Evaluation of *In-silico* and *In-vitro* Acetylcholinesterase Inhibitory Potential of Fulvic Acid and Humic Acid

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Abstract

Alzheimer's disease, commonly known as senile dementia, is the main type of dementia and is an age-related neurodegenerative disease. The cholinergic neurons located in the hippocampus, basal forebrain and cerebral cortex are responsible for cognition and their destruction is responsible for the decrease in cholinergic activity and leads to the occurrence of various cognitive deficits. Acetylcholinesterase is a crucial enzyme that rapidly breaks down the neurotransmitter acetylcholine and ultimately terminates the cholinergic transmission on the postsynaptic membrane. Targeting the acetylcholinesterase enzyme which is responsible for hydrolysis of Ach into choline and acetate has proved of great importance in the management of dementia. Fulvic acid and Humic Acid is a class of organic compounds resulting from the decomposition of biological matter, which is the result of the action of many microorganism. In this present work, a set of organic compounds Fulvic acid and humic acid against AChE enzyme were screened by computational chemistry techniques. The docking results showed a good binding affinity towards AChE. These two compounds were then studied by molecular dynamics simulations. The binding free energy calculation and ligand-protein binding pattern suggested that FA and HA could interact with AChE very well. Since *in-vitro* anti-AChE activity tested for FA and HA it was compared with standard, donepezil. The IC_{50} of standard, donepezil and FA and HA against AChE were 13.45± 0.059 µg/mL 34.69±0.04 µg/mL and 91.87±0.01 µg/mL, respectively. This finding provided that the compound has the potential to be as a therapeutic agent for further anti-AChE drug development in treatment of Alzheimer's disease.

Keywords: *Alzheimer's disease, Acetylcholinesterase, Fulvic acid, Humic acid, Molecular Docking*.

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Introduction:

Alzheimer's disease (AD), commonly known as senile dementia, is the main type of dementia and is an age-related neurodegenerative disease [1]. The incidence of AD gradually increases with age, and the incidence rate can be as high as 50% in individuals over 85 years old [2,3]. With the aging of the global population, the number of patients with AD inevitably increases. According to the statistics re- ported by the World Health Organization, at the end of 2019, more than 50 million people worldwide suffered from AD. The total number of patients with AD globally is estimated to reach 82 million in 2030 and 152 million in 2050 [4,5]

The cholinergic neurons located in the hippocampus, basal forebrain and cerebral cortex are responsible for cognition and their destruction is responsible for the decrease in cholinergic activity and leads to the occurrence of various cognitive deficits [6,7]. Improvement of the activity of cholinergic neurons seems to be the only way to develop potent drugs for the management of AD. It is carried out by modulating the levels of the neurotransmitter acetylcholinesterase (AChE) in the central nervous system. Acetylcholinesterase is a crucial enzyme that rapidly breaks down the neurotransmitter acetylcholine and ultimately terminates the cholinergic transmission on the postsynaptic membrane [8]. Inhibition of AChE causes acetylcholine accumulation in the synapses; this enhances the effects of acetylcholine, enhances the cholinergic nervous system function, and induces intellectual capabilities [9,10]. Targeting the acetylcholinesterase (AChE) enzyme which is responsible for hydrolysis of Ach in to choline and acetate has proved of great importance in the management of dementia [11]. During the past decade, investigation of the crystallographic structures of AChE and its complex with inhibitors has provided information regarding the pharmacophoric features necessary to elucidate the underlying catalytic mechanism and key interactions for the discovery of novel and potent AChEIs [12,13]. Recently, in 2012, Cheung and co- workers presented a high-resolution crystal structure of human AChE (hAChE), in complex with the drug; donepezil (DON), an AD drug; revealing the interactions involved between the ligand and hAChE enzyme and the various binding sites and an choring amino acid residues critical for the inhibition of hAChE [14]. This discovery has brought an important breakthrough in the field of development of anti-AChE agents. As a result, computational simulations coupled with structure activity studies may now be used to mimic interactions within the active site of recombinant hAChE and to under- stand the subtle factors that govern AChE inhibition activity [15,16]. Nowadays, the docking simulations are coupled with other theoretical methods such as: Quantum Mechanics (QM), and Molecular Dynamics (MD). They try to take advantage of each of these methods and to get better results.

