COMPARATIVE STUDY OF HALO-SUBSTITUTED AURONES AGAINST NEUROPROTECTIVE TARGETS OF ALZHEIMER'S DISEASE BY IN-SILICO METHOD AND SYNTHESIS OF FLAVONOIDS WITH IN-VITRO TESTING

SABITHA T B[1]*, B SRIVASTAVA[1], B RAJINIKANTH[2]

[1]School of Pharmaceutical Sciences, Jaipur National University, Jaipur, Rajasthan-302025, INDIA.

[2]Department of Pharmacology, Grace College of Pharmacy, Kodunthirapully P.O, Palakkad-678004, INDIA.

SABITHA THEKKUMPADATH BHASKARAN

PhD Research Scholar,

School of Pharmaceutical Sciences,

Jaipur National University,

Jaipur, Rajasthan-302017,India.

E-mail address: sabitharenjith8489@gmail.com

Orcid ID: 0000-0002-8116-2081

Tel: 8301977886

ABSTRACT:

Alzheimer's disease is a progressive neuronal deterioration due to generalized degeneration of the brain. Flavonoids like aurones show anti-Alzheimer's activity through neuronal protective mechanisms in the brain. so, work aims to study the anti-Alzheimer's activity of various halogen-substituted aurones(flavonoids) through docking to the structural conformation of ligands to the active sites of proteins and to predict the binding affinity. The drug-likeness and the pharmacokinetically activity were identified and compared. The selected compounds were docked by the AutoDock 4.2 to predict Anti-Alzheimer's activity against Acetylcholinesterase, Butyrylcholinesterase and Beta secretase enzymes among the three enzymes aurones show better inhibitory activity towards Acetylcholinesterase enzyme.

Based on molinspiration drug-likeness properties, halo-substituted flavonoids were synthesized. They were tested for in vitro acetylcholinesterase enzyme inhibitory activity and in-vivo anti-Alzheimer's activity. Further, based on the in vitro acetylcholinesterase inhibitory assay, all the synthesized flavonoids possess anti-Alzheimer's activity. Among the nine synthesized flavonoids, 2-chloro flavone showed maximum inhibition at lower concentrations. Further, it is confirmed in in-vivo.

KEYWORDS: Alzheimer's disease, Flavonoids, Molecular docking, Halo-Substituted aurones, docking, Invitro Acetylcholinesterase inhibition.

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INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder, characterized by memory deficit or dementia in advancing age reflected as difficulty in thinking, cognition, decision-making, abrupt behaviour and speech, and makes every task of a day-to-day life complex. The evidence supporting the involvement of increased amyloid beta (AB) production due to genetic polymorphisms, aging, stress, and poor lifestyle AB is produced by a misbalance in two competing pathways amyloidogenic and non-amyloidogenic including the metabolism of the mature amyloid precursor protein (APP). Flavonoids or bioflavonoids are a class of plant secondary metabolites that are polyphenolic compounds. Some of the best-known flavonoids are chalcones and aurones. Aglycones, glycosides, and methylated derivatives all exist as forms of flavonoids [1- 7].

Flavonoids can influence central nervous system activity either by binding to the benzo diazepam site on the GABA receptor resulting in sedation, anxiolytic, or anticonvulsive effects, or by acting as inhibitors of monoamine oxidase a or b there by working as antidepressants and anti-Parkinson's. Molecular docking is an attempt to find the best matching between two molecules or docking is a method that predicts the preferred orientation of one ligand when bound in an active site to form a stable complex. Chemical work is aided by molecular docking. The best-fit orientation of a ligand that binds to a particular protein of interest is described by molecular docking, which can be viewed as an optimization issue. The goal of molecular docking is to reduce the free energy of the entire system by achieving an optimal conformation for the protein and ligand as well as their relative orientation. Docking can be achieved through two interrelated steps: first by sampling confirmations of the ligand in the active site of the protein; then by ranking these confirmations via a scoring function. Induced fit is the term for the conformational shift that results in the overall binding during the docking process between the ligand and the protein in order to create an over-the-best fit [8-13].

Mechanics of Docking

A protein structure is necessary in order to conduct a docking screen. With the help of NMR spectroscopy or X-ray crystallography, protein structure has been identified. The docking program's inputs include databases of possible ligands and the protein structure. The search method and scoring mechanism are crucial to the docking program's success

a) Search algorithm

A stringent search technique would fully explain each and every potential way that ligands and receptors could attach to one another. Monte Carlo (MC) methods, fragment-based methods, distance geometry, matching method, ligand fit method, point complementarity method, blind docking, inverse docking, evolutionary algorithms, and molecular dynamics are a few examples of frequently used search algorithms.

b) Scoring function

The scoring function's goal is to distinguish between the proper postures and wrong poses, or between binders and inactive substances, in a fair amount of calculation time. By using these methods, scoring functions assume numerous assumptions and simplifications when estimating rather than computing the binding affinity between the protein and ligand. Scoring functions can be divided into:

i. Force field-based scoring functions
ii. Empirical-based scoring function
iii. Knowledge-based scoring function

AutoDock

In order to predict the binding interaction between small molecules and the known receptor's three-dimensional structure, the ligand or a subset of the ligand is altered with numerous rotatable bonds using the script-driven flexible automated and random search docking technique known as AutoDock. The Lamarckian genetic algorithm, evolutionary, genetic, and Monte Carlo simulated annealing algorithms are responsible for AutoDock's robustness. AutoDock has demonstrated remarkable effectiveness in the prediction of bound conformations, including those of protein-protein interactions, peptide-antibody complexes, and enzyme-inhibitor complexes. The AMBER force field model, free energy scoring functions, and a vast collection of protein-ligand complexes with established protein-ligand constants are used to assess potential orientations. Side chain edible is present in the most recent unreleased version 4, which. Due to its free academic license, AutoDock has more educational websites than its rivals (Morris *et al*., 1998) [14].

