOR THE MANAGEMENT OF ALZHEIMER'S DISORDER: *In silico* AND *in vitro* APPROACHES

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ABSTRACT

The current therapeutics for Alzheimer's disorder (AD) is aimed at providing the symptomatic relief from AD and it continues to progress steadily despite ongoing therapy. The present study was aimed to identify the herbal molecules that could utilize multiple pathways of AD pathogenesis for better AD management. One hundred herbal molecules were selected and subjected to docking analysis against acetylcholinesterase (AChE) (1EVE), butyrylcholinesterase (BChE) (4B0P), and Tau protein kinase (1J1B). Based on the docking score, RMSD value, inhibition constant (Ki), and amino acids involved, β-carotene, dihydrotanshinone-I, glabridin, liriodenine, morin, N-formylanonaine, quercetin, quercitrin, rutin, sumaflavone, and vitisinol C were found to be the best molecules. These molecules were then subjected to *in vitro* screening for their antioxidant and anti-inflammatory potential. Quercetin and rutin were observed to be the most promising antioxidant and anti-inflammatory molecules which could be beneficial during AD by targeting the oxidative- and inflammatory-stress pathways. The results predicted that quercetin has potential to target multiple pathways of AD pathogenesis so could prove beneficial in treating AD; however, further rigorous analysis is still required.

Keywords: Alzheimer's disease, anti-inflammatory, antioxidant, molecular docking, quercetin, rutin

INTRODUCTION

Rapid socio-economic development across the globe has led to the rise of several lifestyle disorders. Alzheimer's Disorder (AD) is one such lifestyle disorder that has shown a marked increase in recent times, especially in the developed and devolving countries (Walsh *et al.,* 2021). As per World Alzheimer Report (2021), AD and dementia are the 7th leading cause of mortality and are associated with a high level of morbidity, besides leading to the highest healthcare expenditure globally (WHO, 2021). As per conservative estimates, around 57 million people were living with AD and dementia globally in 2019. It is predicted that the population living with AD and dementia will increase to 153 million by 2050, which in itself is a huge healthcare concern (WHO, 2021). AD is a progressive disorder that is associated with severe neurodegeneration and loss of functional neurons in the brain. AD primarily affects the cortex and hippocampus; but as the disease progresses, the entire brain shows degeneration leading to a reduction in the overall brain volume

(Cao *et al.*, 2021). The root cause of the genesis, development, and progression of AD is still unknown; however, several pathways have been identified to contribute to the development and progression of AD. Some prominent pathways include the development of amyloid plaques, tau hyperphosphorylation, oxidative stress-mediated neurodegeneration, neuroinflammation, etc. (Kempuraj *et al.*, 2019; Guo *et al.*, 2021). All these pathways work in harmony and contribute to the disease progression (Marttinen *et al.*, 2018). All the current therapeutic strategies for the management of AD focus on alleviating the AD symptoms and none is capable of preventing the development of AD, halting or reversing the damage that has already been inflicted; besides having multiple side effects (Noori *et al.*, 2021; Hawkins *et al.*, 2021). Considering the current population affected with AD, the future estimated AD population, and the lack of therapeutic strategy to prevent AD genesis and progression, there is an urgent need to identify some molecules which can prevent AD development and progression.

Humans have exploited natural resources for the management of several ailments since the beginning of civilization. Nature has provided us with some promising molecules that are currently utilized in clinical settings for the management of a variety of ailments such as cancer, AD, diabetes, etc. (Forman *et al.*, 2021; Yeung *et al.*, 2021; Gregory *et al.*, 2021). In AD rivastigmine and physostigmine are the best examples of herbal-derived drugs that are used efficiently for the management of AD in clinical settings (Jiang *et al.*, 2021; Gul *et al.*, 2021). Since various pathways work together in the pathogenesis of AD, the best approach could be to identify the potential herbal drug candidates that can target multiple pathways associated with AD development and progression. In present study, we aimed to screen some potential molecules that have the ability to target major pathways involved in the pathogenesis of Alzheimer's disease, which include, acetylcholinesterase (AChE), butyrylcholinesterase (BChE), Tau protein kinase, oxidative stress, and inflammatory stress pathway by employing *in silico* and *in vitro* techniques. For this, we selected 100 herbal molecules from the literature and subjected them to docking on various targets of AD, followed by *in vitro* screening to identify the best potential molecule which can exploit multiple pathways for countering AD pathogenesis.

MATERIALS AND METHODS

Selection and preparation of ligands

In present study, 100 herbal molecules were identified through literature survey which could be beneficial in AD by targeting the various pathological pathways that are crucial for the development and progression of AD. These pathways include oxidative stress pathway, inflammatory stress pathway, neuroprotective pathways, acetylcholinesterase pathway, etc. 2D structure of 100 herbal molecules were generated from the Chemdraw Ultra 7.0 software and converted to 3D structure through Marvin sketch software. Finally, all the molecules were saved in pdb format and converted to pdbqt format for molecular docking studies using AutoDocktools-1.5.6 docking software.

Preparation of target protein molecules

The crystal structure of acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and tau protein kinase were taken from Protein Data Bank (PDB) (*www.rcsb.org*) with PDB ID 1EVE, 4B0P, and 1J1B, respectively. The AutoDock tools was used for the preparation of protein molecules for docking. The docking study was performed as per Trott and Olson (2010) and El-Hachem *et al.* (2017). Briefly, after initiating the Autodock tools, we followed the path to select target.pdb file from the File menu> open> Read Molecule. The crystal 3D structure of protein automatically appeared on screen in 3D format. From the "edit menu" we deleted water molecules, added hydrogens, polar hydrogens, and Kollman charges to the protein. Equal distribution of the charges over the entire protein was confirmed before proceeding further. For this, we followed a path, Edit>

Charges> Check totals on residues> Spread charge deficit over all atom residue> Dismiss. The files were then saved as pdbqt format so that they can be used further. This stored the partial charges and Autodock atom types that are compatible with Autodock grid computing. The grid box was selected from grid menus of AutoDock tools version 1.5.6, centralized, and the size of grid box and its location was adjusted to fit the docking pocket with the coordinates of X = 60, Y = 60, and Z = 60. It was ensured that the grid box covered the entire active docking site of protein and within this grid box docking of ligand and protein was performed. Prepared protein molecules were saved in .pdb format and converted to a pdbqt format for further docking studies.

Molecular docking

A total of 100 herbal molecules were docked on three-protein targets of AD, 1EVE, 4B0P, and 1J1B, by using AutoDocktools-1.5.6 docking software. The specified grid location was set within a protein and the docking score in terms of binding energy (kcal mol^{-1}) was calculated for each prepared ligand and receptor complex. Further, the selected ligand with the lowest binding energy and its protein receptor file was prepared by a selection of grid parameters, docking parameters, and map types were checked for all ligands. The docking parameters and Lamarckian Genetic Algorithm (LGA) were selected and the file was saved as grid log files (glg) and docking ligand files (dlg). After docking, the test structures were examined for docking score, bond length, and amino acid interactions with receptors and visualized by using PyMOL molecular graphics system and Protein-Ligand Interaction Profiler (PLIP) open-source webserver. DruLiTo, an online screening tool, was used to calculate the various structural properties such as hydrogen bond acceptor (HBA), hydrogen bond donor (HBD), total polar surface area (TPSA), partition coefficient (Log P), number of rotatable bonds (nRB), etc. The best-docked molecules were also analyzed for drug-likeness rules which are based on the principles of Lipinski's rule of 5 (Lawal *et al.*, 2021).

