Isolation of flavonoides from *Artemisia macrocephala* anticholinesterase activity: Isolation, characterization and its *in vitro* anticholinesterse activity supported by molecular docking

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Abstract: In this study the flavonoids isolated from *Artemisia macrocephala* were screened out for anticholinesterase activity. The isolated flvanoids were characterized by HNMR, NOESY, COSY, HMBC, HSQC and mass spectroscopy. The compounds (1-4) in appropriate quantities were isolated from chloroform fraction using gravity column chromatography by eluting ethyl acetate/*n*-hexane solvent system. The flavonoids were characterized and resulted in the form of mono substituted methoxy flavones to tri substituted flavones. Ellman's assay techniques were used to find out enzyme inhibition. Operating environment (MOE) software was used for molecular docking studies. Compounds (1), (2) and (3) showed 88.42±2.76, 84.50±1.60 and 90.16±2.98 percent inhibition of the acetyl cholinesterase (AChE) respectively at 1000µg/mL concentrations with IC_{50} value 165, 60, 65µg/mL respectively which were comparable to that of standard galanthamine. While for butyryl cholinesterase (BChE), (1), (2) and (3) showed 91.63±4.32, 81.03±3.53 and 87.69±2.84 percent inhibitions respectively at 1mg/mL as compared to the standard galanthamine which caused 96.50±2.41 percent inhibition at the same concentration. Whereas, compound (4) exhibited moderate activity for both the enzymes. Molecular docking studies confirmed the experimental AChE and BChE inhibitory activities of the test samples by their virtue of multiple interactions with target enzymes. The results confirm that the specie has biologically active constituents that are more useful for the management of several neurodegenerative ailments like ataxia, Parkinson's disease, Alzeimer's disease and some other types of dementia.

Keywords: *Artemisia macrocephala,* isolation, flavonoids, characterization, cholinesterase inhibition, molecular docking (Computational studies).

INTRODUCTION

Alzheimer's disease (AD) is an irreversible and progressive neurodegenerative disorder that affects a huge proportion of the elderly population in the entire globe (Khan *et al.,* 2010). The salient early sign of the disease is loss of short-term memory (amnesia), which usually manifests as minor forgetfulness that becomes gradually more distinct with illness development, with relative conservation of older memories leading to institutionalization and ultimately death (Orhan *et al.,* 2008). Acetylcholine (ACh), a neurotransmitter, deficiency is one of the most common features found in AD patients. This Ach is broken down by Acetyl cholinesterase (AChE) thus reducing its level in the brain region (Jang *et al.,* 2008). According to latest research not only AChE also butyryl cholinesterase (BChE) is responsible for the breakdown of ACh (Giacobini *et al.,* 2002).

The use of anticholinestrase agents in the activation of cholinergic potentials is one of the most important therapeutic strategies. The use of synthetic cholinesterase inhibitors, such as rivastigmine, tacrine, galantamine and donepezil are very common in the treatment of AD. The use of these synthetic drugs is somewhat limited because of their severe adverse effects like gastrointestinal, hepatotoxicity and bioavailability problems (Schulz, 2003; Na *et al.,* 2008). Because of several adverse affects of these synthetic cholinesterase (ChEs) inhibitors, the isolation of better constituents for inhibition of ChEs of natural sources is of prime interest. Various constituents of ChEs inhibition have been discovered including alkaloids (Atta *et al.,* 2001; Claudio *et al.,* 2005; Atta *et al.,* 2002), shikimate derivates, with anolides, farnesylacetone derivatives, pyrazoline derivatives, flavonoids, terpenoids and sterols (Na *et al.,* 2008).

Artemisia macrocephala is a member of family Asteraceae, having vast medicinal significance. It is locally called as "Tarkha" having a height of 20-30cm. It is profusely available in Pakistan northern areas (Zareh, 2005). We have previously reported it for preliminary phytochemical investigation, antispasmodic (Ali *et al.,*

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2011). and antioxidant potentials (Ali *et al.,* 2013). Very recently we have reported its essential oil for AChE and BChE inhibitory potentials with very promising results (Shaoib *et al.,* 2015).

