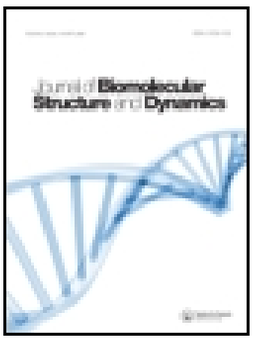

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Exploring 3-Benzyloxyflavones as new lead cholinesterase inhibitors: synthesis, structure–activity relationship and molecular modelling simulations

Ehsan Ullah Mughal[a], Amina Sadiq[b], Momna Ayub[b], Nafeesa Naeem[a], Asif Javid[a], Sajjad Hussain Sumrra[a], Muhammad Naveed Zafar[c], Bilal Ahmad Khan[d], Fouzia Perveen Malik[e] and Ishtiaq Ahmed[f]

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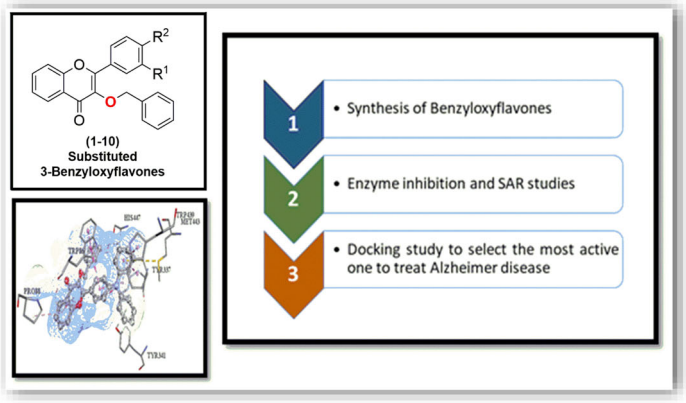
ABSTRACT

In this protocol, a series of 3-benzyloxyflavone derivatives have been designed, synthesized, characterized and investigated *in vitro* as cholinesterase inhibitors. The findings showed that all the synthesized target compounds (**1–10**) are potent dual inhibitors of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes with varying IC_{50} values. In comparison, they are more active against AChE than BChE. Remarkably, amongst the series, the compound **2** was identified as the most active inhibitor of both AChE ($IC_{50} = 0.05 \pm 0.01\ \mu M$) and BChE ($IC_{50} = 0.09 \pm 0.02\ \mu M$) relative to the standard Donepezil ($IC_{50} = 0.09 \pm 0.01$ for AChE and $0.13 \pm 0.04\ \mu M$ for BChE). Moreover, the derivatives **5** ($IC_{50} = 0.07 \pm 0.02\ \mu M$) and **10** ($0.08 \pm 0.02\ \mu M$) exhibited the highest selective inhibition against AChE as compared to the standard. Preliminary structure-activity relationship was established and thus found that cholinesterase inhibitory activities of these compounds are highly dependent on the nature and position of various substituents on Ring-B of the 3-Benzyloxyflavone scaffolds. In order to find out the nature of binding interactions of the compounds and active sites of the enzymes, molecular docking studies were carried out.



HIGHLIGHTS

1. 3-benzyloxyflavone analogues were designed, synthesized and characterized.
2. The target molecules (**1–10**) were evaluated for their inhibitory potential against AChE and BChE inhibitory activities.
3. Limited structure-activity relationship was developed based on the different substituent patterns on aryl part.
4. Molecular docking studies were conducted to correlate the *in vitro* results and to identify possible mode of interactions at the active pocket site of the enzyme.

Abbreviations: AChE: Acetylcholinesterase; Ala: Alanine; AD: Alzheimer disease; AFO: Algar–Flynn–Oyamada; AI: Amyloid-including protein; BChE: Butyrylcholinesterase; DMF: Dimethylformamide; DMSO: Dimethyl sulfoxide; ESI: Electrospray ionization; EtOH: Ethanol; FTIR: Fourier transformed infrared radiations; His: Histamine; H_2O_2: Hydrogen peroxide; IR: Infrared; K_2CO_3: Potassium carbonate; Leu: Leucine; MeOH: Methanol; Met: Methionine; NaOH: Sodium hydroxide; NMR:

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Nuclear magnetic resonance; Pas: Peripheral anionic site; PBD: Protein drug bank; Phe: Phenylalanine; Pro: Proline; Ser: Serine; SAR: Structure-activity relationship; TLC: Thin-layer chromatography; Trp: Tryptophan; Tyr: Tyrosine; UV-VIS: Ultraviolet-Visible

Introduction

Flavonoids represent a group of polyphenolic compounds which are widely distributed throughout the higher plants. They are recognized by possessing 2-phenyl-γ-benzopyrone (C_6-C_3-C_6) as a characteristic skeleton. They are further divided into various sub-classes such as aurones, anthocyanins, chalcones, flavones, flavonols, and isoflavones depending on the saturation level and the absence or presence of the central pyran ring (Patel & Shah, 2017; Sashidhara et al., 2012). Studies performed on these compounds have confirmed that they belong to the class of secondary plant metabolites (Harborne & Williams, 2000; Hostetler et al., 2017) and are well known for having a vast variety of bioactivities such as anti-oxidant (Rice-Evans, 2001), anxiolytic and anti-cancer (Liu et al., 2010), analgesic, anti-inflammatory and anti-microbial (Mishra & Tiwari, 2011), anti-ulcer and thrombosis (Bhatt et al., 2016).