AutoDock 4.2

A semi-empirical free energy force field is used by AutoDock 4.2 to assess conformations during docking simulations. The protein and ligand initially have an unbound configuration. The force field performs a two-step evaluation of binding.

- The transition from this unbound state to the configuration of the ligand and protein in the bound state is estimated for the intramolecular energies in the first step..
- Next, in the second phase, the intermolecular energies of binding the ligand and protein are assessed. The Amber force field is the foundation for the parameters.

MATERIALS:

Chemicals Used: 2-chloro benzaldehyde, 3-chloro benzaldehyde, 4-chloro benzaldehyde, 2-Fluro benzaldehyde, 3-Fluro benzaldehyde, 4-Fluro benzaldehyde, 2-Bromo benzaldehyde, 3-Bromo benzaldehyde, 4-Bromo benzaldehyde, tris-HCl buffer, folin phenol reagent, Acetylcholinesterase, 5,5'-dithiobis-2-nitro benzoic acid, acetylthiocholine iodide, 4 hydroxy acetophenone are purchased from Sigma Aldrich, USA. Sodium hydroxide, hydrochloric acid, dimethyl sulfoxide, iodine crystals, sodium thiosulphate, and ethanol are purchased from HiMedia. Donepezil was obtained from Tanmed Pharmaceuticals, Chennai, India a complimentary sample.

Instruments Used: Digital Balance (Sartorius Ltd., USA), Eppendorf Mini spin, Incubator

(Technico), Jasco FTIR-420 series, Mass spectrometer, Shimadzu-Jasco V-530 UV/Vis Spectrophotometer, ELCO 1/27 pH meter, MR-VIS visual melting range apparatus LAB INDIA), Mass spectrometer, NMR spectra's were recorded on a Bruker 300 MHz NMR Spectrometer.

Softwares Used

- MGL tools-AutoDock 4.2
- Python 2.7.8
- Cygwin 64
- Accelry's Discovery Studio 4.0.1
- Online SMILES translation
- ChemSketch
-

METHODS:

***In-Silico* Docking Studies**

Analysis of the aurone's drug-like qualities:

The drug development process for a new chemical entity benefits from in-silico predictions of the features of a drug-like entity. A compound's drug-likeness property was assessed based on adherence to Lipinski's Rule of Five. Lipinski's rule of five was utilised to test the oral bioavailability using molinspiration. For substances used as oral drugs, Lipinski's rule of five can be used to calculate the oral bioavailability characteristics. Lipinski's rule's parameters, including the compound's molecular weight, log P, hydrogen bond donors, and hydrogen bond acceptors, were computed. The compound cannot be taken into consideration for additional *in-silico* screening studies if it displays any breaches.

ChemSketch was used to construct the potential structural fragments of the aurones, and a molinspiration server was used to test the potential drug-likeness of the fragments. There were no indications of Lipinski's Rule of Five infractions in these aurones.

Molecular Docking Studies

For molecular docking studies, the molecule that passed the drug-likeness tests was taken into consideration. To determine the ability of certain aurones to block the enzymes, Acetylcholinesterase (1EVE), Butyrylcholinesterase (4BDS), and Beta secretase, *in silico* docking tests were conducted (1FKN). AutoDock 4.2 was used to carry out the docking studies. A chemical has better activity if it exhibits lower binding energy in docking experiments compared to the reference compound. The substance will be more active if having low inhibitory constant.

Synthesis of Flavonoid Compounds

Step I: Synthesis of chalcones: Chalcones were synthesized by base-catalyzed Claisen–Schmidt Condensation reaction of substituted acetophenones and substituted benzaldehyde (**Figure 1)**. A mixture of equimolar quantities of substituted benzaldehyde and substituted acetophenone were dissolved in 10 ml rectified spirit in a 250 ml round bottom

flask equipped with a mechanical stirrer and then 10 ml of sodium hydroxide (1 g in 10 ml water) was added dropwise to the reaction mixture. On vigorous stirring for 30 minutes the solution became turbid. The reaction temperature was maintained between 20-25°C with a cold water bath. After vigorous stirring for 2-3 hours, the reaction mixture was neutralized with 0.2 N HCl to favour precipitation. The product obtained was filtered and the crude chalcones were dried in air and recrystallized by using rectified spirit (Choudhari *et al.*, 2011) [15].

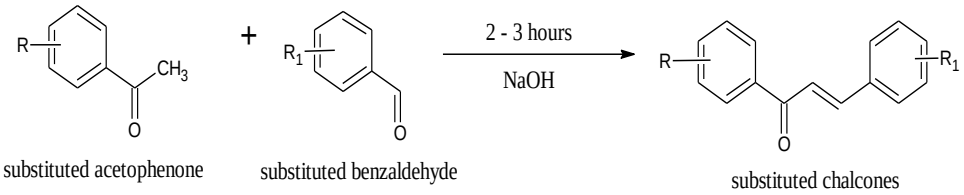


Figure 1: General scheme for the synthesis of chalcones

Step II: Synthesis of flavonoids: Synthesized chalcones (0.01mol) were suspended in DMSO (10ml) and crystals of iodine were added to it. The mixture was refluxed for 30-40 minutes and cooled at room temperature. The reaction mixture was diluted with ice-cold water and the precipitate thus obtained was filtered and washed with 20% sodium thiosulphate. The crude product obtained was recrystallized by using ethanol (Vidhyasagar and Nanda, 2012; Jayapal and Sreedhar, 2011; Wen *et al*, 2006) [16-18] (**Figure 2).**