The role of natural products in the prevention of neurodegenerative diseases and as memory enhancers is of paramount importance. Search for safe, efficient and economical antichlonesterases from renewable natural resources has always been the subject of immense importance for pharmacologists. Based on its rich pharmacological profile and the reported literature, this study is designed to isolate, characterize four flavonoids from *A. macrocephala* and screen them for their possible *in-vitro* AChE and BChE inhibitory potentials supported by molecular docking studies.

MATERIALS AND METHODS

Collection and authentitacation of plant materials

Aerial parts of *A. macrocephala* were collected in April-May, 2014, from Badwan chowk, Dir Lower, Khyber Pakhtunkhwa, Pakistan. The plant was identified by Professor Jahandar Shah. A voucher sample, AM-01-2014, was submitted in Malakand University herbarium.

Extraction and fractionation

Shade-dried (10kg) aerial parts of *A. macrocephala* were pulverized thoroughly. This pulverized mass was then soaked in commercial grade methanol with intermittent stirrering. Filtered the whole suspension after a time period of 20-22 days. Repeated this process thrice. All the three filtrates were combined and concentrated via rotary evaporator at 40ºC under reduced pressure. A residue of greenish-black color (1.2kg) was obtained. A portion of 1 kg of this methanolic greenish-black extract was subjected to successive fractionation starting with *n*-hexane, then chloroform followed by ethyl acetate and *n*-butanol in the last. The crude extract and sub-fractions were stored at 20ºC.

Isolation of compounds

The chloroform fraction was subjected to column chromatography with elution solvent system ethyl acetate: *n*-hexane, starting from pure *n*-hexane with gradual increase of ethyl acetate yielding 12 fractions. Fraction No.7 and 8 were again subjected to chromatography eluted with ethyl acetate: *n*-hexane. The compounds were purified through silica gel column chromatography using 15% ethyl acetate and *n*-hexane solvent system for compounds (1), (2) and 20% for (3), (4).

Anticholinesterase assays

AChE from *Electric eel* and BChE from *Equine* serum were used to find out the enzyme inhibition activity of (1), (2), (3) and (4) using Ellman's assay (Ellman *et al.,* 1961). Test samples were dissolved in DMSO and further diluted in phosphate buffer (0.1M) in various concentrations (62.5-1000µg/mL). AChE (518 U/mg) and BChE (7-16 U/mg) were diluted in 0.1M phosphate buffer (pH 8.0) to get a final concentrations 0.03U/mL for AChE and 0.01U/mL for BChE. Solutions of DTNB (0.2273 mM), ATchI (0.5mM) and BTchI (0.5mM) were prepared in distilled water and kept in the eppendorf in refrigerator (8°C). Added 5µL of enzyme solution to the cuvette followed by test sample (205µL) and DTNB reagent (5µL) for each assay. Added the substrate solution (5µL) subsequently while keeping the solution mixture in a water bath for 15min at 30°C. The absorbance was measured through double beam spectrophotometer at 412 nm. Galanthamine served as positive control. The absorbance and reaction time was taken at 30°C for four minutes. The experiment was carried out in triplicate. The percent enzyme activity and enzyme inhibition by control and test sample was calculated from the rate of absorption with change in time ($V=\Delta Abs/\Delta t$) as follow

Enzyme inhibition (%) = 100 - percent enzyme activity

Enzyme activity (%) = $100 \times V/V_{max}$

(Where V_{max} is enzyme activity in the absence of inhibitor drug).

Molecular docking study

Receptor preparation

In the current study human AChE complex with donepezil inhibitor PDB ID: 4EY7 homodimer having resolution 2.35 Å and human BChE complex with tacrine inhibitor PDB ID: 4BDS monomer having resolution 2.10 Å were used. In case of human AChE chain B was selected for docking implementation. For both AChE and BChE, missing residues were added and optimized by using Molecular operating environment (MOE). Except water involved in the interactions, rest of the water molecules was deleted. In the complex structures the hydrogen atoms were added and energy minimization was performed.

Re-docking setup

Before molecular docking of flavonoid compounds into the active site of AChE (PDB ID: 4EY7) and BChE (PDB ID: 4BDS), redocking was performed to validate the docking software, therefore MOE software was found appropriate. Redocking of co-crystallized ligand was performed into the active site of AChE and BChE. The fitness of each re-docked pose was evaluated on the basis of RMSD (root-mean-square deviation) values.