Fulvic acid (FUL) and Humic Acid (HUM) is a class of organic compounds resulting from the decomposition of biological matter (i.e., plants and animals), which is the result of the action of many microorganism [17]. FUL has been screened for Cardioprotective [18], anti-inflammatory, anti-diabetic [19], antioxidant activity [20,21] and *in-vitro* inhibits aggregation and promotes disassembly of tau fibrils associated with Alzheimer's disease [22] and HUM has been screened for neuroprotective [23], hepatoprotective activity [24] and antioxidant activity [25].

Materials and Methods:

Materials: Fulvic acid was purchased from Suvidhinath Laboratories (Vadodara, Gujarat, India), Humic acid, Acetylcholine esterase (AChE), 5,5'-dithiobis [2] nitrobenzoic acid] DTNB), Acetyl thiocholine (AChI) and Tris [hydroxyl methyl] methane (Tris buffer) were purchased from Sigma Aldrich Pvt ltd, Bangalore.

***In-silico* Molecular Docking and Molecular Dynamic Simulation:**

Ligand Preparation: The Schrödinger Suite LigPrep model was used to prepare the ligands. For energy minimization, LigPrep follows the Optimized Potential Liquid Simulations for All Atoms (OPLS-AA) force field.

Protein Preparation: The Research Collaborator for Structural Bioinformatics (RCSB) Protein Data Bank (http://www.rcsb.org/) was used to retrieve the X-ray crystal structure of the target acetylcholinesterase (AChE) in complex with donepezil (PDB: 4EY7) and AChE in complex with rivastigmine (PDB: 1GQR). The obtained crystal structure of the target was processed by removing the presented ligand molecule and water molecules, the missing hydrogen atoms were attached using protein preparation wizard of GLIDE software

Molecular Docking: Molecular docking technique was applied to study the binding orientation and affinity of the bioactive compounds from Thai herbs toward the binding site of AChE enzyme using AutoDock 4.2 [26,27].

Molecular dynamics (MD) simulations: The molecular dynamic simulation was evaluated to determine the binding stability, conformation and interaction modes between the selected bioactive compounds (ligands) and targets (AChE). The selected ligands-AChE complex files were subjected to molecular dynamics studies using Desmond 2020.1 software. For molecular dynamic simulation, first vacuum was minimized using the steepest descent algorithm for 5000 steps. The complex structure was solvated in a cubic periodic box of 0.5 nm with a simple point charge (SPC) water model. The complex system was subsequently maintained with an appropriate salt concentration of 0.15 M by adding a suitable amount of Na^+ and Cl^- counter ions. Each complex was allowed a simulation time of 50 ns from the NPT (Isothermal-Isobaric, constant number of particles, pressure, and temperature) equilibration was subjected in NPT ensemble for final run. The trajectory analysis of root means square deviation (RMSD) and root mean square fluctuation (RMSF) [28,29].

***In-vitro* Acetylcholinesterase Inhibition Assay:** Based on *in-silico* results, FUL and HUM were selected for their AChE inhibitory activities [30]. AChE activity was measured by using spectrophotometer based on Ellman's method [31]. The enzyme hydrolyses the substrate acetylthiocholine resulting in the product thiocholine which reacts with Ellman's reagent (DTNB) to produce 2-nitrobenzoate-5-mercaptothiocholine and 5-thio-2nitrobenzoate which can be detected at 405 nm. About 1.3 ml of the Tris-HCl buffer (pH 8.0; 50 mM) was treated with 0.4 ml of different concentrations (12.5 – 400 μg/ml) of the compounds (FUL & HUM) and 0.1 ml of the AChE (0.28 U/ml) was added. This mixture was incubated for 15 minutes and 0.3 ml of acetylthiocholine iodide (0.023mg/ml) and 1.9 ml (5,5-dithiobis- (2- nitrobenzoic acid) DTNB (3 mM) solution were added. This final reaction mixture (4.0 ml) was kept for further incubation at room temperature for 30 minutes and the absorbance of the reaction mixture was measured at 405 nm. The assay was done in triplicate and the results were