Flavonols (3-hydroxy-2-phenyl-4*H*-chromen-4-ones) (Figure 1) constitute one of the most important sub-classes of flavonoids. Owing to the structural diversity of flavonols (3-hydroxyflavones) and their ubiquitous occurrence in fruits and vegetables, they have attracted the attention of medicinal chemists because of their importance in a variety of important pharmacological activities including health benefits (Bozdag-Dundar et al., 2005; Graf et al., 2005; Tapas et al., 2008). However, most of the flavonols have not been developed as clinical drugs because of poor bioavailability (less than 5%), toxicity, limited occurrence in nature and induction or inhibition of some metabolic enzymes (Jin et al., 2019; Li et al., 2017).

To overcome those problems, the development of new synthetic methods to produce structurally modified flavones is now considered as an important goal in exploring their diverse roles. This reason has increased the interest of medicinal chemists to further study flavones as lead molecules to treat various diseases. In this context, recently researchers have focused on the synthesis and biological evaluation of flavone-based ethers (Díaz et al., 2017; Imran et al., 2016; Jin et al., 2019; Li et al., 2017; Nhu et al., 2015; Wang et al., 2018). However, the reported natural and synthetic flavone derivatives containing ether linkage mostly present at ring A or ring B, and such motifs have been reported to possess potent enzyme inhibition activities (Imran et al., 2016). Therefore, to the best of our knowledge, exploring the synthesis and inhibitory potential of 3-*O*-benzylflavonol scaffolds against cholinesterase enzymes remain an interesting goal.

Senile Alzheimer's disease (AD) is a severe neurodegenerative disorder with progressive cognitive impairments among the older people in developed and some developing countries that eventually leads to death. AD is responsible for protein aggregation, inflammation, amyloid-including protein (AI), oxidative stress, and acetylcholine signalling dysfunction in the basal forebrain. The development of chemical agents for the treatment of AD has been of great interest for several years (Rampa et al., 1998). The enzymes such as AChE and BChE were identified as essential targets for efficient AD management by increasing the production of acetylcholine in the brain and reducing the AI deposition as each of these enzymes play an essential role at the beginning of geriatric plaque development (Mughal et al., 2018; 2019). Deposition of β-amyloid in the brain is hypothesized as the main cause of neuronal cell death in AD patients, thus preventing the accumulation of β-amyloid represents an alternative therapeutic strategy that specifically targets AD pathogenesis. Several small molecules can interact with the mechanism of β-amyloid aggregation and the functionalization of small aromatic molecules can be used as the logical nature of inhibitors of aggregation. Although there is a significant amount of literature reporting on AChE and BChE inhibitors (Agbo et al., 2019; Asghar et al., 2020; Bajda et al., 2011; Barai et al., 2019; Cavdar et al., 2019; Chen et al., 2017; Mughal, Sadiq, Khan, et al., 2017; Mughal, Sadiq, Murtaza, et al., 2017; Faraji et al., 2019; Mathew et al., 2019; Mishra et al., 2017; Mphahlele et al., 2018; Mughal et al., 2018; Mughal et al., 2019; Özil et al., 2019; Saxena & Dubey, 2019; Shaik et al., 2016; Shaikh et al., 2020; Sun et al., 2019; Xie et al., 2016; Yerdelen et al., 2015), but it has been observed that a great deal of attention has been paid in recent years to the hunt for new AChE inhibitors for the treatment of AD (Loizzo et al., 2008). In this connection, during recent years, natural flavonoids and their synthetic analogues have been identified as potential inhibitors of cholinesterase enzymes, as they have the benefits of being more tolerable, inexpensive and easier to occur in the natural environment (Agbo et al., 2019; Anand & Singh, 2013; Daglia, 2012; Mughal, Sadiq, Khan, et al., 2017; Mughal, Sadiq, Murtaza, et al., 2017; Uriarte-Pueyo & I Calvo, 2011). Furthermore, flavonoids and related compounds were reported in the literature as potent antimicrobial agents (Genoux et al., 2011; Ibrahim, 2014; Kamlesh et al., 2017; Mahmoud et al., 2017; Mughal et al., 2006; Sarbu et al., 2019; Shakhatreh et al., 2016). Due to their unique ability to modulate different enzyme systems, the envisioned compounds exhibit a great diversity in their biological activities and therapeutic functions (Dymarska et al., 2018). Additionally, the substitution pattern in flavone derivatives plays a vital role in their biochemical and pharmacological properties.