Figure 2: General scheme for the synthesis of flavonoids

***In Vitro* Acetylcholinesterase Inhibitory Assay**

Based on Ellman's approach, the assay for measuring enzyme inhibition was conducted (Ellman *et al.*, 1961), modified by Sacan and Yanardag (2010), and carried out [19, 20]. The reaction mixture's final volume (4.0 ml) is made up of 1.3 ml of Tris-HCl buffer (pH 8.0; 50 mM), 0.4 ml of drug solution in various doses (ranging from 0.2 to 51.2 g/ml), and 0.1 ml of AChE (0.28 U/ml). 0.3 ml of acetylthiocholine iodide (0.023 mg/ml) and 1.9 ml of (5, 5'-dithiobis-(2-nitrobenzoic acid) DTNB (3 mM) solution were added to this combination after it had been incubating for 15 minutes. The finished reaction mixture was then incubated for an additional 30 minutes at room temperature, and the reaction mixture's absorbance was measured at 405 nm.

The test compounds were compared with the measurements of the standard drug, donepezil. The control was prepared by replacing the drug with a suitable solvent. The blank was prepared by replacing all the reagents with the solvent (DMSO) to nullify the effect of

the colour of the tested flavonoids. All determinations of the assay were done in triplicate and the results were expressed as the standard error of the mean (Sacan and Yanardag, 2010). The percentage inhibition was calculated using the formula:

$$\%\ \mathbf{Acetylcholinesteraseinhibition} = \frac{(\mathbf{A_0} - \mathbf{A_1})}{\mathbf{A_0}} \times \mathbf{100}$$

A_0= Absorbance of the control

A_1= Absorbance of the standard/compounds.

RESULTS:

Molecular Docking of Aurones with Acetylcholinesterase Enzyme

Aurones with chlorine, bromine, and fluorine substitutions were docked with acetylcholinesterase using AutoDock 4.2.

- In chlorine substituted aurones, 2nd, 3rd, and 4th positions of aurones were used for the substitution of the chloro group. All three chloro-substituted aurones showed great binding energy which was comparable to the standard drug Donepezil, at 10 different poses and binding energy ranging from -8.76 to -6.91, in which 2-chloro substituted aurone compound showed excellent binding energy -8.76kcal/mol. With an inhibitory constant of 0.377µM, and standard Donepezil showed -8.51kcal/mol and KI 0.578µM (**Figure 3)**.

  These results proved that the anti-Alzheimer's activity of the selected aurones.

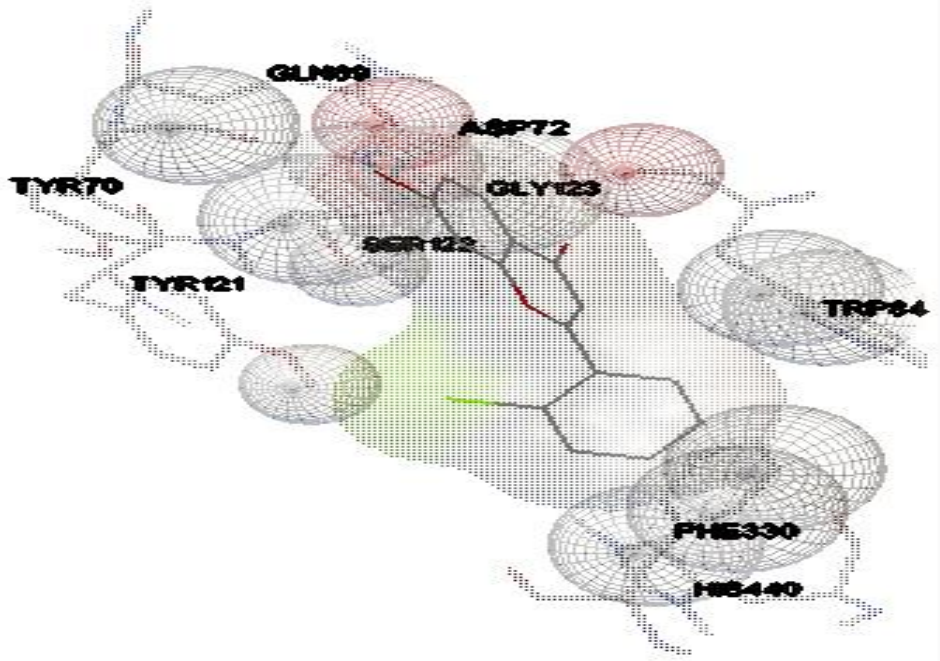


Figure 3: 2-chloro derivative with acetylcholinesterase enzyme

- In Bromine substituted aurones, 2nd 3rd and 4th positions of aurones were used for the substitution of the bromo group. All three bromo-substituted aurones showed good binding energy with all ten different poses and binding energy ranging from -

7.51 to -7.07. With -7.51 kcal/mol and KI 3.31 M, the 4-Bromo derivative exhibited excellent binding energy .

- In fluorine substituted aurones 2nd, 3rd and 4th positions of aurones were used for the substitution of the fluro group. All three fluro-substituted aurones showed good binding energy with all ten different poses, and binding energy ranges from -6.75 to -6.39. In which the 2-fluro derivative showed excellent binding energy with -6.75 kcal/mol and KI 11.18µM.

- Overall, 2-chloro derivative showed excellent binding energy of -8.76kcal/mol and KI of 0.377 µM, which was greater, compared to standard Donepezil with binding energy of -8.51kcal/mol and KI 0.578 µM, with acetylcholinesterase enzyme.