In vitro evaluation

Screening of herbal molecules for their potential against various targets of AD was performed through docking studies. The best-screened molecules were then subjected to *in vitro* tests on various prominent pathways that are involved in the pathogenesis of AD to narrow down the screening process. In present study, we focused on two major pathways associated with pathogenesis and progression of AD, oxidative stress pathway (antioxidant assay), and inflammatory stress pathway (anti-inflammatory assay), and screened herbal molecules for their antioxidant and anti-inflammatory potential through various *in vitro* methods. For the preparation of stock solution, we used four solvents, *viz*., DMSO, methanol, ethanol, and water. Herbal molecules were dissolved in solvent in which they showed good solubility. The required quantity of this stock solution was diluted in 95% methanol to give the desired working concentration. Further, IC_{50} values for each assay were calculated from the regression equation derived after plotting a concentration vs % inhibition graph.

Evaluation of antioxidant activity

2,2-diphenyl-1-picrylhydrazyl (DPPH) assay: DPPH assay is one of the well-established methods to determine the anti-oxidant potential of a test drug. The assay was performed as per Mehta *et al.* (2016), with slight modifications. Briefly, DPPH solution of 0.4 mM concentration was freshly prepared in 95% methanol and stored at 4°C in the dark until used. Different concentrations of test molecules (0.625, 1.25, 2.50, and 5.00 µM) were prepared from a 10 µM stock solution by diluting the required quantity in 95% methanol. The test solution (10 mL) was transferred to the reaction tube and 3 mL of freshly prepared DPPH solution was added to it. The control reaction mixture consisted of 10 mL 95% methanol and 3 mL DPPH solution. The reaction mixture was mixed and then allowed to stand for 30 min in dark at room temperature after which the absorbance of samples was recorded at 517 nm in triplicate by using a UV spectrophotometer (Thermo Scientific 1510 Multiskan Go). DPPH radical scavenging activity (%) was calculated through recorded absorbance (Abs) using the following equation after which the IC_{50} value of each molecule was determined.

$$\%\ \text{DPPH radical scavenging activity} = \frac{(Abs\ of\ control - Abs\ of\ test)}{Abs\ of\ control} \times 100$$

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assay: ABTS assay is another efficient method to determine the antioxidant potential of test samples. This assay was performed as per Liu *et al.* (2021), with slight modification. Briefly, a 7 mM aqueous solution of ABTS was prepared and then oxidized with 2.45 mM potassium peroxodisulfate for 16 h at room temperature in dark. This led to the generation of coloured chromatophhore ($ABTS^{\bullet+}$), which were then used in the experiment. Different concentrations of test molecules (0.625, 1.25, 2.50, and 5.00 µM) were prepared from 10 µM stock solution by diluting the required quantity in 95% methanol. The reaction was performed in a 96-well plate and the reaction mixture consisted of 180 µL $ABTS^{\bullet+}$ solution, to which 20 µL test solution was added. The Control reaction mixture consisted of 180 µL $ABTS^{\bullet+}$ and 20 µL methanol. The reaction mixture was allowed to stand in dark at room temperature for 30 min and then the absorbance of samples was recorded at 734 nm in triplicate by using UV spectrophotometer. ABTS radical scavenging activity (%) was calculated using the following equation after which the IC_{50} value of each molecule was determined.

$$\%\ \text{ABTS radical scavenging activity} = \frac{(Abs\ of\ control - Abs\ of\ test)}{Abs\ of\ control} \times 100$$

Nitric oxide (NO) radical scavenging assay: NO scavenging assay was performed as per Liu *et al.* (2021), by using Griess reagent, with slight modifications. Briefly, different concentrations of test molecules (0.625, 1.25, 2.5, and 5.00 µM) were prepared from 10 µM stock solution by diluting the required quantity in 95% methanol. The reaction mixture consisted of 75 µL test sample and 75 µL sodium nitroprusside. The control reaction mixture consisted of 75 µL methanol and 75 µL sodium nitroprusside. The reaction mixture was placed under visible polychromatic light for 60 min after which 150 µL Griess reagent was added to each reaction mixture and absorbance was recorded at 550 nm in triplicate by using a UV spectrophotometer. The %NO scavenging activity was calculated using the following equation after which the IC_{50} value of each molecule was determined.

$$\%\ \text{NO radical scavenging activity} = \frac{(Abs\ of\ control - Abs\ of\ test)}{Abs\ of\ control} \times 100$$

Evaluation of anti-inflammatory activity

Heat-induced hemolysis: The anti-inflammatory potential of test molecules was determined by evaluating their potential to inhibit heat-induced hemolysis as per Paul *et al.* (2021), with slight modifications as per the requirement. Fresh goat blood from the slaughterhouse was collected in sterile glass tubes having 5% EDTA solution to prevent blood clotting. Blood was centrifuged for 20 min at 5000 rpm, the supernatant drained out and the sediment having RBCs washed with normal saline. The process was repeated thrice and then 10% v/v RBC suspension was prepared for experimentation. Different concentrations of test molecules (0.625, 1.25, 2.5, and 5.0 µM) were prepared from 10 µM stock solution by diluting the required quantity in normal saline. The reaction mixture consisted of 1 mL test sample and 1 mL RBC suspension. In control, an equal volume of normal saline was used. Reaction mixtures were subjected to heat-induced hemolysis by incubating them at 57°C for 30 min over a water bath. After cooling, the reaction mixtures were centrifuged at 5000 rpm for 5 min and absorbance of the supernatant was recorded spectrophotometrically at 560 nm. The experiment was performed in triplicate. The %inhibition of heat-induced hemolysis was calculated by using the following equation and IC_{50} value of each molecule determined.

$$\%\ \text{inhibition of heat} - \text{induced hemolysis} = \frac{(Abs\ of\ control - Abs\ of\ test)}{Abs\ of\ control} \times 100$$

Inhibition of protein denaturation: *In vitro* anti-inflammatory potential of test molecules was evaluated by investigating their potential to inhibit albumin denaturation, as per Ullah *et al.* (2014), with slight modifications as per the experimental requirement. Briefly, different concentrations of test molecules (0.625, 1.25, 2.5, and 5.0 µM) were prepared from 10 µM stock solution by diluting

the required quantity in 0.1 M phosphate-buffered saline (PBS; pH 6.4). The reaction mixture consisted of 100 µL test drug and 500 µL bovine serum albumin (1% prepared in PBS). In control reaction, 100 µL PBS was used instead of test drug. The reaction mixture was incubated for 15 min at 37°C, followed by incubation for 5 min at 70ºC. The reaction mixture was allowed to cool and then the absorbance of samples was noted spectrophotometrically at 660 nm. The entire experiment was performed in triplicate. The % inhibition of albumin denaturation was calculated by following equation, and IC_{50} value of each molecule was determined.

$$\%\ \text{inhibition of albumin denaturation} = \frac{(Abs\ of\ control - Abs\ of\ test)}{Abs\ of\ control} \times 100$$

Statistical analysis

Data in the present study is expressed as mean ± standard deviation (SD) of triplicate recordings using XLSTAT 2015.1 and Graphpad prism 5. One-way analysis of variance (ANOVA), followed by Duncan's multiple comparison test were used to analyze the data. Significance among samples were expressed at significance level of 0.05.