Encouraged by above-mentioned biological potential of flavone as a privileged scaffold and following our study on exploring new structural motifs against cholinesterase enzymes (Mughal, Sadiq, Khan, et al.2017; Mughal, Sadiq, Murtaza, et al., 2017; Mughal et al., 2018; Mughal et al., 2019), we have designed and synthesized a new set of 3-*O*-benzylflavonol ethers with different functional groups for determining their inhibitory capacity against the previously

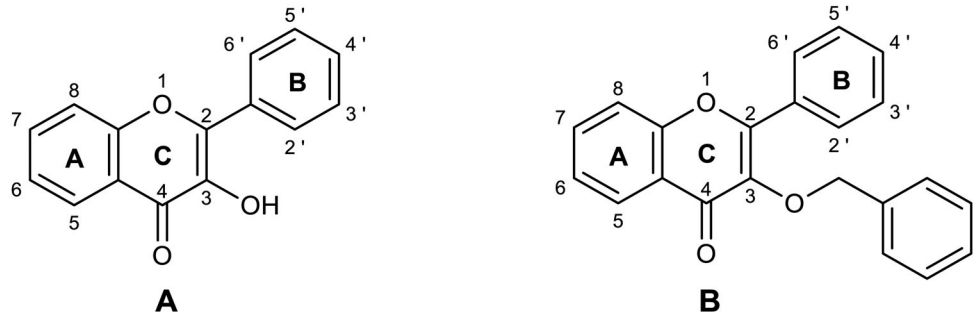


Figure 1. Representative structures of Flavonol (A) and 3-*O*-benzylflavonol or 3-benzyloxyflavone (B).

mentioned targets. The role of synthetic flavones as possible inhibitors of cholinesterases remains an important target and a constant endeavour.

Materials and methods

All the commercially available reagents were obtained from Sigma-Aldrich and Merck and used as supplied. Melting points were determined on an Electro-thermal melting point apparatus and uncorrected. IR spectra were taken as KBr discs using Bio-Rad spectrophotometer. NMR (^{1}H, 300 MHz, ^{13}C, 75 MHz) spectra were recorded on a Bruker spectrometer with TMS as the internal standard. EI-MS spectrometric analysis was obtained using a Fisons VG sector-field instrument. The IR values and chemical shifts are expressed in $\bar{\nu}$ units and parts per million respectively. Reaction progress was monitored using thin-layer chromatography (TLC) on silica gel pre-coated plates and spots were detected under UV light. The absorption spectra have been recorded on the Jasco UV-VIS V-670 instrument using QUARTZ cell in very dilute solutions prepared in different solvents.

General procedures for the syntheses of 3-hydroxyflavone and 3-benzyloxyflavone derivatives

Synthesis of 3-hydroxyflavone derivatives (F_1-F_{10})

A mixture of 2′-hydroxyacetophenone (1.2 mL, 10.0 mmol) and 10 mL of an aqueous solution of NaOH (30%) was stirred in MeOH (25 mL) for 30 min accompanied by dropwise addition of substituted benzaldehyde (10.0 mmol). The reaction mixture was stirred further at ambient temperature for 5-6 h. The progress of reaction was checked by comparative TLC. Chalcone thus formed, *in situ*, was cyclized further by the addition of 1.5 mL of 35% H_2O_2 solution followed by stirring for an additional 1 h at the same temperature. The reaction mixture was neutralized by hydrochloric acid (HCl, 10%). The precipitates formed were filtered, washed thoroughly with water, dried and crystallized by ethanol to give pure Flavonol (**F_1-F_{10}**).

Synthesis of 3-benzyloxyflavone derivatives (1–10)

The pure synthesized flavonol product (1.0 mmol) was dissolved in dimethylformamide (5.0 mL) containing anhydrous K_2CO_3 (5.0 mmol), and tetra-butyl ammonium bromide (3.0 mmol). After stirring the reaction mixture at 50-60 °C for 30 min, benzyl chloride (2.0 mmol) was added and the reaction mixture was stirred further at the same temperature for 24 to 48 h. After reaction completion (analysed by TLC), the reaction mixture was quenched by addition of water and neutralized with HCl (10%). The phases were separated and the aqueous phase was extracted with chloroform (2 × 25 mL). The combined organic phase was washed with brine, dried over anhydrous Na_2SO_4 and the solvent was removed under reduced pressure. The crude product was purified by recrystallization in ethanol to afford the desired product in pure form.

Enzyme inhibition assay

AChE and BChE inhibition activities were measured spectrophotometrically by the Ellman method with minor adjustment. Five different concentrations of standard and test compounds were prepared by diluting their stock solutions immediately before use. 100 µL of each sample was combined with 50 µL AChE/BChE enzyme and allowed to stand for 10 min. 50 µL of substratum *i.e.* acetylthiocholine iodide (0.71 mM) for acetylcholinesterase or butyrylthiocholine chloride (0.2 mM) for butyrylcholinesterase, 50 µL (0.5 mM) of DTNB and 500 µL phosphate buffer of pH 8 were added to the above-mentioned mixture and the mixture was incubated at retention time for 15 min at 37 °C. The solution becomes yellow due to the hydrolysis of substrate causing the formation of 5-thio-2-nitrobenzoate anion. The substrate hydrolysis was determined using spectrophotometer by measuring the increase in absorbance at 400 nm and 412 nm for acetylcholinesterase and butyrylcholinesterase, respectively. The percent enzyme inhibition (%) was calculated using the following equation:

$$(\%)\ \text{Inhibition} = \frac{B-A}{B} \times 100$$

Here A = enzyme absorbance with test sample; B = enzyme absorbance without test sample. Every experiment was performed in triplicate and the average value was taken. IC_{50} values were estimated by linear regression analysis. Donepezil was used as a standard (Agbo et al., 2019; Asghar et al., 2020; Barai et al., 2019; Mughal, Sadiq, Khan, et al., 2017; Mughal, Sadiq, Murtaza, et al., 2017; Faraji et al., 2019; Mishra et al., 2017; Mphahlele et al., 2018; Mughal et al., 2018; Mughal et al., 2019; Özil et al., 2019; Shaikh et al., 2020; Sun et al., 2019).

Molecular docking studies

Docking assay was conducted to determine the enzyme-ligand interactions. AChE (PDB ID: 4BDT) and BChE (PDB ID: 4BDS) crystal structures were obtained from ACDz Chemsketch protein database (RCSB) and 3 D Pro 12.0 was used to optimize the compounds in 3 D orientation and processed as SYBYL mol 2 file format. The AutoDock software v1.5.6 has been used for docking purposes. Discovery Studio Visualizer v 4.0 was used to represent the most active and best position of compounds under analysis (Mughal, Sadiq, Khan, et al.2017; Mughal, Sadiq, Murtaza, et al., 2017; Mughal et al., 2018; Mughal et al., 2019).

Results and discussion

Chemistry

Given the extensive literature on the Algar–Flynn–Oyamada (AFO) reaction and the reported synthesis of close analogues to those we required (Mughal et al., 2018; Nhu et al., 2015), our initial attempts to prepare 3-*O*-Benzylated flavonol derivatives (**1–10**) employed the conventional two-step one-pot approach: generation of the requisite 2′-hydroxychalcone as key intermediates via the Claisen–Schmidt condensation, and subsequent oxidative cyclization with basic hydrogen peroxide (35%) solution (Scheme 1). In the event, 2′-hydroxychalcones were obtained through base-catalysed condensation of 2′-hydroxyacetophenone with different aryl aldehydes in methanol-sodium hydroxide solution. The resultant 2′-hydroxychalcone derivatives were then subjected to conventional AFO conditions (hydrogen peroxide and sodium hydroxide) in the same solvent to produce the flavonols (**F_1-F_{10}**) as outlined in Scheme 1. These intermediate compounds were purified through recrystallization by ethanol and thereafter analysed by FTIR, UV-Vis and NMR spectroscopic techniques only. The desired compounds (**1–10**) were synthesized through a single-step reaction of 3-hydroxyflavone (flavonol) with benzyl chloride in the presence of K_2CO_3 dissolved in DMF. All 3-benzyloxyflavone derivatives (**1–10**) were obtained in moderate to good yields and purified by recrystallization in EtOH.

The structures of all newly synthesized compounds were deduced from various spectroscopic techniques (UV-Vis, FTIR, NMR spectroscopic techniques). Their molecular masses were confirmed by Electrospray Ionization (ESI) method. For instance, in the IR spectrum, there are signs of a successful benzyl group attachment with chromone part by the disappearance of peak around 3300 cm^{-1} due to the 3-OH and the appearance of new peak around 1300 cm^{-1} due to benzylic moiety. Similarly, the count of a number of protons and the number of carbon resonances in their ^{1}H-NMR and ^{13}C-NMR spectra, respectively, were also in agreement with the suggested molecular formulas. There is one characteristic singlet peak because of oxymethylene (-OCH_2-) group appearing around δ 5.00 ppm in ^{1}H-NMR spectra of the envisioned compounds. Additionally, the ^{1}H NMR spectra showed downfield peaks for aromatic protons ranging from 8.23 to 7.15 ppm. Furthermore, their ^{13}C NMR spectra manifested two characteristics signals around δ 173.0 ppm and 75.0 ppm for ketonic and oxymethylene (-OCH_2-) structural features, respectively. Likewise, the structures of other derivatives were also characterized by a similar strategy. In spite of having all NMR data, the complete assignment of each signal was not achieved. Nevertheless, all spectroscopic data are in good agreement with assumed structures of the desired compounds.