expressed as mean ± SEM. Donepezil was used as standard [32]. The percentage inhibition was calculated for the compounds (FUL, HUM and Donepezil) using the following formula,

$$\%\ Inhibition = \frac{A0 - A1}{A0} \times 100$$

where,

A0 is Absorbance of control

A1 is Absorbance of standard

The control was prepared by replacing the drug with the suitable solvent. The blank was prepared by replacing all the reagents with the solvent (water) to nullify the effect of color of the tested. All determinations of the assay were done in triplicate and the results were expressed as standard error of mean [33,34]

Result:

***In-silico* Molecular Docking and Molecular Dynamic Simulation:**

The binding affinity scores of fulvic acid, humic acid and standard drugs, donepezil (DON) and rivastigmine (REV) are shown in table 1.

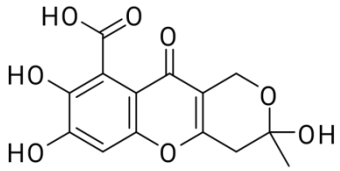
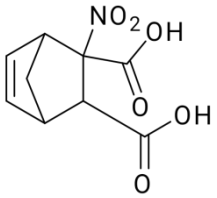
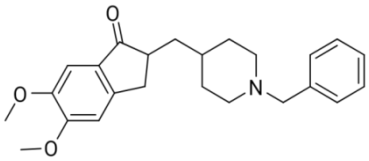
Compound	Structure	PDB ID: 4EY7			PDB ID: 1GQR		
		S (kcal/mol)	RMSD-Refine(Å)	Rg Values	S (kcal/mol	RMSD Refine	Rg Value
Fulvic Acid		-7.02	2.21	23.1	-6.78	2.17	23.2
Humic Acid		-5.62	2.31	23.4	-5.46	2.28	23.3
Donepezil		-8.49	2.11	22.89	-	-	-
Rivastigmine		-	-	-	-7.02	2.61	23.4

Table 1. Molecular docking scores (kcal/mol) of Fulvic acid, Humic acid with two standard drugs against two different human target proteins associated with AD

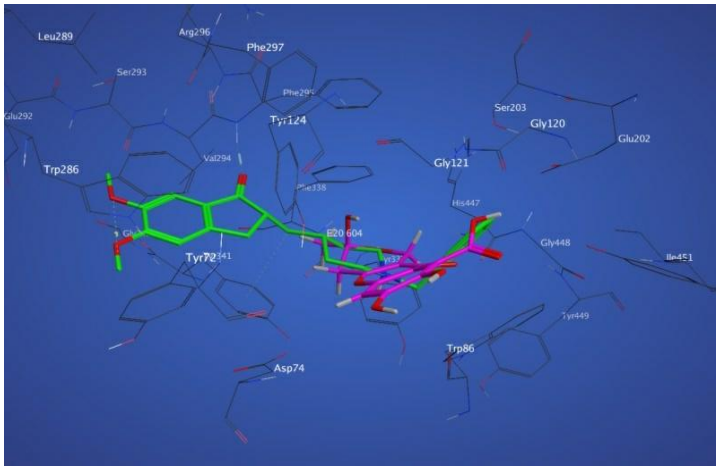


Figure 1: 3D interaction of Donepezil (green) and FA (Pink) in the active site of 4EY7