Ligand preparation

Three dimensional structures of four flavonoids were sketched using MOE. MMFF94 charges were applied and energy minimization was performed.

Docking of acetyl and butyryl cholinesterase inhibitors

Docking studies result in appropriate confirmation of ligand within the binding sites of proteins. The scoring

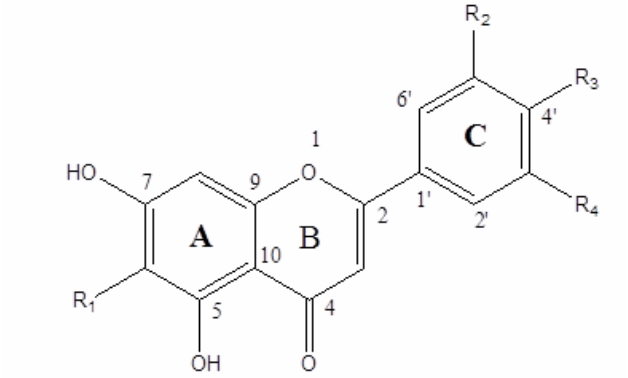
function satisfactorily describe best pose through the mathematical numbers. Compounds were docked in the binding pocket of AChE (PDB ID: 4EY7) and BChE (PDB ID: 4BDS) through MOE. For each ligand, 30 poses were generated. Best conformation was selected through scoring function and interaction pattern of ligand and protein.

RESULTS

Spectroscopic data

ISM-1(1)

Pale yellow amorphous powder, IR (KBr, Cm^{-1}), 3394, 3267, 1652, 1511, UV λ_{MeOH} max nm: 340 and 346; ^{1}H-NMR (500 MHz, acetone-d_6): δ 13.13 (s, H-5/OH), 7.21 (br s, 2H, , H-6'/H-2'), 6.70 (s, 1H, H-2), 6.64 (s, 1H, H-8), 3.98 (s, 3H, H-5'/OCH_3), 3.87 (s, 3H, H-4'/OCH_3), 3.86 (s, 3H, H-6/OCH_3), δ EI-MS: *m/z* 360 (M^+), 342, 317, 69 [13].



	R1	R2	R3	R4
1	OMe	OMe	OMe	OH
2	OMe	H	OMe	OMe
3	H	OMe	OMe	OH
4	H	H	OMe	H

Fig. 1: Structures of compounds 1 - 4.

ISM-4 (2)

Pale yellow amorphous powder, IR (KBr, Cm^{-1}), 3391, 3263, 1659, 1519. UV λ_{MeOH} max nm: 342 and 347; ^{1}H-NMR (500 MHz, CD_3OD): δ 7.59 (d, 1H, $J_{6',5'}$ =8.0 Hz, H-6'), 7.10 (br s, 1H, H-2'), 7.09 (d, 1H, $J_{5',6'}$ =8.0 Hz, H-5'), 6.63 (s, 1H, H-2), 6.52 (s, 1H, H-8), 3.92 (s, 3H, H-4'/OCH_3), 3.90 (s, 3H, H-3'/OCH_3), 3.86 (s, 3H, H-6/OCH_3), δ EI-MS: *m/z* 344 (M^+), 329, 326, 301 [14].

ISM-5(3)

Pale yellow amorphous powder, ^{1}H-NMR (300 MHz, acetone-d_6): δ 12.89 (s, 1H, H-5/OH), 7.20 (s, 2H, H-2'/H-6'), 6.68 (br s, 1H, H-3), 6.53 (d, 1H, $J_{8,6}$ = 2.0 Hz, H-8), 6.24 (d, 1H, $J_{6,8}$=2.0 Hz, H-6), 3.97 (s, 3H, H-3'/OCH_3), 3.85 (s, 3H, H-4'/OCH_3), δ EI-MS: *m/z* 330 (M^+), 315.