The spectral data of all the synthesized flavonols except **F_2** are given in the literature as well as in supporting information (SI) (Gunduz et al., 2012; Gupta et al., 2014; Khanna et al., 2015; Singh et al., 2017; You et al., 2020). However, the complete spectroscopic data of their ether derivatives **(1–10)** and the flavonol (**F_2**) are as under:

2-(4-(Diphenylamino) phenyl)-3-hydroxy-4H-chromen-4-one (F_2)

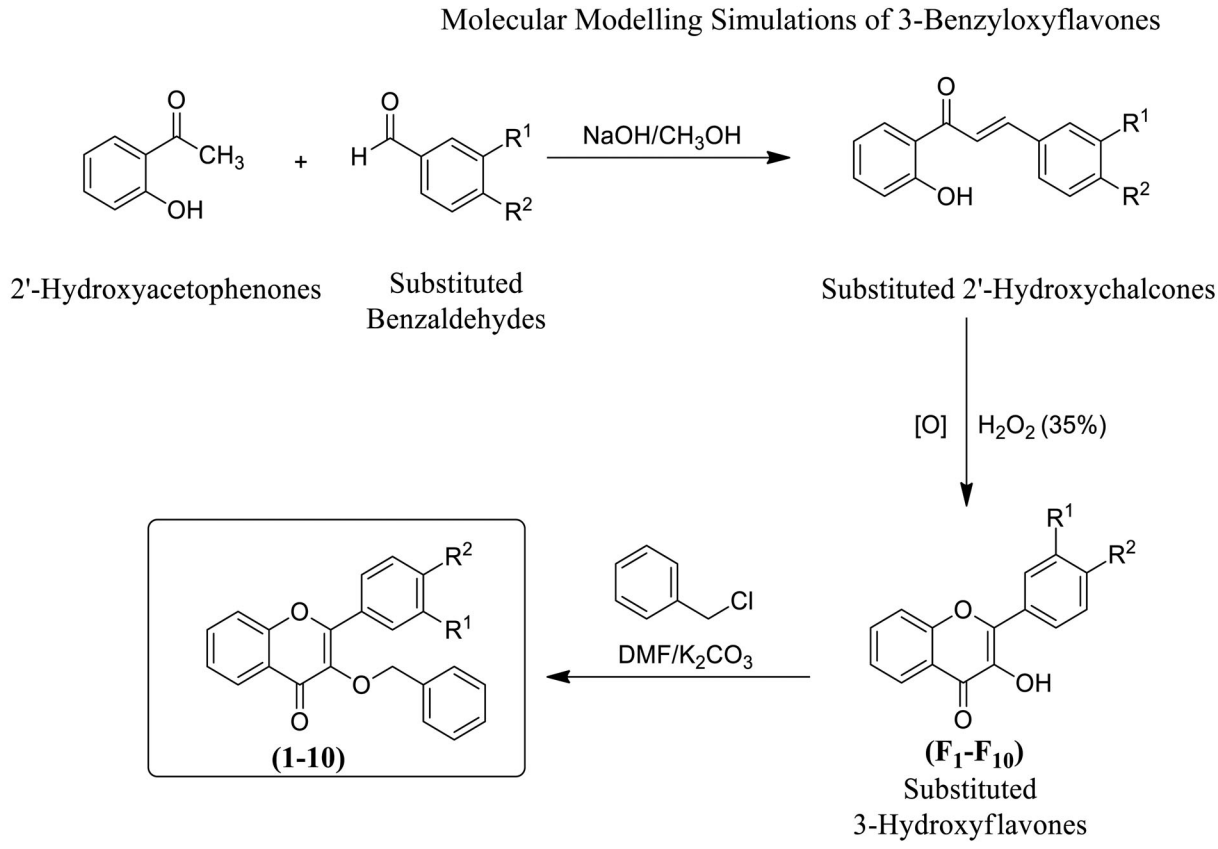
Mustard-yellow crystalline solid; Yield: 90%; m.p. 188-190 °C; R_f (Ethyl acetate: *n*-hexane 1:3) = 0.7; UV λ_{max} = 293, 393 nm (CH_3OH); FTIR (cm^{-1}): 3221, 1686, 1481, 1410, 1329, 1154, 1134, 824; ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.74 (s, 1H, OH), 7.63–7.58 (m, 2H, Ar-H), 7.30–7.24 (m, 6H, Ar-H), 7.12-7.07 (m, 8H, Ar-H), 6.96 (d, J = 9.0 Hz, 2H, Ar-H), ^{13}C NMR (75 MHz, $CDCl_3$): δ 181.5, 153.4, 146.1, 131.4, 129.7, 129.3, 126.3, 126.2, 125.0, 118.3, the other carbons are isochronous; accurate mass (EI-MS) of $[M]^{+\cdot}$: Calcd. for $C_{27}H_{19}NO_3$ 405.13649; found 405.13640.