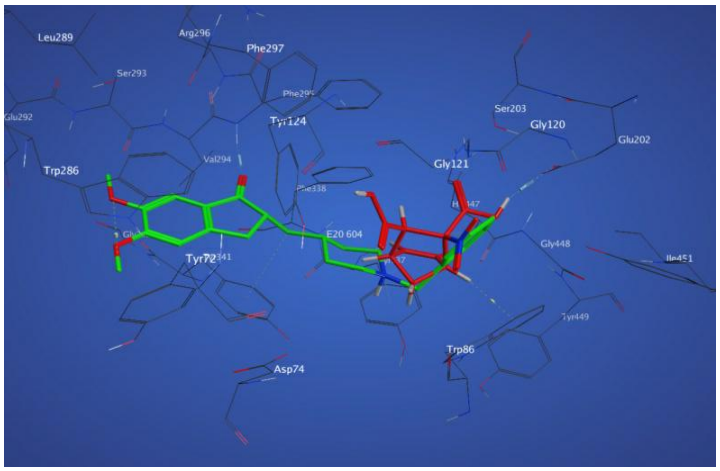


Figure 2: 3D interaction of Donepezil (green) and FA (Red) in the active site of 4EY7

Figure 3. 3D interaction of Donepezil (green), FA (Pink) and HA (Red) in the active site of 4EY7

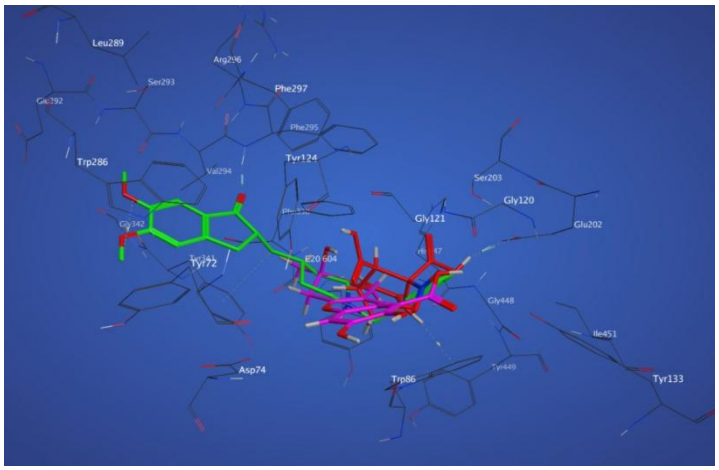
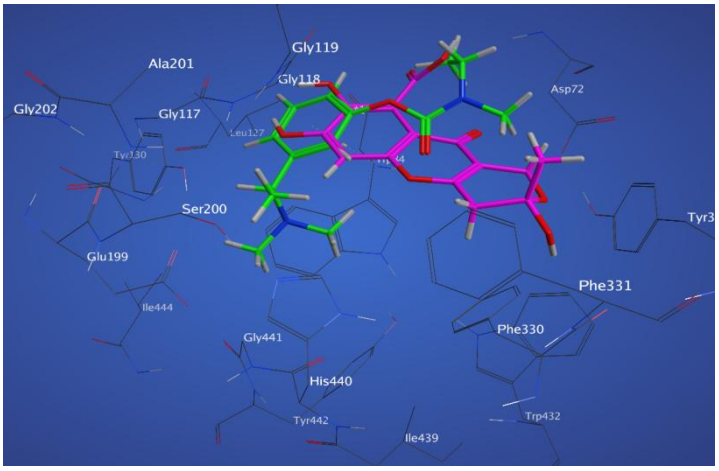


Figure 4. 3D interaction of rivastigmine (green) and fulvic acid (pink) in the active site of 1GQR



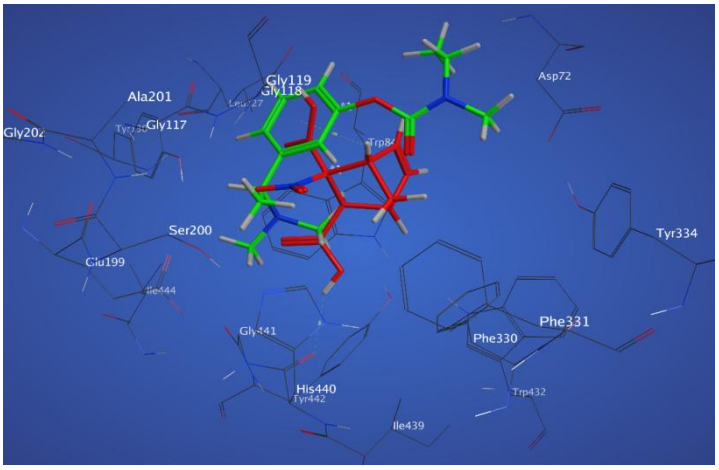


Figure 5. 3D interaction of rivastigmine (green) and humic acid (red) in the active site of 1GQR