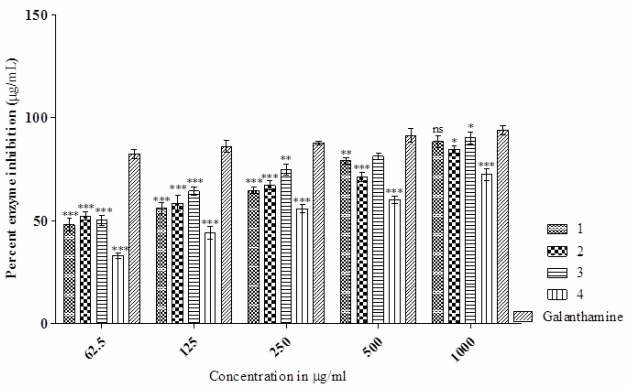


Fig. 2: Percent acetylcholinesterase activity of the tested compounds at different concentrations. Results are expressed as mean ± SEM (n=3). Data was analyzed by two way ANOVA followed by Bonferoni test.

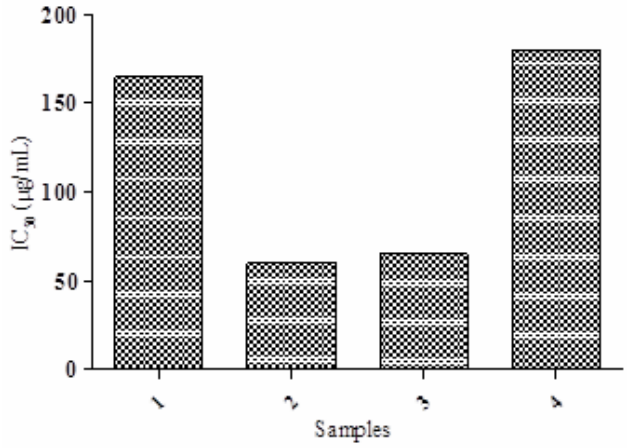


Fig. 3: IC_{50} values of the test compound for acetyl cholinesterase

ISM-6 (4)

Pale yellow amorphous powder, ^{1}H-NMR (500 MHz, acetone-d_6): δ 12.98 (s, 1H, H-5/OH), 7.96 (d, 2H, $J_{2',3'/6',5'}$ = 8.0 Hz, H-2/H-6), 7.03 (d, 2H, $J_{3',2'/5',6'}$ = 8.0 Hz, H-3/H-5), 6.68 (s, 1H, H-3), 6.66 (d, 1H, $J_{8,6}$ = 2.0 Hz, H-8), 6.32 (d, 1H, $J_{6,8}$ = 2.0 Hz, H-6), 3.92 (s, 3H, H-4'/OCH_3), δ EI-MS: *m/z* 284 (M^+), 255.

Anticholinesterase assay

To find potential AChE inhibitors from *Artemisa macrocephala*, flavonoids were isolated from its chloroform fraction. They were docked against the said enzymes. The percent inhibition results of all the isolated compounds are summarized in fig. 2. All the tested samples showed percent enzyme inhibition activity in a concentration dependent way. Compounds (1), (2) and (3) were found to be much more effective than (4). They caused 88.42±2.76, 84.50±1.60 and 90.16±2.98 percent

inhibition of the acetyl cholinesterase respectively at 1000 µg/mL concentrations. These results were comparable with that of standard galanthamine. The IC_{50} for these two compound were to be 165, 60, 65µg/mL respectively. Compound (4) showed moderate activity with IC_{50} value 180µg/mL as shown in fig. 3. All the flavonoids showed significant AChE inhibitory activity.

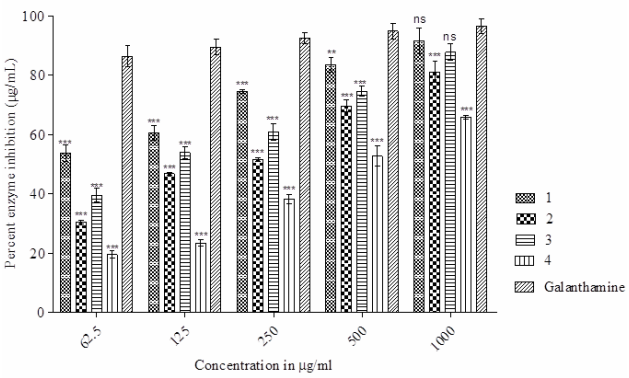


Fig. 4: Percent buterylcholinesterase activity of the tested compounds at different concentrations. Results are expressed as mean ± SEM (n=3). Data was analyzed by two way ANOVA followed by Bonferoni test.