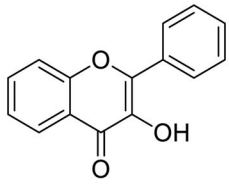
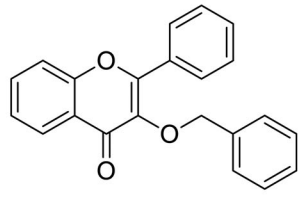
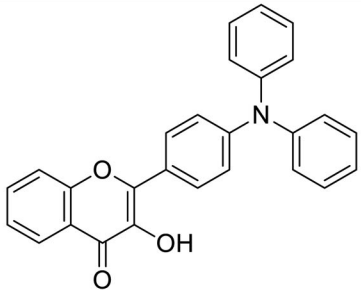
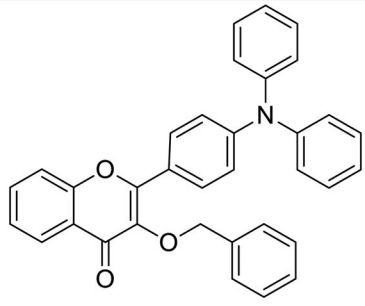
3-(Benzyloxy)-2-phenyl-4H-chromen-4-one (1) (*Peres et al., 2017*)

Dark-brown crystalline solid; Yield: 86%; m.p. 116–118 °C; R_f (Ethyl acetate: *n*-hexane 1:3) = 0.8; UV λ_{max} = 296 nm (EtOAc); FTIR (cm^{-1}): 3029, 2895, 1719, 1596, 1282; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.70–7.65 (m, 4H, Ar-H), 7.42-7.25 (m, 5H, Ar-H), 7.25–7.15 (m, 5H, Ar-H), 5.05 (s, 2H, $\underline{CH_2}$-Ph); ^{13}C NMR (75 MHz, $CDCl_3$): δ 175.5, 157.3, 156.0, 142.1, 136.4, 135.0, 132.4, 131.3, 130.4, 129.2, 129.0, 128.7, 128.4, 127.6, 127.3, 127.0, 125.4, 124.7, 123.8, 117.7, 73.5; accurate mass (ESI, AcCN, +ve) of $[M+H]^+$: Calcd. for $C_{22}H_{17}O_3$ 329.11776; found 329.11770.

3-(Benzyloxy)-2-(4-diphenylamino) phenyl)-4H-chromen-4-one (2)

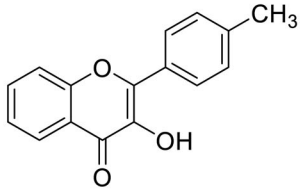
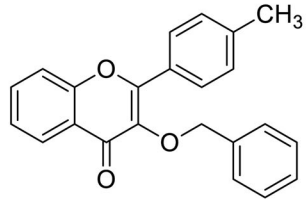
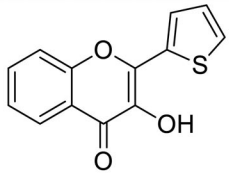
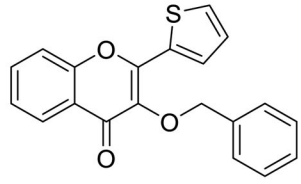
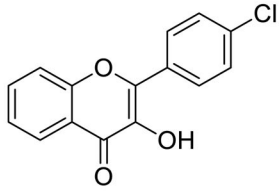
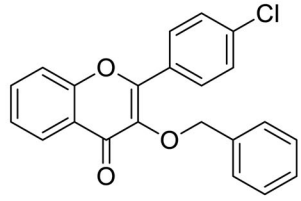
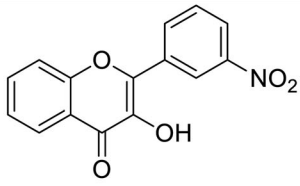
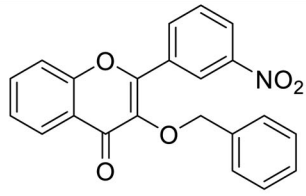
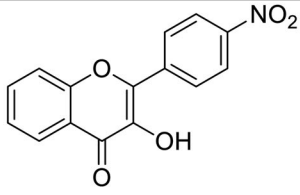
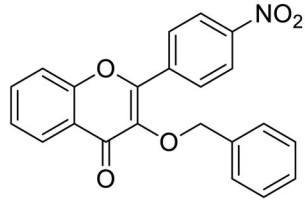
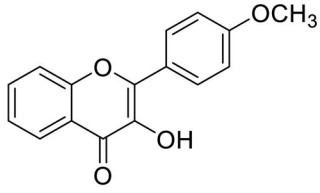
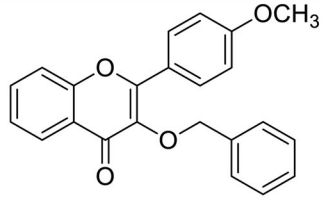
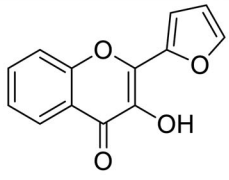
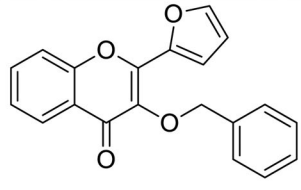
Yellow crystalline solid; Yield: 80%; m.p. 147–150 °C; R_f (Ethyl acetate: *n*-hexane) = 0.9; UV λ_{max} = 298 nm (EtOAc); FTIR (cm^{-1}): 3110, 2850, 1685, 1501, 1402, 1328, 1284;^{1}H NMR (300 MHz, $CDCl_3$): δ 7.67–7.60 (m, 4H, Ar-H), 7.40–7.26 (m, 6H, Ar-H), 7.22–7.15 (m, 8H, Ar-H), 7.26-7.15 (m, 5H, Ar-H), 5.06 (s, 2H, $\underline{CH_2}$-Ph); ^{13}C NMR (75 MHz, $CDCl_3$): δ 179.7, 155.2, 148.3, 135.7, 133.7, 132.6, 132.1, 131.0, 130.6, 130.2, 129.8, 129.1, 128.7, 128.4, 128.1, 128.0, 126.7, 125.8, 124.7,124.2, 123.7, 123 118.4, 74.2, the other carbons are isochronous; accurate mass (ESI, AcCN, +ve) of $[M+H]^+$: Calcd. for $C_{34}H_{26}NO_3$ 496.19126; found 496.19116.