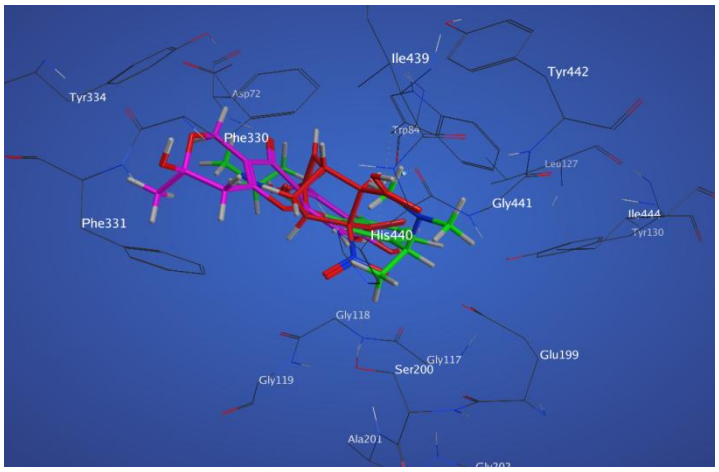


Figure 6. 3D interaction of rivastigmine (green), fulvic acid (pink) and humic acid (red) in the active site of 1GQR

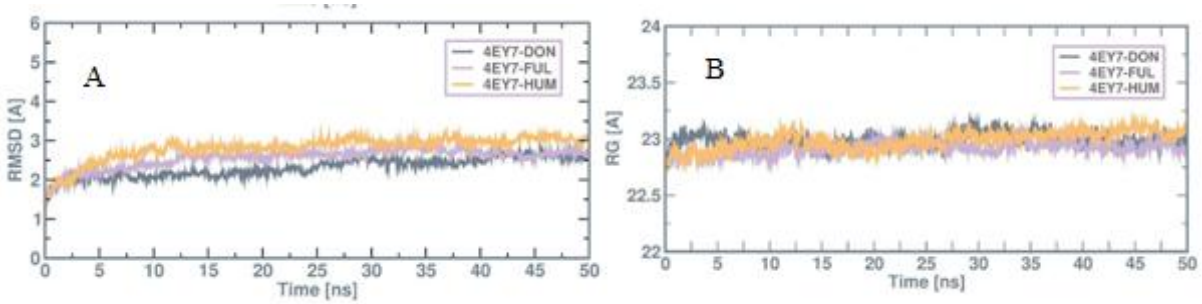


Figure 8. Generated RMSD plot and Rg plot from MD simulation at 100 ns. (A), RMSD plot of 4EY7-DON, 4EY7-FUL, 4EY7-HUM and (B), Rg plot of 4EY7-DON, 4EY7-FUL, 4EY7-HUM