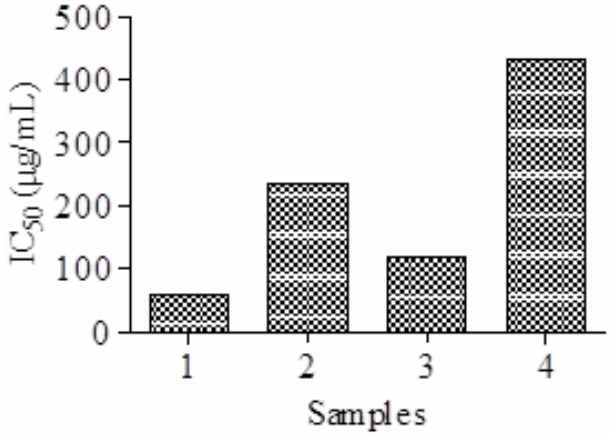


Fig. 5: IC_{50} values of the test compound for butyrylcholinesterase.

compared to the reference standard galanthamine, while (4) caused moderate inhibitory activity as shown in fig. 4. (1), (2) and (3) caused 91.63±4.32, 81.03±3.53 and 87.69±2.84 percent inhibition at 1mg/mL concentration respectively as compared to 96.50±2.41 percent inhibition caused by reference standard galanthamine at the same concentration. Flavonoid (4) caused 65.74±0.56 percent inhibition at 1 mg/mL concentration which is moderate as compared to the standard and other three flavonoids.

Validation of docking protocol

To validate the software redocked pose to the co-crystallized ligand was correlated. For AChE complex RMSD value between the redocked pose of PDB ID: 4EY7 and its bound conformation was found to be 0.33Å, fig. 6(A), while for BChE complex the RMSD value between the redocked pose of PDB: 4BDS and its bound conformation was found to be 0.72 Å, fig. 6(B). Approximately similar interactions with cognate ligand were observed. The MOE-dock results depicting that MOE software can be utilized for further docking of flavonoid compounds.

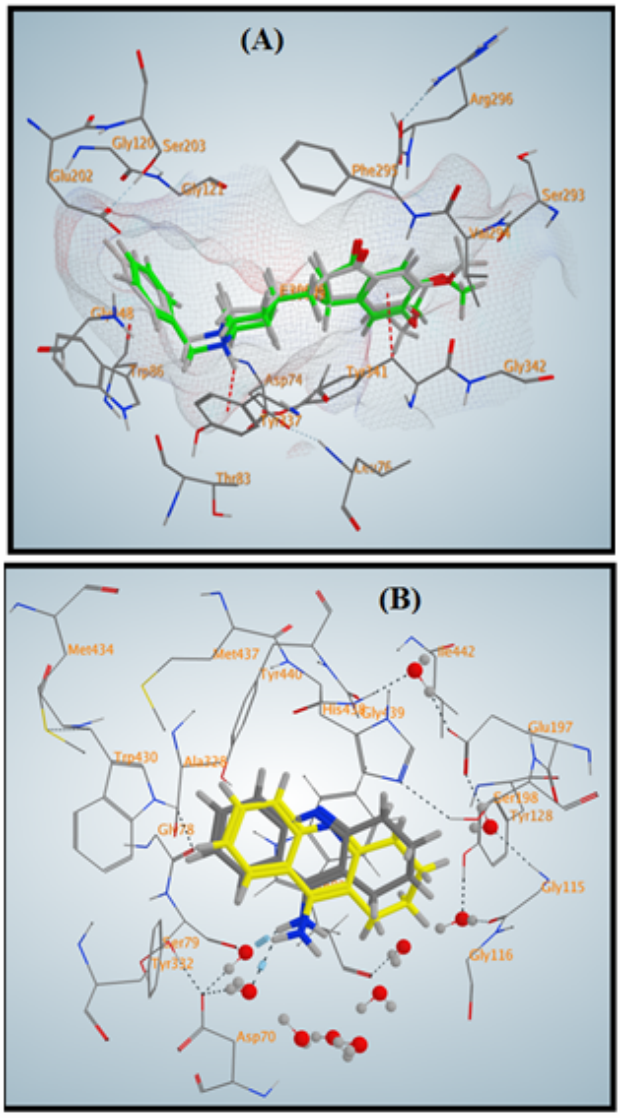


Fig. 6: Selected best re-docked pose of AChE (A, presented in green) and re-docked pose of BChE (B, presented in yellow) against the crystallized ligand E20605 (depicted in grey) in the binding site of Acetyl and Butyryl cholinesterase. Active site residues are presented as wire model. Light blue color line is for hydrogen bonding, and red color line is for pi-pi hydrophobic Interactions.