Molecular Docking of Aurones with Butyrylcholinesterase Enzyme

Aurones with chlorine, bromine, and fluorine substitutions were docked with Butyrylcholinesterase using AutoDock 4.2

- In chlorine substituted aurones, 2nd,3rd and 4th positions of aurones were used for the substitution of the chloro group. All three chloro-substituted aurones showed good binding energy with all ten different poses and binding energy ranging from -6.71 to -6.34. In which the 2-chloro derivative showed excellent binding energy with -6.71 kcal/mol and KI of 12.13µM **(Figure 4)**.

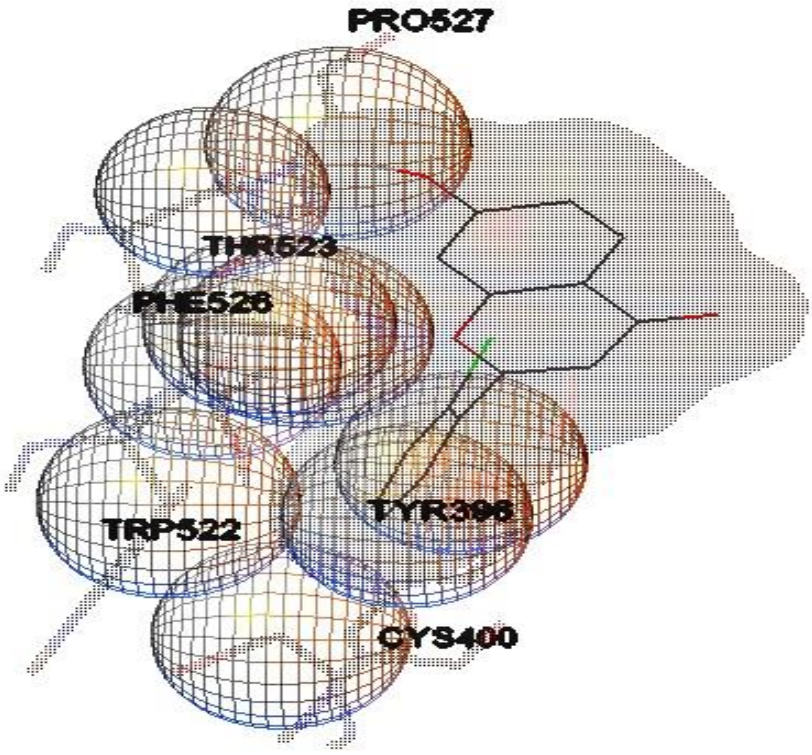


Figure 4: 2-chloro derivative with Butyrylcholinesterase enzyme

- In Bromine substituted aurones, 2nd, 3rd and 4th positions of aurones were used for the substitution of the Bromo group. All three bromo-substituted aurones showed good binding energy with all ten poses and binding energy ranging from -7.12 to -6.60. In which the 3-Bromo derivative showed excellent binding energy with -7.12 kcal/mol and KI of 6.04µ.
- In fluorine substituted aurones, 2nd, 3rd and 4th positions of aurones were used for the substitution of the fluro group. All three fluro-substituted aurones showed good binding energy with all ten poses and binding energy ranging from -6.31 to -6.14. The 3-Fluro derivative showed excellent binding energy with -6.31 kcal/mol and KI of 23.82µM.
- Overall 3-Bromo derivatives have an excellent comparable binding energy of -7.12 kcal/mol and KI 6.04µM. This was greater when compared to the standard Donepezil of binding energy -6.61kcal/mol and KI of 14.35µM, with Butyrylcholinesterase enzyme.

Molecular Docking of Aurones with Beta-Secretase Enzyme

Aurones with chlorine, bromine, and fluorine substitutions were docked with Beta-secretase, using AutoDock 4.2

- In chlorine substituted aurones, 2nd, 3rd and 4th positions of aurones were used for substitution of the chloro group, and binding energy ranging from -6.19 to -5.45 in which 3-chloro derivative showed good binding energy with -6.19 kcal/mol and KI of 29.17µM.
- In Bromine substituted aurones, 2nd, 3rd and 4th positions of aurones were used for the substitution of Bromo group and binding energy ranging from -6.61 to -5.95 in which 3-Bromo derivative showed good binding energy with -6.61 kcal/mol and KI of 14.36 µM.
- In fluro substituted aurones, 2nd,3rd and 4th positions of aurones were used for substitution of fluro group and binding energy ranging from -5.89 to -5.81 in which the 3-Fluro derivative showed good binding energy with -5.89 kcal/mol and KI of 48.45 µM
- Overall 3-Bromo derivative showed excellent binding energy of -6.61 kcal/mol and KI of 14.36µM, which was greater when compared to the standard Donepezil of binding energy of -6.51 kcal/mol and KI of 16.91µM, with Beta-secretase enzyme.

Overall Docking Result

Individual docking score for each ligand towards each enzyme is given in **Table 1.**

Table1: Docking result

LIGAND MOLECULE	ACE (acetylcholinesterase)		BCE (butyrylcholinesterase)		SEC (Beta-secretase)	
	BE	KI	BE	KI	BE	KI

	(kcal/mol)	(µM)	(kcal/mol)	(µM)	(kcal/mol)	(µM)
2-chloro	-8.76	377.16 (nM)	-6.71	12.13 (uM)	-5.45	101.13 (uM)
3-chloro	-8.48	605.59 (nM)	-6.48	17.65 (uM)	-6.19	29.17 (uM)
4-chloro	-6.91	8.64 (uM)	-6.34	22.68 (uM)	-5.99	40.74 (uM)
2-bromo	-7.07	6.62 (uM)	-6.88	8.98 (uM)	-5.95	43.37 (uM)
3-bromo	-7.32	4.34 (uM)	-7.12	6.04 (uM)	-6.61	14.36 (uM)
4-bromo	-7.51	3.13 (uM)	-6.60	14.52 (uM)	-6.11	33.01 (uM)
2-fluro	-6.75	11.18 (uM)	-6.14	31.72 (uM)	-5.86	50.65 (uM)
3-fluro	-6.69	12.4 (uM)	-6.31	23.82 (uM)	-5.89	48.45 (uM)
4-fluro	-6.39	20.54 (uM)	-6.19	29.07 (uM)	-5.81	55.31 (uM)
Donepezil	-8.51	578.19 (nM)	-6.61	14.35(uM)	-6.51	16.91 (uM)