RESULTS AND DISCUSSION

Molecular docking study

Molecular docking is an efficient computational tool extensively used to screen molecules for their specific activity against various drug targets. The outcome of docking study provides us the crucial information on whether a molecule can interact with the target receptor or not, and possess potential activity against that receptor by evaluating ligand-protein interaction energy (kcal mol^{-1}), the number of interactions, bond length, root mean square deviation (RMSD) values, amino acids involved in the interaction, etc., based on which protein specific activity of a test ligand compound was predicted (El-Hachem *et al.,* 2017). In present study, 100 ligands (herbal molecules) were subjected to molecular docking screening against AChE, BChE, and Tau protein kinase (PDB ID: 1EVE, 4B0P and 1J1B, respectively) by using AutoDock tools. In present study, we used Donepezil as an internal standard for all receptors to avoid any variations in the standard for comparison. The selected molecules along with their docking score are given in Supplementary Table 1.

The levels and activity of acetylcholine (ACh) in hippocampus diminishes during AD and therefore drugs inhibit the activity of AChE enzyme, aid in upregulating ACh levels, thereby alleviate AD symptoms and improve AD progression (Athar *et al.,* 2021). Current AD therapy is mostly based on decreasing the activity of AChE enzyme in AD brain, which enhances cholinergic transmission, however, relief is temporary and AD continues to progress with age (Marucci *et al.,* 2021). In present study, we selected this drug target. The docking results of best herbal molecules against AChE (PDB 1EVE) are depicted in Table 1 and the images depicting ligand-protein interactions are depicted in Fig. 1. The results suggest that GLU199, PHE331, PHE330, TRP84, TYR334, ILE287, TYR121, and GLY118 are major amino acids involved in the interaction of internal standard (Donepezil) with protein and molecules showing interactions with these amino acids were predicted to be efficient in targeting AChE. These molecules include β-carotene, dihydrotanshinone-I, glabridin, liriodenine, morin, N-formylanonaine, quercetin, quercitrin, rutin, sumaflavone, and vitisinol C. The results demonstrated sumaflavone and rutin as the best ligands to target AChE with docking score of -13.8 and -12.4 kcal mol^{-1}, respectively. The docking score of other herbal molecules (and donepezil) that demonstrated good interaction with AChE (1EVE) were in the range of -10.2 to -11.8 kcal mol^{-1}, suggesting good interaction of these ligands with the target protein. The RMSD values of these molecules were in the range of 0.14 to 0.94, which was less than or comparable to donepezil, which demonstrated RMSD of 1.00, suggesting good interaction. Moreover, these molecules demonstrated Ki in the range of 9.33 nm to 4.89 µM in comparison to

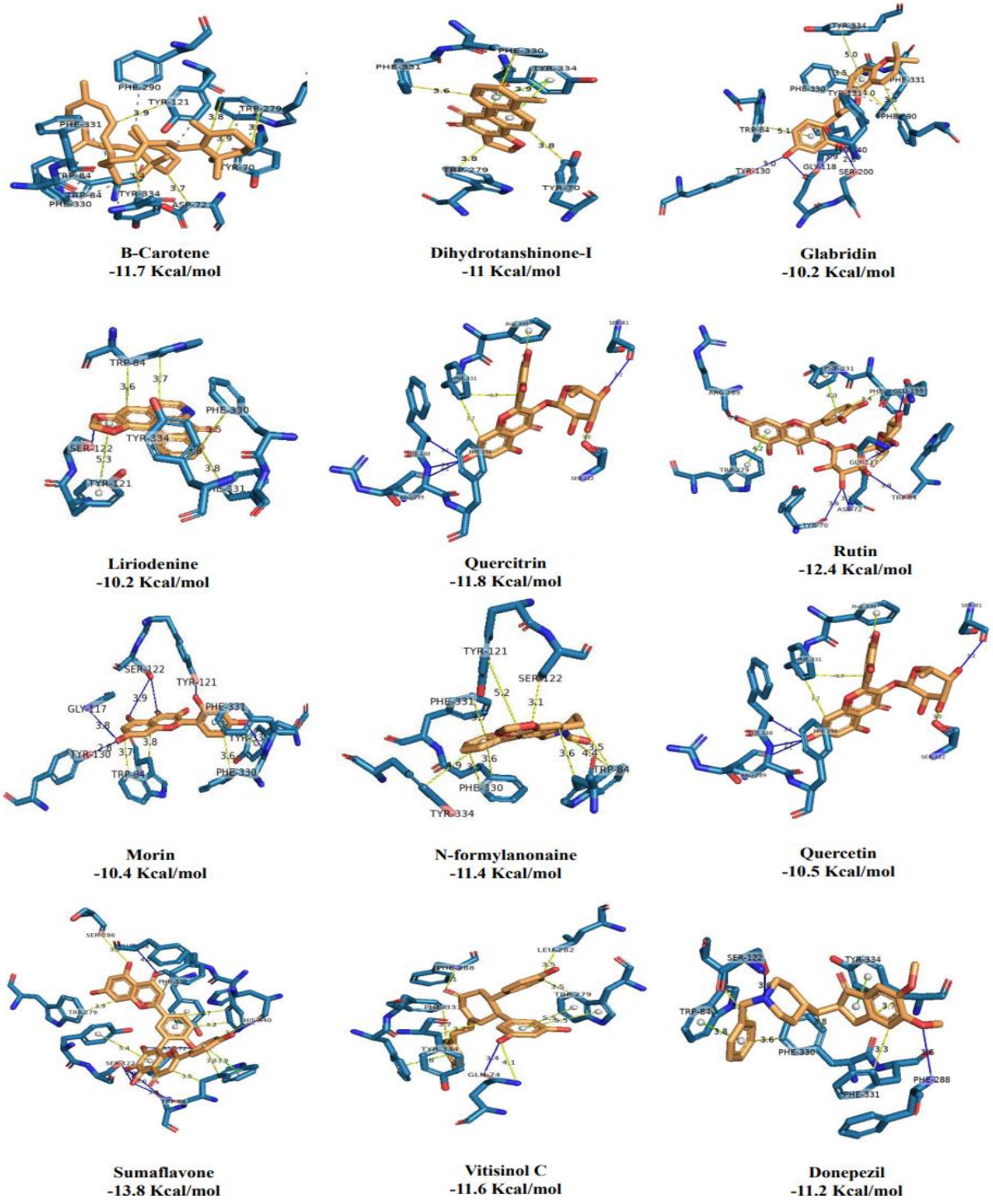

Fig. 1: Docking interaction of β-carotene, dihydrotanshinone I, glabridin, liriodenine, morin, N-formylanonaine, quercetin, quercitrin, rutin, sumaflavone, vitisinol C, and donepezil on acetylcholinesterase protein (PDB: 1EVE). Images depict ligand-protein interaction, amino acids involved, and the bond length of the interaction.