Flavonols	Structures	Target Compounds	Structures
F_1		1	
F_2		2	

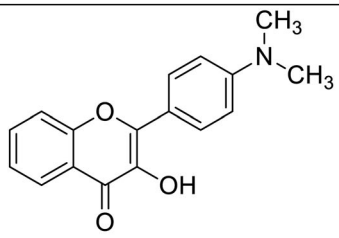
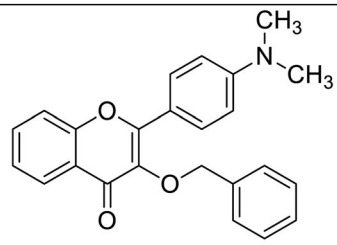
Scheme 1. Synthesis of flavonols (F_1-F_{10}) and 3-*O*-benzylflavonol derivatives (**1**–**10**).

Molecular Modelling Simulations of 3-Benzyloxyflavones

F_3		**3**	
F_4		**4**	
F_5		**5**	
F_6		**6**	
F_7		**7**	
F_8		**8**	
F_9		**9**	

Scheme 1. Continued.

Molecular Modelling Simulations of 3-Benzyloxyflavones

F_{10}		10	

Scheme 1. Continued.

3-(Benzyloxy)-2-(4-methylphenyl)-4H-chromen-4-one (3)

Off-white solid; Yield: 75%; m.p. 110–112 °C; R_f (Ethyl acetate: *n*-hexane 1:3) = 0.85; UV λ_{max} = 316 nm (EtOAc); FTIR (cm^{-1}): 3031, 2995, 2887, 1718, 1597, 1293; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.23 (dd, *J* = 3.0, 9.0 Hz, 1H, Ar-H), 7.91–7.86 (m, 2H, Ar-H), 7.64–7.57 (m, 1H, Ar-H), 7.46–7.43 (m, 1H, Ar-H), 7.36–7.27 (m, 3H, Ar-H), 7.23-7.15 (m, 5H, Ar-H), 5.05 (s, 2H, CH_2-Ph), 2.36 (s, 3H, Me); ^{13}C NMR (75 MHz, $CDCl_3$): δ 175.0, 156.5, 155.3, 141.0, 139.7, 136.7, 133.4, 129.0, 128.93, 128.90, 128.8, 128.3, 128.2, 128.1, 125.7, 124.4, 124.2, 118.0, 74.2, 24.3,the other carbons are isochronous; accurate mass (ESI, AcCN, +ve) of $[M+H]^+$: Calcd. for $C_{23}H_{19}O_3$ 343.13342; found 343.13328.

3-(Benzyloxy)-2-(thiophen-1-yl)-4H-chromen-4-one (4) (*Kamboj et al., 2013*)

Orange solid; Yield: 80%; m.p. 154–156 °C; R_f (Ethyl acetate: *n*-hexane 1:3) = 0.7; UV λ_{max}= 340 nm (EtOAc); FTIR (cm^{-1}): 3029, 2960, 1964, 1720, 1598, 1288, 1024; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.20–7.98 (m, 3H, Ar-H), 7.87–7.66 (m, 2H, Ar-H), 7.47–7.30 (m, 2H, Ar-H), 7.24–7.15 (m, 5H, Ar-H), 5.03 (s, 2H, CH_2-Ph); ^{13}C NMR (75 MHz, $CDCl_3$): δ 175.2, 155.1, 144.5, 135.7, 131.7, 130.5, 129.3, 129.0, 128.9, 128.7, 128.4, 128.1, 127.9, 127.4, 127.0, 125.8, 125.3, 123.5, 118.6, 74.18; accurate mass (ESI, AcCN, +ve) of $[M+H]^+$: Calcd. for $C_{20}H_{17}O_3S$ 337.08984; found 337.08971.