Docking Orientations of the Selected Aurones and Donepezil with Acetylcholinesterase Enzyme

Table 2: Docking orientations with AChE

SUBSTITUTIONS	BINDING INTERACTION WITH AMINO ACID RESIDUE
2-CHLORO	TYR70, TYR121, ASP72, GLY123, GLN99, PHE330, HIS440
4-BROMO	ASN85, ASP72, TRP84, PHE331, PHE288, TYR121
2-FLURO	ARG289, SER286, TYR121, PHE288, PHE331, TYR334
DONEPEZIL	TYR121, HIS440, PHE331, ILE287, SER286, TYR334

Docking orientations of the selected aurones and donepezil with acetylcholinesterase enzyme is given through the **Table 2.** Based on the aforementioned investigation, all of the aurones chosen displayed improved anti-Alzheimer activity. The 2-chloro derivative of the chosen aurones had excellent binding interactions and orientations with the acetylcholinesterase enzyme.

Docking Orientations of the Selected Aurones and Donepezil with Butyrylcholinesterase Enzyme

Table 3: Docking orientations with BChE

SUBSTITUTIONS	BINDING INTERACTION WITH AMINO ACID RESIDUE
2-CHLORO	PRO527, THR523, PHE526, TRP522, TYR398, CYS400
3-BROMO	VAL529, PRO527, PHE526, TRP522, TYR396, CYS400, PRO401
3-FLURO	PRO527, PHE526, TRP522, TYR396, CYS400
DONEPEZIL	PHE396, GLY117, ILE69, GLN71, ASP70

Docking orientations of the selected aurones and donepezil with butyrylcholinesterase enzyme is given through the **Table 3.** Based on the aforementioned study, all of the chosen aurones had greater anti-efficacy; Alzheimer's however, the 3-Bromo derivative particularly excelled in binding with and orienting toward the butyrylcholinesterase enzyme.

Docking Orientations of the Selected Aurones and Donepezil with Beta-Secretase Enzyme

Table 4: Docking orientations with BSE

SUBSTITUTIONS	BINDING INTERACTION WITH AMINO ACID RESIDUE
3-CHLORO	PRO369, CYS380, GLU371, ASP379, ASP372, VAL373, SR376,
3-BROMO	PRO319, PHE38, VAL329, CYS330, TRP331
3-FLURO	GLY291, TYR132, THR133, GLN134
DONEPEZIL	GLY334, SER376, ASP379, CYS380, ASP372, GLU371, PRO369

Docking orientations of the selected aurones and donepezil with beta-secretase enzyme is given through the **Table 4.** Based on the aforementioned study, all aurones that were chosen showed greater anti-activity. Alzheimer's specifically, 3-Bromo derivatives of the aurones that were chosen had excellent binding contacts and orientations towards the beta-secretase enzyme.

Synthesis of Flavonoids

The syntheses of chalcones were carried out by using the Claisen-Schmidt condensation method and various flavonoids were synthesized from chalcones. All the synthesised compounds had a yield that varied from 69 to 81%. Utilizing MR-VIS visual melting range equipment, the melting point was ascertained. By utilising the solvent chloroform: water, the R_f values of the synthesised flavonoids ranged from 0.64 to 0.88, respectively (9:1)**.** Percentage yield, melting point, and R_f value of synthesized flavonoids are given in **Table 5**.

Table 5: Percentage yield, melting point, and R_f value of synthesized flavonoids

Compound code	Percentage yield	Melting point	R_f Value
C1 (2-chloro)	81%	187.2°C	0.88
C2 (3-chloro)	72%	187.2°C	0.87
C3 (4-chloro)	78%	187.2°C	0.88
C4 (2-fluoro)	75%	152.4°C	0.77
C5 (3-fluoro)	69%	152.4°C	0.76
C6 (4-fluoro)	74%	152.4°C	0.76
C7 (2-bromo)	77%	195.3°C	0.82
C8 (3-bromo)	80%	195.3°C	0.82
C9 (4-bromo)	79%	195.3°C	0.83

Spectral Studies of Compounds

Based on chemical information, UV, IR, MASS, and H-NMR spectroscopy, the substances produced during the current experiment were identified.

a. UV spectra of synthesized flavonoids

UV spectral analyses of synthesized flavonoids were carried out and the UV spectral data is given in **Table 6.**