donepezil (Ki = 1.43 µM). Reports suggest that molecules demonstrating good docking interaction with AChE are capable of imparting a beneficial effect on the progression of AD (Almaz *et al.*, 2021). The evidence from clinical setting also suggests that targeting AChE is a potential strategy for the

Table 1: Binding energies (kcal mol^{-1}), RMSD values, and inhibition constant (Ki) of herbal molecules with 1EVE protein

Ligands	PDB: 1EVE (Acetylcholinesterase)		
	Docking score (kcal mol^{-1})	Reference RMSD	Estimated inhibition constant (Ki)
B-carotene	-11.7	0.76	9.33 nM
Dihydrotanshinone I	-11.0	0.64	1.75 µM
Glabridin	-10.2	0.47	65.49 nM
Liriodenine	-10.9	0.28	3.26 µM
Morin	-10.4	0.14	1.88 µM
N-formylanonaine	-11.4	0.47	2.02 µM
Quercetin	-10.5	0.46	1.46 µM
Quercitrin	-11.8	0.76	4.89 µM
Rutin	-12.4	0.94	1.10 µM
Sumaflavone	-13.8	0.94	3.06 µM
Vitisinol C	-11.6	0.71	94.78 nM
Donepezil	-11.2	1.00	1.43 µM

management of AD pathogenesis (Uddin *et al.,* 2021). Therefore, these predicted molecules could prove beneficial in modulating the pathogenesis of AD, however, further investigation is required.

BChE is another enzyme associated with the regulation of Ach levels in brain (Kinchen *et al.*, 2021) and is normally found in association with pathology such as β-amyloid plaques deposition, particularly in the cerebral cortex (Xiong *et al.,* 2021). Reports reveal that some newer therapeutic strategies for the management of AD target BChE and deliver some promising results (Xing *et al.*, 2021). The docking results of the best herbal molecules against BChE (PDB 4B0P) are depicted in Table 2 and the images depicting ligand-protein interactions are in Fig. 2.

Table 2: Binding energies (kcal mol^{-1}), RMSD values, and inhibition constant (Ki) of herbal molecules with 4B0P protein

Ligands	PDB: 4B0P (Butyrylcholinesterase)		
	Docking score (kcal mol^{-1})	RMSD value	Estimated inhibition constant (Ki)
β Carotene	-10.0	0.94	80.76 nM
Dihydrotanshinone I	-10.1	0.51	7.29 µM
Glabridin	-10.2	0.41	3.43 µM
Liriodenine	-11.1	0.65	2.85 µM
Morin	-9.8	0.76	5.30 µM
N-formylanonaine	-10.4	0.51	3.00 µM
Quercetin	-9.8	0.82	6.21 µM
Quercitrin	-12.1	0.86	1.60 µM
Rutin	-11.2	0.76	67.50 µM
Sumaflavone	-12.0	1.00	24.81 µM
Vitisinol C	-11.4	0.94	390.25 nM
Donepezil	-9.7	1.00	30.95 µM

Our results suggest that TRP82, THR120, TYR332, and ASP70 are major amino acids involved in the interaction of internal standard (donepezil) with proteins and the molecules showing interactions with these amino acids predicted to be efficient in targeting BChE. These molecules include β-carotene, dihydrotanshinone I, glabridin, liriodenine, morin, n-formylanonaine, quercetin, quercitrin, rutin, sumaflavone, and vitisinol C. The quercetrin, vitisinol c, rutin, and liriodenine were adjudged as best ligand to interact with BChE with docking score of -12.1, -11.4, -11.2 and -11.1 kcal mol^{-1}, respectively. Other herbal molecules also demonstrated good interaction with BChE (4B0P) and their docking score were in the range of -9.8 to -10.4 kcal mol^{-1}, which was better than the docking score of internal standard (-9.7 kcal mol^{-1}). The RMSD values of these molecules were