Docking results of acetylcholinesterase inhibitors

Isolated flavonoid derivatives were subjected to docking for the exploration of binding pattern against AChE. Most active compound (2) showed multiple interactions, fig.

7(A) along with a crucial interaction with Tyr341 of AChE validating the high experimental activity. Sigma-pi interaction between hydroxyl of its chromen-4-one moiety and phenyl of Trp286 of AChE and CH3 of its chromen-4-one moiety mediating vander Waal interaction with Tyr341 of AChE. Methoxy of its phenol moiety also makes vander Waal interaction with O atom of Asp74 AChE. Least active compound (4), fig. 7(B) lacks the crucial interaction with Tyr431 of AChE. Only hydroxyl of its phenol comprising sigma-pi interaction with Tyr337 and its pyran-4-one comprises pi-pi interaction with Gly121 of AChE. Hydroxyl of phenol of compound (1) is making hydrogen bond interaction with Asp74 of AChE while hydroxyl of its chromen-4-one is forming vander Waals interaction with Tyr341 of AChE. Hydroxyl of chromen-4-one in compound (3) makes vander Waals interaction with Tyr341 of AChE and carbonyl O atom of its pyrane-4-one moiety establishing pi-pi interaction with Phe 297 of AChE.

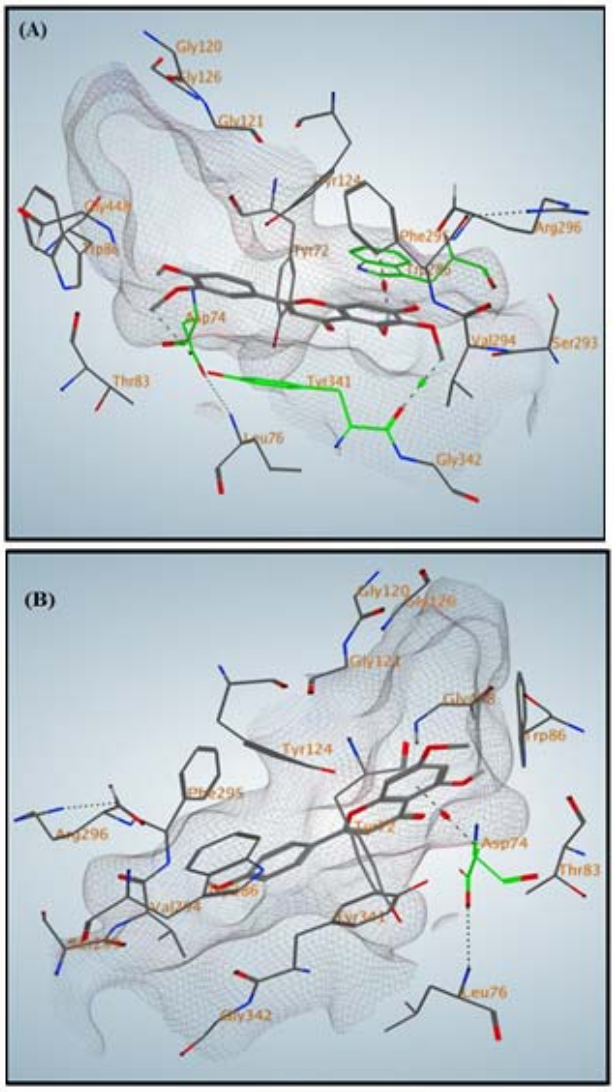


Fig. 7: (A) shows the docking interactions of the most active, compound (2). B. shows the interactions of least active, compound (4). Residues involved in the interactions are represented as green. We can observe that compound (2) comprises the large no. of interactions, while compound (4) contains less number of interactions.