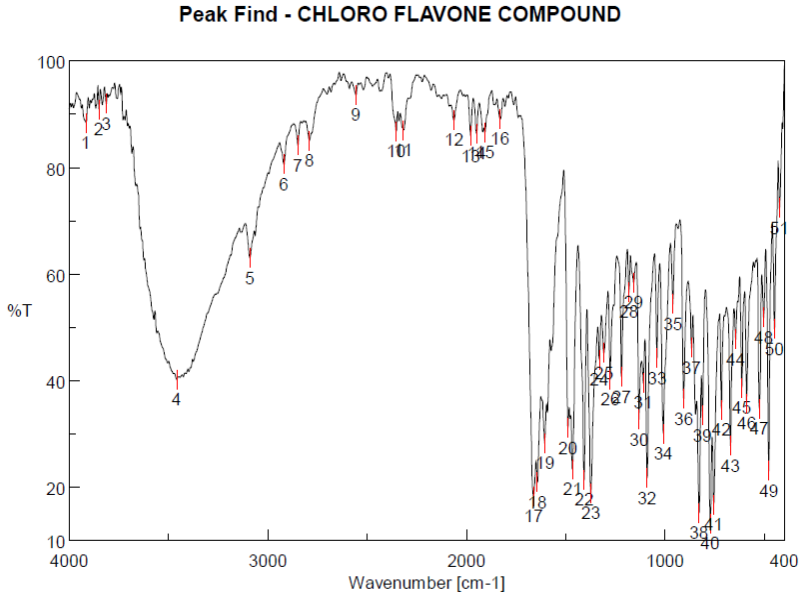
Table 6: UV Spectral data of synthesized flavonoids

Compound code	λ max (nm)
C1 (2-chloro)	297
C2 (3-chloro)	297
C3 (4-chloro)	297
C4 (2-fluoro)	294
C5 (3-fluoro)	294
C6 (4-fluoro)	294
C7 (2-Bromo)	277
C8 (3-Bromo)	277

C9 (4-Bromo) 278

b. IR spectrum (KBr pellet method)

IR spectral analyses of synthesized flavonoids were carried out by using KBr pressed pellet technique. IR spectral data is given in **Table 7 and Figure 5** shows the IR spectra of chloro-flavone.



[Comments]

Sample name	CHLORO FLAVONE COMPOUND
Comment	FT-IR SPECTRUM
User	
Division	
Company	SRIPMS

[Result of Peak Picking]

No.	Position	Intensity	No.	Position	Intensity
1	3915.75	88.4137	2	3850.18	90.9337
3	3813.54	91.9747	4	3459.67	40.2078
5	3090.37	63.0638	6	2918.73	80.5814
7	2848.35	84.1332	8	2790.49	85.0376
9	2557.15	93.6497	10	2353.69	86.7519
11	2317.05	86.9845	12	2063.46	88.8527
13	1979.57	85.9512	14	1949.68	86.4385
15	1907.25	86.5573	16	1830.12	89.0839
17	1663.3	18.3559	18	1643.05	20.947
19	1606.41	28.2585	20	1489.74	31.1609
21	1466.6	23.3963	22	1407.78	21.4159
23	1374.03	18.8983	24	1330.64	43.9551
25	1308.46	45.1453	26	1276.65	40.2482
27	1219.76	40.7234	28	1182.15	56.6144
29	1158.04	58.3675	30	1132.01	32.7138

Figure 5: IR spectra of C1 (chloro-flavone)

Table 7: IR spectral data of C1

S. No	Type of stretching	Frequency(cm^{-1})
1	Aromatic C-H stretching	3090

2	Aromatic C-OH stretching	3459
3	C=O stretching	1663
4	C-O-C stretching	1219
5	C=C Ring stretching	1466
6	Aromatic C-Cl stretching	754

c. NMR spectra of Chloro-flavone (C1)

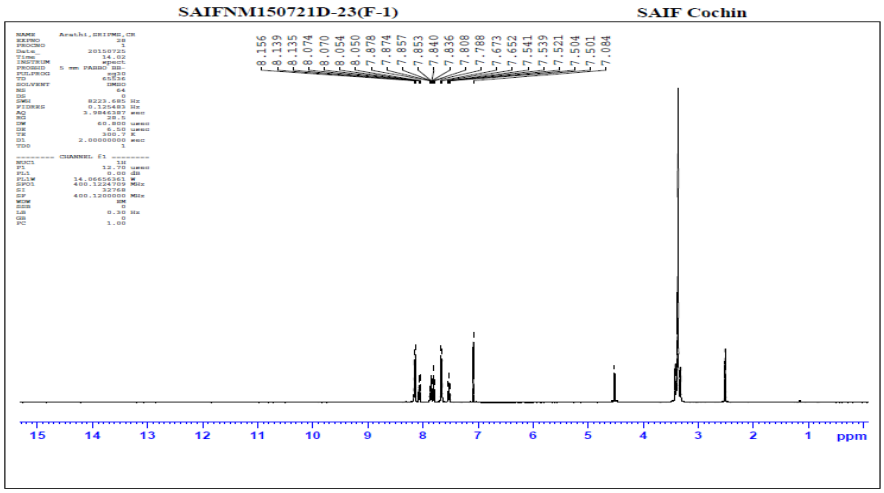


Figure 6: ^{1}H-NMR spectra of C1 (Chloro-flavone)

Table 8: ^{1}H-NMR spectral data of F1 compound

Type of proton	Chemical shift	No. of proton
Aromatic proton	7.501 to 8.156	7
-OH proton	2.521	1
Cyclic alkene	4.510	1