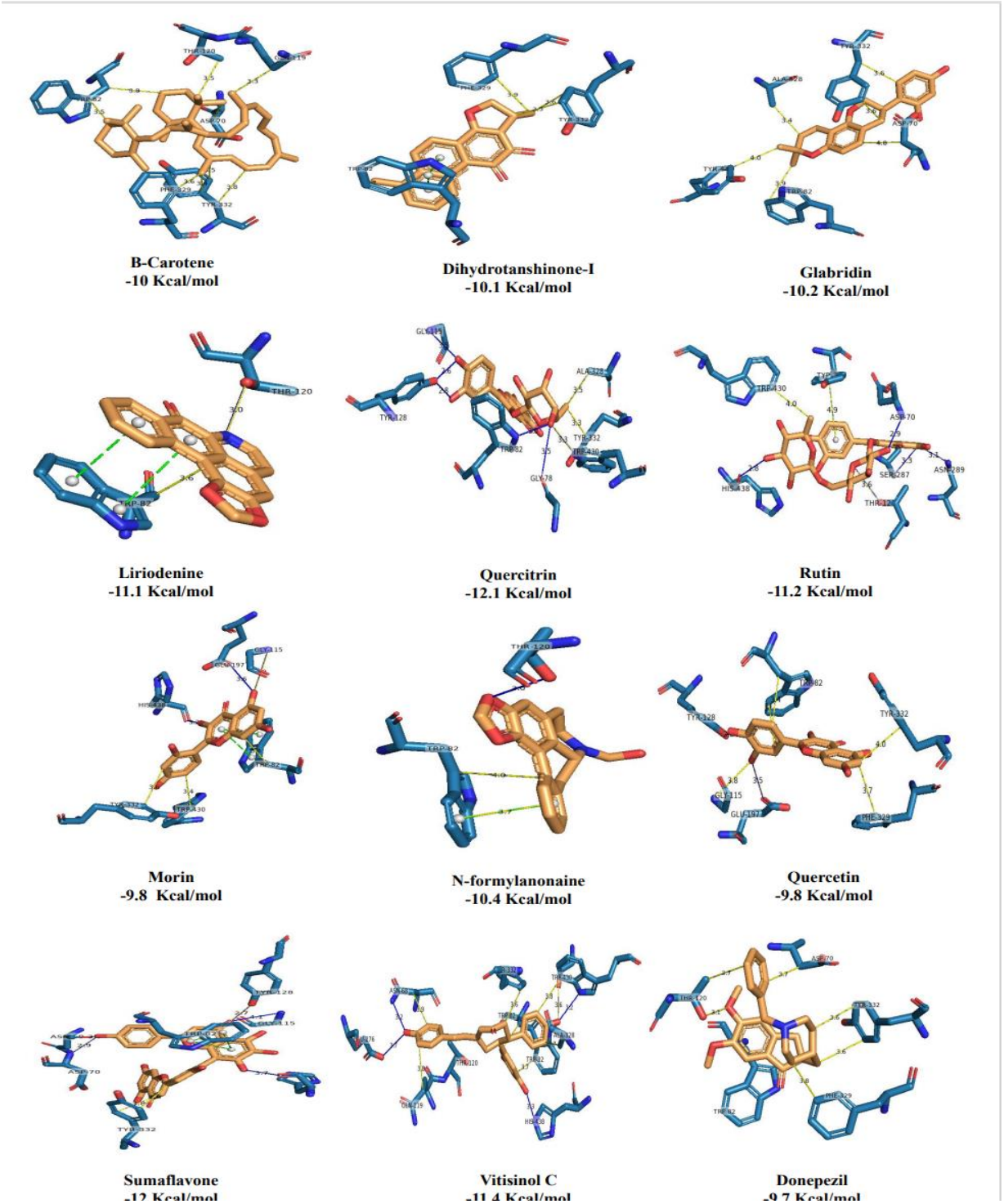

Fig. 2: Docking interaction of β-carotene, dihydrotanshinone I, glabridin, liriodenine, morin, N-formylanonaine, quercetin, quercitrin, rutin, sumaflavone, vitisinol C, and donepezil on butyrylcholinesterase protein (PDB: 4B0P). Images depict ligand-protein interaction, amino acids involved, and the bond length of the interaction.

in the range (0.41 to 1.00) comparable to the internal standard that demonstrated RMSD of 1.00. Moreover, these molecules demonstrated Ki in the range of 80.76 nm to 67.50 µM. Ki values of all the tested molecules were better than donepezil (Ki = 30.95 µM), with an exception of rutin (Ki =

67.50 µM). Reports also suggest that molecules that demonstrate good docking interaction with BChE are capable of imparting beneficial effect on the progression of AD (Nguyen *et al.,* 2021). The evidence from the clinical setting suggests that targeting BChE is a potential strategy for the management of AD pathogenesis (Zhang *et al.,* 2021).

In AD, one of the major pathological pathways responsible for the development and progression of AD is hyperphosphorylation of Tau proteins (Dai *et al.,* 2021). Tau protein kinases enzyme is responsible for hyper-phosphorylation of tau, leading to the deposition of neurofibrillary tangles and ultimately leading to AD (Wang *et al.,* 2021). The present study suggests that GLN685, GLY565, GLY568, ILE562, LEU688, LYS585, PHE567, THR638, VAL570, and VAL635 are the major amino acids involved in the interaction of internal standard with the protein and molecules showing interactions with these amino acids were predicted to be efficient in targeting Tau Protein Kinase. Our results demonstrated dihydrotanshinone I as the best ligand against Tau protein kinase (1J1B) with the docking score of -10.1 kcal/mol. The docking score of the other herbal molecules that demonstrated better interaction with the Tau Protein Kinase (1J1E) in comparison to Donepezil (-8.0 kcal mol^{-1}) are depicted in Table 3 and the images depicting ligand-protein interactions in Fig. 3. The RMSD values of these molecules were in the range of 0.45 to 1.00, which was less than and comparable to the internal standard (RMSD = 1.00). Moreover, these molecules demonstrated inhibition constants (Ki) in the range of 616.65 nm to 49.46 µM. In comparison to Donepezil (Ki = 27.59 µM), all molecules demonstrated better Ki values with an exception of Rutin (Ki = 49.46 µM). Reports suggest that molecules that demonstrate good docking interaction with Tau Protein Kinase are capable of imparting beneficial effects on the progression of AD in pre-clinical settings (Maurya *et al.,* 2021). Interestingly, accumulated evidence from the clinical setting also suggests that Tau Protein Kinase could be a potential target in managing the pathogenesis of AD (Kim *et al.,* 2021). These predicted molecules could prove beneficial in modulating the pathogenesis of AD, however, further investigation is required.

The results of the docking screening suggest that β-carotene, dihydrotanshinone-I, glabridin, liriodenine, morin, N-formylanonaine, quercetin, quercitrin, rutin, sumaflavone, and vitisinol C are capable of targeting AChE, BChE, and Tau protein kinase, which are the major pathways involved in the pathogenesis of AD. Therefore, we strongly recommend these molecules be further investigated for the management of AD through *in vitro* and *in vivo* approaches that could provide us with some crucial scientific base for the application of these molecules during AD.

Drug likeness properties

The herbal molecules screened from the docking study were subjected to a test for drug-likeness properties along with donepezil. This test is based on Lipinski's rule of five and the results are

Table 3: Binding energies (kcal mol^{-1}), RMSD values, and inhibition constant (Ki) of herbal molecules with 1J1B protein receptor

Ligands	PDB: 1J1B (Tau protein kinase)		
	Docking score (kcal mol^{-1})	RMSD value	Estimated inhibition constant (Ki)
β-carotene	-9.1	0.94	9.63 µM
Dihydrotanshinone I	-10.1	0.45	1.63 µM
Glabridin	-9.6	0.94	2.52 µM
Liriodenine	-9.6	0.59	6.45 µM
Morin	-8.4	0.80	2.95 µM
N-Formylanonaine	-9.2	0.45	6.84 µM
Quercetin	-8.6	1.00	832.25 µM
Quercitrin	-9.9	0.86	3.82 µM
Rutin	-9.6	0.94	49.46 µM
Sumaflavone	-9.6	1.00	11.17 µM
Vitisinol C	-9.5	0.94	616.65 µM
Donepezil	-8.0	1.00	27.59 µM