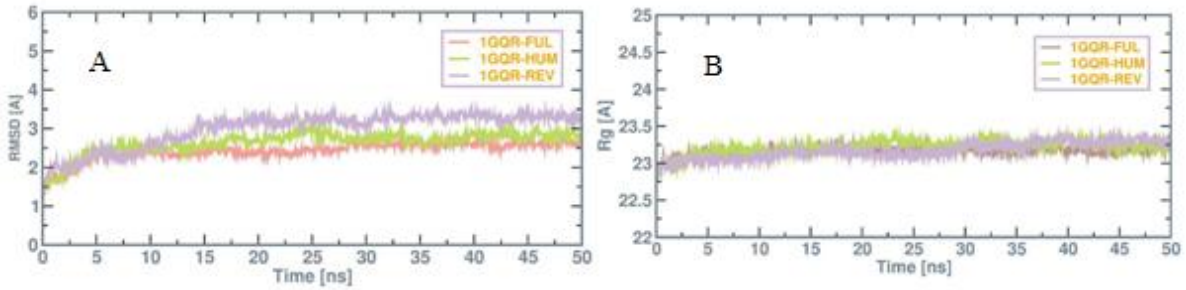


Figure 7. Generated RMSD plot and Rg plot from MD simulation at 100 ns. (A), RMSD plot of 1GQR-REV, 1GQR-FUL, 1GQR-HUM and (B), Rg plot of 1GQR-REV, 1GQR-FUL, 1GQR-HUM

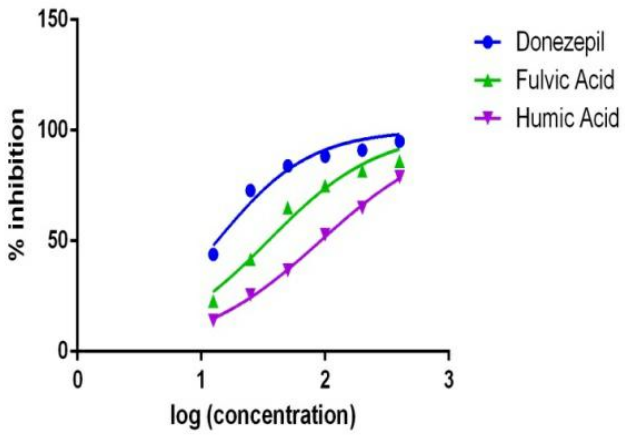
In-vitro Acetylcholinesterase Inhibition Assay

Table 2. *In-vitro* AChE inhibitory effect of Donepezil, Fulvic acid and Humic acid

Concentration (µg/ml)	% Inhibition of AChE by Donepezil Hydrochloride	% Inhibition of AChE by Fulvic acid	% Inhibition of AChE by Humic acid
12.5µg/ml	43.89 ± 2.940	22.77±2.421	13.88 ±2.00
25 µg/ml	72.78 ± 0.553	41.66±0.961	25.55 ±1.47
50 µg/ml	83.88 ± 0.556	65.00±1.923	36.66±1.92
100 µg/ml	88.33 ± 0.961	75.00±0.964	52.77±2.00
200 µg/ml	91.11 ± 1.470	81.67±0.961	64.88±1.83
400 µg/ml	95.00 ± 0.964	86.11±0.556	78.88 ±0.88
IC_{50}	13.45 ± 0.05 µg/ml	34.69±0.04 µg/ml	91.87±0.012 µg/ml

Results are presented as mean ± SEM (n =3).*p < 0.05 denotes significant difference compared to the donepezil.

Figure 9. In-*vitro* AChE inhibitory effect of Donepezil, Fulvic acid and Humic acid



Discussion:

FUL and HUM, natural compounds investigated for the *in-silico* AChE binding affinity (S score on Table 1) properties. Fulvic acid exhibited a binding affinity of -7.02 kcal/mol against 4EY7 and -6.78 kcal/mol against 1GQR. Humic acid exhibited a docking score of -5.62 kcal/mol against 4EY7 and -5.46 kcal / mol against 1GQR. While donepezil and rivastigmine used as the reference standard had binding affinity of -8.49 kcal/mol (4EY7) and -7.02 kcal/mol (1GQR) respectively. Fulvic acid showed a better binding profile than humic acid. The binding energy of fulvic acid was close to the binding energy of the reference compound Donepezil. Donepezil showed a higher docking score as compared to rivastigmine.

Further analysis of generated docking conformations (Figure 1-6) suggests that FUL and HUM shares a similar binding orientation and interaction in comparison to donepezil and rivastigmine. These *in-silico* results revealed that the natural compounds (fulvic acid and humic acid) bind and interact with AChE almost at the same binding site and their interaction pattern is more similar to donepezil (Figure 3 and 4).