d. Mass spectra of C1

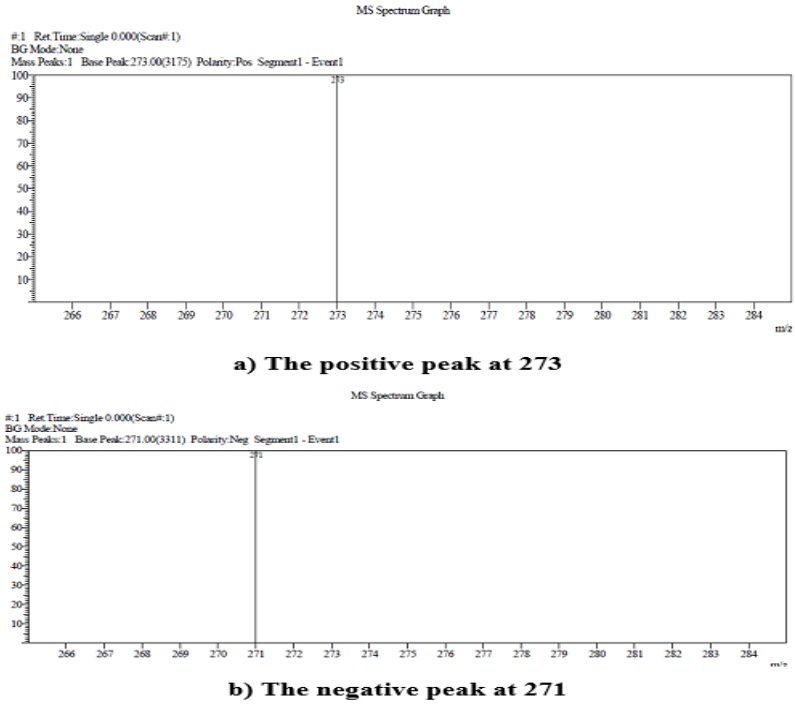


Figure 7: Mass spectra of C1 (Chloro-flavone)

***In Vitro* Acetylcholinesterase Inhibitory Assay**

The acetylcholinesterase inhibitory efficacies of the produced halo-substituted flavonoids were tested. When the concentrations were increased from 0.2 to 51.2µg/ml, the absorbance of all the synthetic halo-substituted flavonoids (C1 to C9) and the reference substance donepezil dropped in a dose-dependent way. All of the produced halo-substituted flavonoid compounds (C1 to C9) and donepezil's percentage inhibition were calculated. For the donepezil, a dose-dependent rise in percentage inhibition was seen for compounds C1 through C9. The range of donepezil's percentage inhibition was 15.43 to 165.82%, and the IC50 value was determined to be 1.136µg/ml. The proportion of inhibition for C1 (2-chloro) ranged from 19.07 to 117.17%, and its IC50 value was discovered to be 2.108µg/ml. The percentage inhibition for the C2 (3-chloro) ranged from 13.36 to 97.34%. C2 had IC50 of 2.872µg/ml. The C3 (4-chloro) compound's ability to inhibit the acetylcholinesterase enzyme ranged from 1.70 to 70.65%, and the IC50 was 24.392µg/ml. The IC50 value for the chemical C4 (2-fluro) was 18.391µg/ml, while the percentage of inhibition ranged from 2.27 to 73.10%. The percentage of inhibition for C5 (3-fluro) ranged from 5.60 to 75.69%, and the IC50 value was 17.541µg/ml. The percentage inhibition for C6 (4-fluro) varied from 1.22 to 68.64%. C2 has an IC50 of 27.837µg/ml. The IC50 value for the chemical C7 (2-Bromo) was 17.554µg/ml, and the range of its percentage inhibition was 2.38 to 74.67%. The percentage of inhibition for C8 (3-Bromo) ranged from 1.95 to 71.38%, and the IC50 value was discovered to be 22.443µg/ml. The percentage inhibition for C9 (4-Bromo) varied from 2.88 to 73.37%. C9 has an IC50 of 8.973µg/ml.

Among the nine halo-substituted flavonoid compounds, C1 had a greater percentage of inhibition at concentrations of 51.2µg/ml with 117.17% activity. C1 had excellent inhibitory

activity. The order of potency of flavonoid compound in acetylcholinesterase inhibitory assay was;

C1 > C2 > C9 > C5 > C7 >C4>C8>C3>C6.

Table 9: *In vitro* acetylcholinesterase inhibitory assay

Conc. in µg/ml	Percentage inhibition of AChE									
	C1	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**	**C9**	**DPZ**
0.2	19.07 ± 0.08	13.36 ± 0.13	1.70 ± 0.12	2.27 ± 0.36	5.60 ± 0.12	1.22 ± 0.08	2.38 ± 0.04	1.95 ± 0.03	2.88 ± 0.09	15.43 ± 0.07
0.4	33.35 ± 0.01	23.67 ± 0.16	2.86 ± 0.07	3.62 ± 0.11	7.46 ± 0.10	2.55 ± 0.46	3.87 ± 0.25	3.12 ± 0.05	8.36 ± 0.07	25.62 ± 0.03
0.8	39.53 ± 0.16	37.79 ± 0.04	4.43 ± 0.34	4.98 ± 0.06	14.56 ± 0.16	4.17 ± 0.24	5.82 ± 0.18	4.79 ± 0.14	15.48 ± 0.12	48.30 ± 0.08
1.6	47.19 ± 0.13	43.71 ± 0.02	12.76 ± 0.15	15.32 ± 0.02	20.86 ± 0.08	11.73 ± 0.03	16.72 ± 0.32	13.02 ± 0.25	25.54 ± 0.06	61.91 ± 0.05
3.2	60.11 ± 0.16	57.43 ± 0.01	19.20 ± 0.03	21.56 ± 0.16	26.37 ± 0.05	18.85 ± 0.11	22.10 ± 0.41	20.61 ± 0.17	34.66 ± 0.09	67.36 ± 0.02
6.4	69.76 ± 0.05	66.51 ± 0.04	31.87 ± 0.27	33.34 ± 0.07	31.61 ± 0.04	30.98 ± 0.32	35.37 ± 0.23	32.85 ± 0.02	42.47 ± 0.02	100.13 ± 0.12
12.8	89.17 ± 0.03	78.18 ± 0.07	43.94 ± 0.01	46.02 ± 0.22	40.94 ± 0.08	42.53 ± 0.41	47.08 ± 0.14	44.70 ± 0.09	61.71 ± 0.05	118.73 ± 0.01
25.6	95.83 ± 0.07	91.18 ± 0.02	58.44 ± 0.21	60.51 ± 0.14	64.31 ± 0.09	57.16 ± 0.03	61.23 ± 0.03	59.11 ± 0.21	66.5 ± 0.13	133.73 ± 0.28
51.2	117.17 ± 0.1	97.34 ± 0.04	70.65 ± 0.14	73.10 ± 0.30	75.69 ± 0.06	68.64 ± 0.07	74.67 ± 0.05	71.38 ± 0.16	73.37 ± 0.03	165.82 ± 0.13
IC_{50}	**2.108 ± 0.05**	**2.872 ± 0.16**	**24.392 ± 0.04**	**18.391 ± 0.13**	**17.541 ± 0.03**	**27.832 ± 0.06**	**17.55 ± 0.08**	**22.433 ± 0.21**	**8.973 ± 0.05**	**1.136 ± 0.05**