Docking results of butyryl cholinesterase (BChE) inhibitors

Flavonoid derivatives were subjected to docking for the exploration of binding pattern against BChE and their interactions were analyzed carefully. Most active compound for BChE, (1) showed multiple interactions, fig. 8 (A) validating its high experimental activity. Compound (1) against BChE comprises vander Waal interaction between its carbonyl oxygen of chromen-4-one and Gly116 of BChE, and a hydrogen bond was observed between the hydroxyl of its chromen-4-one moiety and Ala328 of BChE. Least active compound (4), fig. 8 (B) contains no interaction against BChE validating the experimental finding. Compound (3) comprises hydrogen bond interaction between the hydroxyl of its phenol moiety and Gly116 of BChE. Compound (2) mediates hydrogen bond interaction between the CH3 of its phenol moiety and Ser198 of BChE.

DISCUSSION

Among the several approaches used for the management of AD, the one of prime importance is to augment the level of acetylcholine through acetyl cholinesterase inhibitors (Enz *et al.,* 1993). Several inhibitors of AChE have been in used for its management. Anyhow, Food and Drug Administration in the United States has approved only galanthamine, donezepil, tacrine and rivastigmine. But due to several side effects and some problems in bioavailability of the present available drugs, a great interest of searching alternative inhibitors of AChE is still of great interest (Zhang *et al.,* 1992). Nature is a huge reservoir of chemical and biological constituents. The chemical synthesis of these complex and unique structures of natural products is not an easy job. A huge proportion of plant species is used in traditional medicines in the world. A selective, reversible and potent inhibitor of AChE, the natural compound Huperzine A was isolated for the first time in 1986 from Chinese medicine *Huperzia serrata* (Jose *et al.,* 2006). Flavonoids are interesting compounds having diverse biological activities like anti-inflammatory, neuro-protective, anti-genotoxic, anti-cancer, antiglycative and anti-Alzheimer's activity (Cao *et al.,* 2013; (Cho *et al.,* 2011; Yixie *et al.,* 2012). These are among the other classes like lkaloids, monoterpenes, coumarins, triterpenes, benzenoids, diterpenes, oxygen heterocycles, sesquiterpenes and ztilbenes reported for AChE inhibitory activity (Jose *et al.,* 2006).

Flavonoids are one of the potential natural constituents used for the management of AD. AChE inhibitors are most important medicaments used for the management of AD.

Therefore, flavonoids having AChE inhibitory potentials and also promising antioxidant potentials could be the new multi potent medicaments for management of AD (Uriate and Calyo, 2011). The results of these isolated flavonoids as AChE inhibitors are of high significance and can be further evaluated in animals for *in-vivo* study.

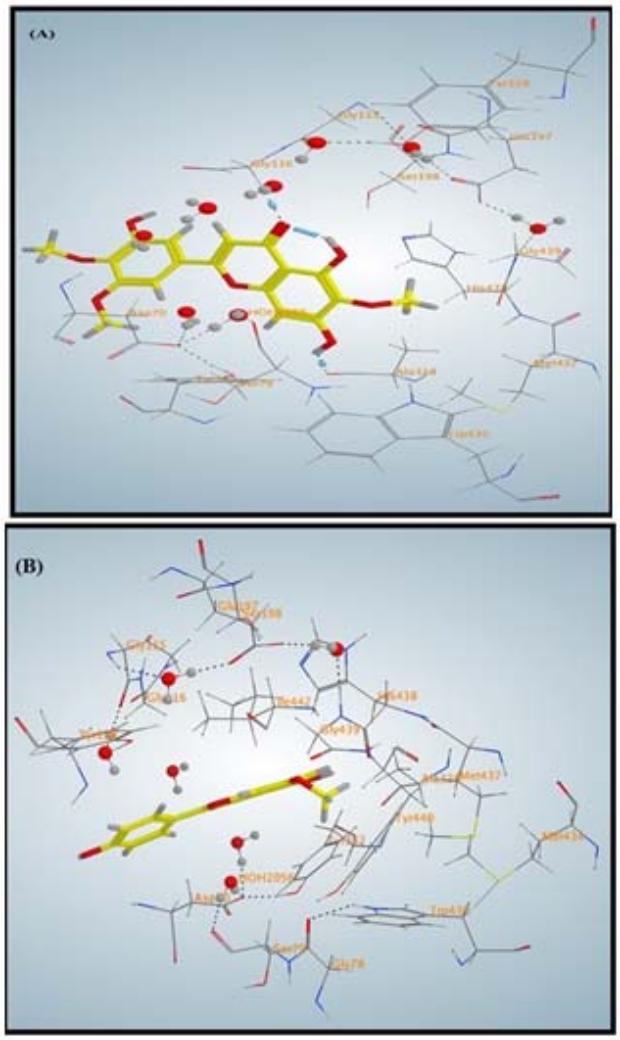


Fig. 8: (A) shows the docking interactions of the most active, compound (1) against BChE. B. shows the interactions of least active, compound (4). We can observe that compound (1) comprises the large number of interactions, while compound (4) contains no interactions.