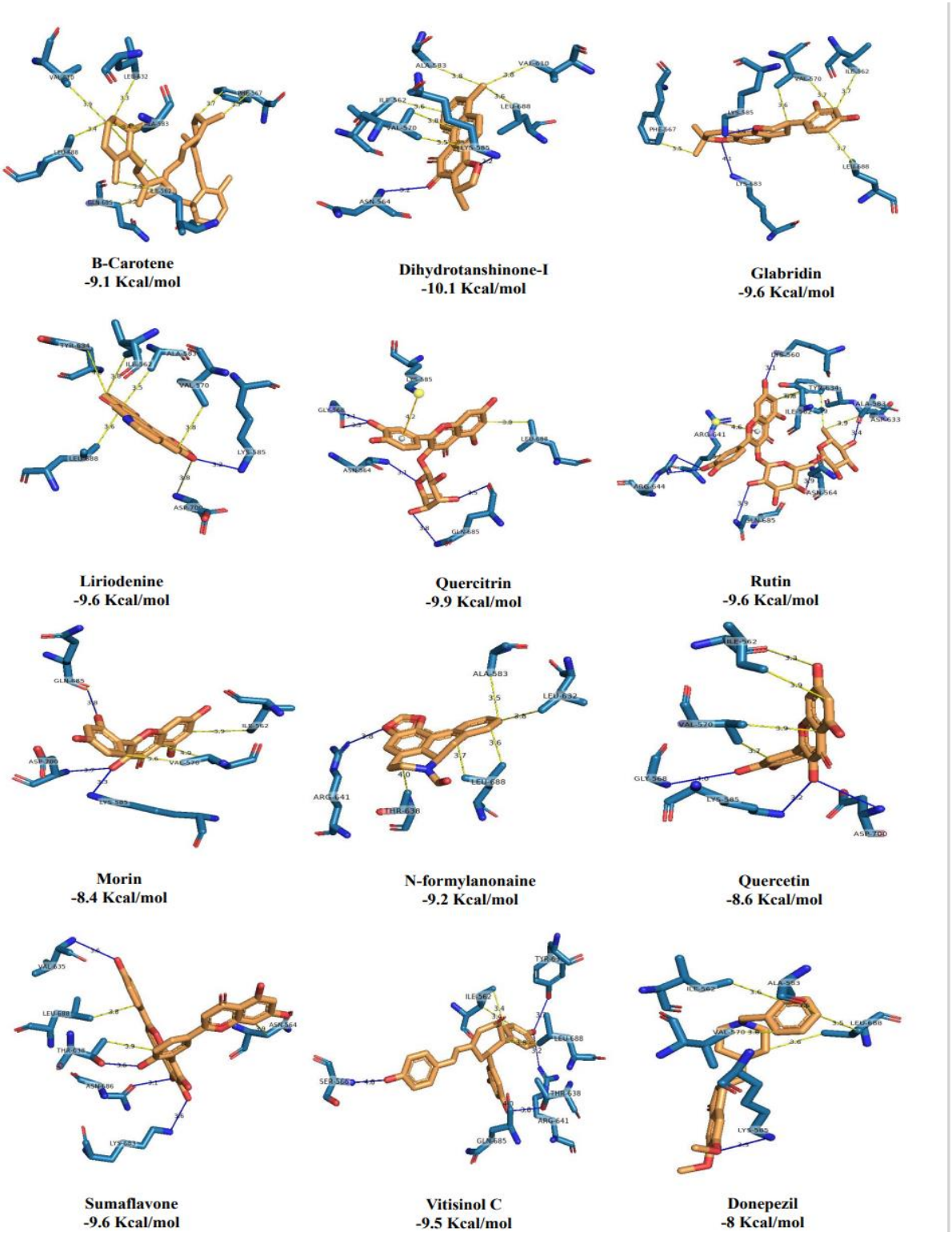

Fig. 3: Docking interaction of β-carotene, dihydrotanshinone I, glabridin, liriodenine, morin, N-formylanonaine, quercetin, quercitrin, rutin, sumaflavone, vitisinol C, and donepezil on tau protein kinase protein (PDB: 1J1B). Images depict ligand-protein interaction, amino acids involved, and the bond length of the interaction.

depicted in Table 4. According to this rule, the molecule should have a molecular weight ≤ 500, a Log P ≤ 5, HBD sites ≤ 5, and HBA sites ≤ 10 to make them likely to be effective via oral administration. The rule suggests that the molecules violating more than one of these rules may have problems with bioavailability (Sarkar *et al.,* 2021). Despite the best binding energy, some herbal molecules like sumaflavone, rutin, β-carotene, and quercitrin violated more than one of these rules, suggesting a potential problem in oral bioavailability. Likewise, as per Veber's rule TPSA values (Shahid *et al.,* 2021) of quercitrin, and rutin were higher than 140 Å, which suggests that these two compounds have lower oral bioavailability. These findings justify the reason that herbal molecules may seem to be effective in preliminary screening but the same results are not obtained in the *in vivo* or clinical settings, which could be due to the lower bioavailability of these molecules. Therefore, to utilize the full therapeutic efficiency of the herbal molecules we need to consider enhancing the bioavailability of these molecules which could lead to better results.

Table 4: Drug likeness properties of the herbal molecules and donepezil

Ligands	MW	Log P	HBA	HBD	nRB	nAtom	TPSA
β-carotene	536.44	14.734	0	0	10	96	0
Dihydrotanshinone I	278.09	2.572	3	0	0	35	43.37
Glabridin	324.14	2.266	4	2	1	44	58.92
Liriodenine	275.06	1.235	4	0	0	30	47.89
Morin	302.04	1.405	7	5	1	32	127.45
N-Formylanonaine	293.11	1.601	4	0	1	37	38.77
Quercetin	302.04	1.834	7	5	1	32	127.45
Quercitrin	448.1	0.802	11	7	3	52	186.37
Rutin	610.15	-0.735	16	10	6	73	265.52
Sumaflavone	535.94	2.807	11	0	3	41	52.6
Vitisinol C	428.16	2.702	5	4	4	56	97.99
Donepezil	379.21	2.633	4	0	6	57	38.77

MW: Molecular weight; Log P: Log of octanol-water partition coefficient; HBA: Hydrogen bond acceptor; HBD: Hydrogen bond donor; nRB: Number of rotatable bonds; nAtom: Number of atoms; TPSA: Topological polar surface area

Antioxidant activity

In the present study, the antioxidant potential of 11 herbal molecules showing the best results in docking study against various targets of AD was evaluated using DPPH, ABTS, and NO radical scavenging assay. These methods are well-established and used to screen the potential of plant extracts/ herbal molecules against oxidative stress with high reliability and accuracy (Apak *et al.,* 2016). DPPH assay is based on the principle of measuring the content of colored DPPH free radical in the methanolic solution spectrophotometrically at 517 nm. In presence of an anti-oxidant molecule, DPPH free radicals get converted to a more stable and colorless form after gaining electrons from the potent antioxidant. This implies that the greater the antioxidant potential of a molecule, the more discoloration will be produced (Digiacomo *et al.,* 2015). In the present study, a strong dose-dependent antioxidant potential of β-carotene, quercetin, quercetrin, rutin, sumaflavone, and vitisinol C. The IC_{50} values of these molecules were significantly ($p < 0.05$) higher than the other molecules and were observed in the range of 0.38 ± 0.004 to 0.86 ± 0.12 µM, with quercetin demonstrating the best antioxidant potential (IC_{50} value of 0.38 ± 0.004 µM). The results of the DPPH radical scavenging assay are depicted in Fig. 4 (A).

ABTS assay is based on the spectrophotometric estimation of ABTS chromatophore (cation) that is generated by the reaction of ABTS and potassium persulfate. In presence of an antioxidant moiety, the generation of ABTS chromatophore is reduced depending on the antioxidant potential of an antioxidant. The extent of ABTS discoloration correlates to the antioxidant potential of a test molecule (Rahman *et al.,* 2018). In present study, a strong dose-dependent ABTS scavenging potential for quercetin, sumaflavone, quercetrin, and rutin with their IC_{50} values in the range of 1.38

± 0.04 to 2.45 ± 0.02 µM were observed (Fig. 4B). The IC_{50} values of these molecules were significantly higher than other tested molecules. In this assay, quercetin was found most potent anti-oxidant with IC_{50} value of 1.38 ± 0.04 µM. Other tested molecules also demonstrated dose-dependent antioxidant potential but were not as efficient as quercetin, sumaflavone, quercetrin, and rutin.