IC_{50} = The half maximal inhibitory concentration
DPZ= Donepezil Hydrochloride
C1= 2-Chloro derivative
C2= 3-Chloro derivative
C3= 4-Chloro derivative
C4= 2-Fluoro derivative
C5= 3-Fluoro derivative
C6= 4-Fluoro derivative
C7= 2-Bromo derivative
C8= 3-Bromo derivative
C9= 4-Bromo derivative
AchE= Acetylcholinesterase enzyme

DISCUSSION:

A new chemical entity's drug development process can benefit from *in silico* forecasts of the qualities that make a substance similar to a medication. It aids in locating possible lead compounds while minimising side effects. A compound's drug-likeness property was assessed based on adherence to Lipinski's Rule of Five. Lipinski's rule of five was utilised to test the oral bioavailability using molinspiration. For substances used as oral drugs, Lipinski's Rule of Five makes it possible to calculate the oral bioavailability characteristics. For Lipinski's Rule, the parameters of the compound's molecular weight, log P, hydrogen bond donors, and hydrogen bond acceptors were computed. The compound cannot be selected for additional in silico screening experiments if it displays any violations. ChemSketch was used to construct the potential structural pieces of the halo substituted flavonoids (approximately 09 flavonoids), and the Molinspiration service was used to test their drug-likeness. Selected halo-substituted aurones were proven to exhibit Anti-Alzheimer's activity by docking studies when compared to the standard drug Donepezil.

In the Acetylcholinesterase enzyme, 2-chloro-substituted aurone exhibited highest B.E with the Acetylcholinesterase enzyme. 2-chloro derivative showed maximum inhibition at the lower concentration. The B.E of the 2-chloro derivative was higher than that of the standard drug Donepezil in Acetylcholinesterase enzyme.

In the Butyrylcholinesterase enzyme, 3-bromo substituted aurone exhibited highest B.E with the Butyrylcholinesterase enzyme. 3-Bromo derivative showed maximum inhibition at lower concentrations. The B.E of the 3-Bromo derivative is higher than that of the standard drug Donepezil in the Butyrylcholinesterase enzyme.

In the Beta-secretase enzyme, the 3-Bromo derivative exhibits a higher B.E with the Beta-secretase enzyme. 3-Bromo derivative shows maximum inhibition at a lower concentration. The B.E of the 3-Bromo derivative is higher than that of the standard drug Donepezil in Beta-secretase enzyme.

Finally, we conclude that among the three enzymes (Acetylcholinesterase, Butyrylcholinesterase, and Beta-secretase), Aurones show better inhibitory activity towards the Acetylcholinesterase enzyme.

From the halo-substituted aurones, 2-chloro-substituted aurones show maximum inhibition towards the Acetylcholinesterase enzyme compared to the standard drug Donepezil. So aurone produces excellent Anti-Alzheimer's activity by the inhibition of the Acetylcholinesterase enzyme.

Further anti-Alzheimer's activity of halo-substituted aurones towards these enzymes is found by *in-vitro* and *in-vivo* methods.

CONCLUSION:

Flavonoids are used in the treatment of various diseases including Alzheimer's disease. In recent years, there has been an increase in interest in alternative medicine and the healing

capabilities of natural remedies made from plants. Several flavonoids were created, and their structural identity was determined using spectroscopic methods such as UV, IR, mass, and ^{1}H-NMR.. Flavonoids were reported to possess various pharmacological activities such as neuroprotective activity, anti-diabetic activity anti-inflammatory activity, anti-arthritic activity, anti-platelet activity, vasorelaxant activity, and anti-cancer activity. Therefore, the identification and characterization of flavonoids like moieties is a promising approach to fighting illnesses such as Alzheimer's disease. Fast and simple synthesis or chemical modifications of flavonoids allow rapid generation of a large number of novel compounds.
The present study was focused on crediting the protein-ligand interactions, design, and interaction of certain aurones for possible Anti-Alzheimer's activity using docking studies. The selected compounds were docked by AutoDock 4.2 and the docking results confirm the possibility of aurones to possess Anti-Alzheimer's activity against Acetylcholinesterase, Butyrylcholinesterase, and Beta secretase enzymes

Halo-substituted flavonoids were created using molinspiration-drug similarity qualities as a guide. They underwent tests to see whether they have any in-vivo anti-Alzheimer's action and in-vitro acetylcholinesterase enzyme inhibitory activity. In addition, every synthetic flavonoid has anti-Alzheimer's action according to an in-vitro assay for acetylcholinesterase inhibitors. Inhibition was greatest at lower concentrations for 2-chloro flavone among the nine synthetic flavonoids.
In-vivo tests have further verified it.

CONFLICT OF INTEREST:

Regarding this study, there are no conflicts of interest for the authors.

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