Scientifically it has been proved that inhibition of BChE is an efficient way in the management of AD and dementias (Schwarz *et al.,* 1995). A significant large quantity of BChE is found in Alzheimer plaques compared to plaques of normal age related non-demented brains. Generally it is viewed as a backup for the homologus AChE and to work as a scavenger for anticholinesterase constituents (Lisa *et al.,* 1999). Having rich structural diversity, flavonoids isolated from plants have found to be efficient inhibitors of BChE (Cho *et al.,* 2012; Atia *et al.,* 2007) indicating their possible role in symptomatic treatment of AD. Based on the results of these four isolated flavonoids and reported literature, it can be concluded that these flavonoids are potential candidates for in-vivo inhibition of BChE.

Molecular docking studies of the isolated flavonoids for AChE confirm our experimental results. By comparing structure (1) and (3), the presence of MeO group at para position of ring A decreases the activity. It is shown in the docking studies that hydroxyl of chromen-4-one and carbonyl O atom of pyrane-4-one moiety in compound (3) make Vander Waals interaction with Tyr341 and pi-pi interaction with Phe 297 of AChE respectively, validating the in-vitro experimental results. By comparing structure (4) with (1) (2) and (3), it shows that by increasing the number of MeO groups at ring C enhances the activity.

Its hydroxyl and CH_3 of chromen-4-one show sigma-pi and Vander Waal interactions with phenyl of Trp286 and Tyr341 of AChE respectively. Similarly, methoxy of its phenol moiety shows Vander Waal interaction with O atom of Asp74 AChE. The incorporation of OH group in ring (C) results a decrease in the activity as shown in structure (3).The, hydroxyl of phenol of compound (1) makes hydrogen bond interaction with Asp74 and hydroxyl of its chromen-4-one is forming Vander Waals interaction with Tyr341 of AChE respectively, confirming its experimental value. On other hand, docking studies for BChE inhibition reveals that most active compound (1) shows multiple interactions with target BChE, validating its high experimental activity. The presence of OH group on ring C enhances the activity i.e structure (1) and (3) are having OH group on ring C show enhance activity than structures (2) and (4). This is clear from the docking studies that (1) comprises Vander Waal interaction between carbonyl oxygen of chromen-4-one and Gly116 of BChE, and a hydrogen bond was observed between the Hydroxyl of chromen-4-one moiety and Ala328 of BChE. The removal of MeO group at ring C reduces the activity i.e structure (3) possess lower activity than structure (1) in the docking study the compound (3) comprises hydrogen bond interaction between the hydroxyl of phenol moiety and Gly116 and (2) mediates hydrogen bond interaction between the CH3 of phenol moiety and Ser198 of BChE respectively. Comparison of structure (4) with structures (1), (2) and (3) shows that increasing the number of MeO groups on ring C enhances the activity. From the docking study compound (4) contains no interaction against BChE which is the least active compound validating the experimental finding.

CONCLUSION

The inhibition of the AChE and BChE has been of prime importance in prevention of many neurodegenerative diseases. Active constituents from medicinal plants have been the best alternatives for inhibition of AChE and BChE because of their less toxicity and enhanced

efficacy. Results of the present study show that the isolated flavonoids possess excellent potentials against AChE and BChE inhibition that were confirmed by molecular docking studies. It can be concluded from the results that these active constituents are more useful and can be the lead molecules for the management of several neurodegenerative diseases like ataxia, Parkinson's disease, Alzeimer,s disease and several other types of dementia. Our research group is committed to investigate the *in-vivo* anti-AChE and anti-BChE activity in animal model and possible mechanism of these isolated flavonoids in order to provide a base on scientific knowledge.

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