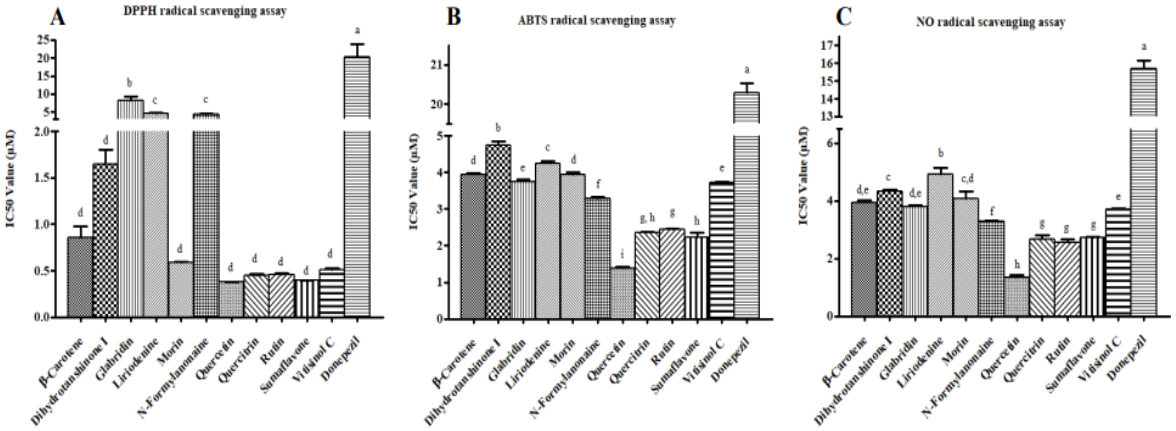

Fig. 4: IC_{50} values for antioxidant activity of β-carotene, dihydrotanshinone I, glabridin, liriodenine, morin, N-formylanonaine, quercetin, quercitrin, rutin, sumaflavone, vitisinol C, and donepezil. A): DPPH radical scavenging assay; B) ABTS radical scavenging assay; C) NO radical scavenging assay. Data are expressed as means ± standard deviation (n = 3). Different letters above the error bar of histograms indicate significant variation at $p < 0.05$ by Duncan's multiple range test.

NO is an essential free radical involved in normal physiological functioning of mammalian cells, including neurons (Madhusoodanan and Murad, 2007). During AD and diabetic disorders NO is generated excessively and gets converted to various reactive nitrogen species like peroxynitrite radicals (ONOO), which are known to be associated with AD pathogenesis (Poon *et al.*, 2004). NO possess a very short half-life in an aqueous solution. The levels of NO were quantified in terms of the final oxidized products nitrite (NO^{2-}) and nitrate (NO^{3-}) by using Griess reagent (Udayabanu *et al.*, 2008). In the present study, a strong dose-dependent NO radical scavenging potential of quercetin, sumaflavone, quercetrin, and rutin was observed (Fig. 4C) with IC_{50} values between 1.35 ± 0.08 and 2.72 ± 0.03 µM ($p < 0.05$ when compared to other molecules). Moderate antioxidant potential was noted for other molecules but quercetin proved best with IC_{50} value of 1.35 ± 0.08 µM.

Antioxidant assays suggest that quercetin, sumaflavone, quercetrin and rutin have very strong antioxidant potential and can interfere in oxidative stress pathway, which is one of the major path-ways in the development and progression of AD and neurodegeneration. Although, donepezil is used in clinical settings for the management of AD, but it fails to produce any significant antioxidant effect. Donepezil only acts by inhibiting AChE activity (Abdel-Salam *et al.*, 2016) and does not have any effect on the oxidative stress pathway during AD, due to which AD may continue to progress slowly despite therapy with donepezil (Li *et al.*, 2018). Herbal molecules showed good interactions with various targets of AD in docking study and have excellent potential to counter oxidative stress; therefore, these molecules could target multiple pathways involved in pathogenesis and could prove beneficial during AD. Oxidative stress exposure to brain is reported to lead to the development and progression of AD (Ahmed and Braidy, 2021). Excessive oxidative stress is depicted by the generation of excessive reactive oxygen and nitrogen species (ROS/RNS) and reduction or saturation in the levels of natural antioxidants of brain such as catalase, total thiol, SOD, etc. (Sharma *et al.*, 2021). Excessive ROS and RNS induce oxidative damage to brain tissue, protein, lipid, and DNA, thus compromising the normal physiology and function of brain and play a major role in the progression of neurodegeneration and cognitive impairment (Buccellato *et al.*, 2021). Numerous evidence suggest that oxidative stress and its products show deleterious effects on

many cellular targets in AD (Lanzillotta *et al.*, 2021; Misrani *et al.*, 2021). In AD brains there is an increase in lipid peroxidation in temporal lobe which can be observed with histopathological alterations (Ramsden *et al.*, 2021). The molecules like asiaticin, bacosides A, scentellin and centellicin reportedly have beneficial effect on the progression of AD and neurodegeneration by targeting the oxidative stress in brain (Mehla *et al.*, 2020). Therefore, quercetin, sumaflavone, quercetrin, and rutin can prove beneficial during AD and neurodegenerative conditions by reducing oxidative damage in brain.