On the basis of docking results, we selected 1GQR-FUL, 1GQR-HUM, 1GQR-REV and 4EY7-DON, 4EY7-FUL, 4EY7-HUM protein to perform molecular dynamics simulation study. The simulation was carried out on protein-ligand complexes, Vera–bound systems to study the dynamic behavior of the targeted protein. The average RMSDs for 4EY7-FUL, 4EY7-HUM revealed 2.21 +/- 0.02 Å and 2.31 +/- 0.02 Å respectively, while 4EY7-DON protein complex showed 2.11 +/- 0.02 Å against target protein, 4EY7. The average RMSDs from 0 to 50 ns for 1GQR-FUL, 1GQR-HUM, and 1GQR-REV protein complex proteins were 2.17 +/- 0.03 Å, 2.28 +/- 0.01 Å, 2.61 +/- 0.02 Å respectively. The RMSD value of fulvic acid was closer to the reference compound donepezil.

Root mean square deviation (RMSD) of the 1GQR-FUL, 1GQR-HUM, and 1GQR-REV complexes did not show any significant deviation (Figures 8A). These RMSD results represent the relative stability of compounds complex throughout the simulation. Overall, the RMSD results indicate that 4EY7-DON, 4EY7-FUL, 4EY7-HUM protein complexes considered were relatively stable when compared to 1GQR-FUL, 1GQR-HUM, and 1GQR-REV protein complexes throughout the simulation. The differences between the complexes were small, suggesting that they are very similar in terms of their stability. This is likely due to the fact that they all contain similar components in their structures. Therefore, the RMSD values are a useful metric for comparing the stability of different protein complexes.

The radius of gyration can be described as the mass-weighted root mean square distance of atoms from their center of mass. The competence, shape folding of the overall structure at different time points during the trajectory can be seen in the Rg plot illustrated in Figure 7B, 8B. Throughout the simulation, 1GQR-FUL, 1GQR-HUM, 1GQR-REV and 4EY7-DON, 4EY7-FUL, 4EY7-HUM complexes exhibited a similar pattern of Rg value. The average RG value from 0 to 100 ns for 1GQR-FUL, 1GQR-HUM, 1GQR-REV protein complex proteins were 23.2 +/- 0.01 A, 23.3 +/- 0.02 A, 23.4 +/- 0.02 A and for protein complex, 4EY7-DON, 4EY7-FUL, 4EY7-HUM were 22.89 +/- 0.01 A, 23.1 +/- 0.02 A, 23.4 +/- 0.02 A and respectively. The Rg values of the protein complex indicate that there is no significant difference between the

shapes folding of the complex at different time points during the trajectory. This suggests that the protein complex is structurally stable throughout the simulation.

Reduction of ACh in the hippocampus and cortex of the brain is one of the most important remarkable changes observed in AD. The critical role of cholinesterase in neural transmission makes them a key target of a large number of cholinesterase-inhibiting drugs relevant to the treatment of neurodegenerative disorders, including AD. To evaluate the potential of the FUL and HUM as an anti-AD drug, its AChE inhibitory activities were quantified. As shown in table-2 the FUL and HUM showed significant AChE inhibitory effects when compared to the standards and the cholinesterase inhibitory activity occurred in a dose-dependent manner. FUL and HUM was found to inhibit AChE activity by 86.11% and 78.88% at a concentration of 200 µg/mL, while donepezil, used as the reference standard in this study, inhibited the AChE activity by 95 % under the same experimental condition. The IC_{50} of standard donepezil and FUL and HUM against AChE were 13.45 ± 0.059 µg/mL, 34.69 ± 0.04 µg/mL and 91.87±0.01 µg/mL, respectively (Table 2). Fulvic acid was found to exhibit a better AChE inhibitory activity as compared to standard, donepezil.

Conclusion:

The *in-silico* and *in-vitro* findings of this study confirm that fulvic acid and humic acid are potential inhibitors of acetylcholinesterase enzyme. Further studies are required to confirm the therapeutic potential of fulvic acid and humic acid.

Conflict of Interest:

The authors have no conflict of interest regarding this investigation.

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