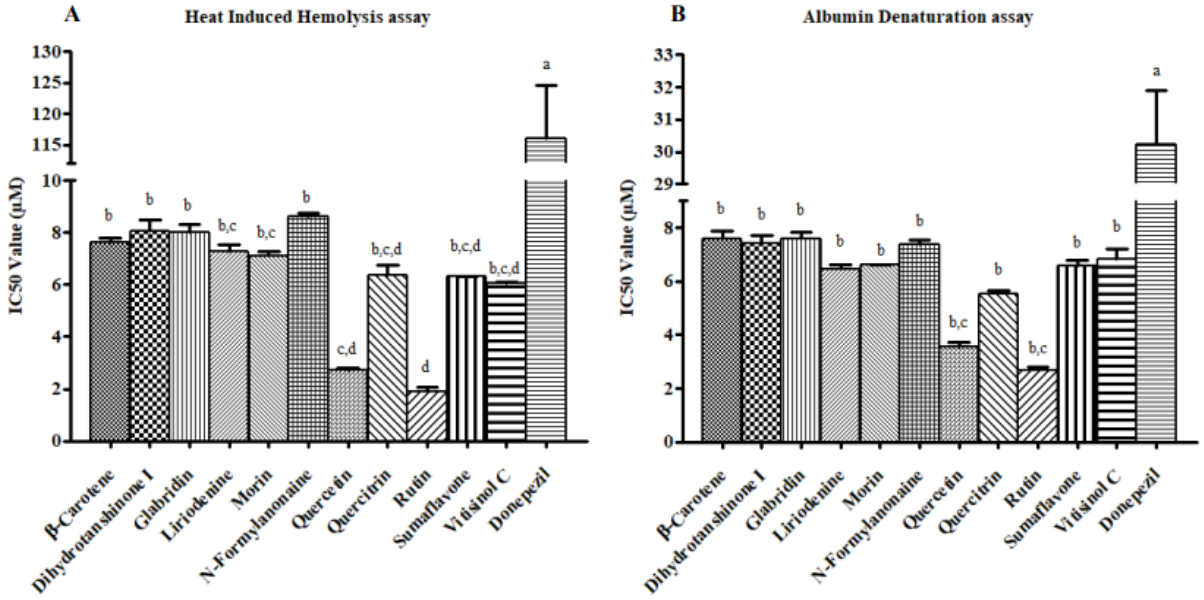

Fig. 5: IC_{50} values for the anti-inflammatory activity of β-carotene, dihydrotanshinone I, glabridin, liriodenine, morin, N-formylanonaine, quercetin, quercitrin, rutin, sumaflavone, vitisinol C, and donepezil. A: Inhibition of heat induced haemolysis; (B) Inhibition of protein denaturation. Data are expressed as means ± standard deviation (n = 3). Different letters above the error bar of histograms indicate significant different at $p < 0.05$ by Duncan's multiple range test.

Anti-inflammatory assay

The anti-inflammatory potential of 11 herbal molecules which showed the best results in docking study against various targets of AD was evaluated using inhibition of heat-induced hemolysis and albumin denaturation assay, which are well-established methods to screen molecules for *in-vitro* anti-inflammatory potential with good reliability (Nashaat *et al.*, 2021). An inflammatory response in the mammalian cell is initiated by cell membrane by generating arachidonic acid from membrane phospholipids which, in turn, lead to the generation of various inflammatory mediators through cyclooxygenase and lipoxygenase pathways. Hence, it may be assumed that the stabilization of the cellular membrane will lead to the reduction in the generation of inflammatory mediators; thereby may suppress the inflammation (Zhang and Cui, 2021). Inhibition of heat-induced hemolysis is based on the principle of stabilizing cell membrane and thereby could correlate to suppression of inflammation and aid in the *in-vitro* screening of molecules for anti-inflammatory potential. Since the cell membrane of RBC is structurally and functionally identical to the lysosomal membrane, therefore, it is used in *in-vitro* anti-inflammatory assays (Paul *et al.*, 2021). It has been reported that several anti-inflammatory drugs stabilize the lysosomal membrane to prevent the release of hydrolytic enzymes responsible for inflammation (Narendhirakannan *et al.*, 2021). In the present study, rutin (IC_{50} = 1.89 ± 0.16 µM) and quercetin (IC_{50} = 2.71 ± 0.11 µM) were the most potent molecules to prevent heat-induced hemolysis of RBC in a dose-dependent manner ($p < 0.05$ when compared to other molecules), suggesting that these molecules could have strong anti-inflammatory potential (Fig. 5A). *In vitro* anti-inflammatory potential of other molecules in terms of preventing

heat-induced hemolysis was found in the range of 6.06 ± 0.04 to 8.61 ± 0.15 µM, so demonstrating the moderate potential to prevent heat-induced hemolysis of RBCs.

Proteins perform specific and well-defined functions within the living system and their activity is governed by their structural integrity to a great extent (Wang and Wu, 2018). Various internal and external factors and stressors could result in the loss of structural integrity of protein molecules and hence, compromise the normal physiological functions governed by these proteins (Lim *et al.,* 2005). Alteration in the structural integrity of tau protein in CNS leads to the development of AD and this is one such example wherein altering the structural integrity of protein leads to disorders and compromised functioning (Taccola *et al.,* 2021). Denaturation of proteins is closely associated with inflammation. Reports suggest that non-steroidal anti-inflammatory drugs show good potential to prevent protein denaturation (Salaria *et al.,* 2021). On this background, we evaluated the *in vitro* anti-inflammatory potential of herbal molecules to prevent denaturation. The results suggest that rutin and quercetin are capable of preventing albumin denaturation in a dose-dependent manner and their IC_{50} values were significantly ($p < 0.05$) higher than the other molecules and were observed in the range of 2.69 ± 0.12 and 3.54 ± 0.2 µM, respectively (Fig. 5B). The IC_{50} values of other herbal molecules were in the range of 5.53 ± 0.12 to 7.60 ± 0.26 µM in a dose-dependent manner, suggesting their moderate potential to prevent the denaturation of albumin.

The *in-vitro* anti-inflammatory assays suggested that quercetin and rutin have very strong anti-inflammatory potential; thus can interfere in the inflammatory stress pathway, which is one of the major pathways in the development and progression of AD and neurodegeneration. Donepezil failed to produce any significant anti-inflammatory effect. Donepezil acts by inhibiting AChE activity which explains its beneficial effect during AD (Masurkar *et al.,* 2021). Herbal molecules interacted well with various targets of AD in docking study and have excellent potential to counter oxidative and inflammatory stress, thus these molecules could target multiple pathways involved in AD pathogenesis and could prove beneficial during AD.

Blood Brain Barrier (BBB) is a crucial factor to be considered for any molecule to be biologically active within the CNS. Our findings are further strengthened by previous reports where quercetin and rutin have been demonstrated to cross BBB to exert their beneficial effects in the CNS, which include neuroprotection, alleviation of neuronal oxidative stress, neuronal proliferation, etc. (de Boer *et al.,* 2005; Faria *et al.,* 2010; Huebbe *et al.,* 2010; Ishisaka *et al.,* 2011; Habtemariam *et al.,* 2016).

Conclusion: The study suggests that herbal molecules are capable of targeting multiple pathways involved in AD pathogenesis so could prove beneficial in AD management. Docking study of 100 herbal molecules on AChE, BChE, and Tau protein kinase predicted that β-carotene, dihydrotanshinone-I, glabridin, liriodenine, morin, N-formylanonaine, quercetin, quercitrin, rutin, sumaflavone, and vitisinol-C have good affinity to bind to these targets, and have good RMSD and Ki values that were better or comparable to the internal standard. The study suggests that these herbal molecules can target AChE, BChE, and Tau protein kinase to impart beneficial effects. On narrowing down the screening process through *in vitro* antioxidant and anti-inflammatory assay, we concluded that sumaflavone, quercetin, and rutin have potential to counter oxidative and inflammatory stress pathways of AD pathogenesis. The drug likeliness properties evaluated through Lipinski's rule of five suggest that sumaflavone and rutin could demonstrate an issue with oral bioavailability. Considering the potential of quercetin to cross BBB along with our findings, quercetin appears to be the best molecule that could target AChE, BChE, Tau protein kinase, oxidative stress pathway, and inflammatory stress pathway with good drug likeliness capacity. However, these preliminary findings need preclinical studies on the best molecules to reach a decisive conclusion about their potential against AD pathogenesis.

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