3-(Benzyloxy)-2-(4-chlorophenyl) -4H-chromen-4-one (5)

Off-white solid; Yield: 82%; m.p. 143–145 °C; R_f (Ethyl acetate: *n*-hexane 1:3) = 0.81; UV λ_{max} = 350 nm (EtOAc); FTIR (cm^{-1}): 3025, 2920, 1720, 1571, 1289, 752;^{1}H NMR (300 MHz, $CDCl_3$): δ 8.24 (d, *J* = 3.0, 9.0 Hz, 1H, Ar-H), 7.88 (m, 2H, Ar-H), 7.65-7.59 (m, 1H, Ar-H), 7.46-7.43 (m, 1H, Ar-H), 7.38–7.33 (m, 3H, Ar-H), 7.25-7.15 (m, 5H, Ar-H), 5.08 (s, 2H, CH_2-Ph); ^{13}C NMR (75 MHz, $CDCl_3$): δ 175.1, 155.2, 138.1, 136.6, 135.6, 133.4, 129.7, 127.3, 125.2, 124.5, 122.0, 118.2,72.1, other carbons are isochronous; accurate mass (ESI, AcCN, +ve) of $[M+H]^+$: Calcd. for $C_{22}H_{16}ClO_3$ 363.07879; found 363.07865.

3-(Benzyloxy)-2-(3-nitrophenyl)-4H-chromen-4-one (6)

Light-brown solid; Yield: 74%; m.p. 123–125 °C; R_f (Ethyl acetate: *n*-hexane) = 0.6; UV λ_{max} = 298 nm (EtOAc); FTIR (cm^{-1}): 3084, 2925, 1724, 1527, 1497, 1288; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.80 (d, *J* = 3.0 Hz, 1H, Ar-H), 8.48 (m, 1H, Ar-H), 8.39 (t, *J* = 9.0 Hz, 1H, Ar-H), 8.00 (d, *J* = 9.0 Hz, 1H, H-5), 7.82-7.76 (m, 4H, Ar-H), 7.30-7.21 (m, 5H, Ar-H), 5.03 (s, 2H, CH_2-Ph); ^{13}C NMR (75 MHz, $CDCl_3$): δ 175.5, 158.4, 155.1, 146.2, 134.7, 132.7, 131.4, 130.5, 130.0, 129.6, 128.8, 128.5, 128.3, 127.8, 127.1, 126.0 (CH, 125.5, 125.0, 124.0, 122.8, 118.1, 74.2; accurate mass (ESI, AcCN, +ve) of $[M+H]^+$: Calcd. for $C_{22}H_{16}NO_5$ 374.10284; found 374.10275.

3-(Benzyloxy)-2-(4-nitrophenyl)-4H-chromen-4-one (7)

Brown solid; Yield 58%; m.p. 104 °C; R_f (Ethyl acetate: *n*-hexane 1:3) = 0.65; UV λ_{max} = 274 nm (EtOAc); FTIR (cm^{-1}): 3124, 2800, 1722, 1567, 1522, 1280; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.38 (d, *J* = 9.0 Hz, 2H, Ar-H), 8.29 (*J* = 9.0 Hz, 2H, Ar-H), 8.12–8.01 (m, 2H, Ar-H), 7.90–7.80 (m, 5H, Ar-H), 5.06 (s, 2H, CH_2-Ph); ^{13}C NMR (75 MHz, $CDCl_3$): δ 175.4, 159.8, 156.7,148.8, 137.7, 135.1, 130.2, 129.8, 129.6, 129.0, 128.8, 128.7, 128.5, 128.1, 127.9, 127.2, 125.8, 125.5, 124.7,124.0, 118.1, 74.2; accurate mass (ESI, AcCN, +ve) of $[M+H]^+$: Calcd. for $C_{22}H_{16}NO_5$ 374.10284; found 374.10271.

3-(Benzyloxy)-2-(4-methoxyphenyl)-4H-chromen-4-one (8) (*Kavitha, 2012*; *Rao & Kumar, 2014*)

Orange solid; Yield: 71%; m.p.135–137 °C; R_f (Ethyl acetate: *n*-hexane 1:3) = 0.7; UV λ_{max} = 280 nm (EtOAc); FTIR (cm^{-1}): 3028, 2880, 1720, 1501, 1423, 1200; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.02 (d, *J* = 9.0 Hz, 2H, Ar-H), 7.98 (d, *J* = 9.0 Hz, 2H, Ar-H), 7.80 (m, 1H, Ar-H), 7.67–7.47 (m, 3H, Ar-H), 5.04 (s, 2H, CH_2-Ph);^{13}C NMR (75 MHz, $CDCl_3$): δ 175.2, 160.3,159.7,155.2, 135.4, 134.3, 130.4, 129.5, 129.1, 128.8, 128.5, 128.2, 128.0, 127.5,127.3, 127.0, 126.0 125.6,124.8, 123.1,118.1, 74.3, 55.6; accurate mass (ESI, AcCN, +ve) of $[M+H]^+$: Calcd. for $C_{23}H_{19}O_4$ 359.12833; found 359.12820.

3-(Benzyloxy)-2-(furan-2-yl)-4H-chromen-4-one (9)

Dark-brown solid; Yield: 66%; m.p. 95–97 °C; R_f (Ethyl acetate: *n*-hexane 1:3) = 0.6; UV λ_{max} = 329 nm (EtOAc); FTIR (cm^{-1}): 3021, 2789, 1709, 1592, 1235, 1170; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.35–8.25 (m, 1H, Ar-H), 8.05–7.98 (m, 2H, Ar-H), 7.77–7.50 (m, 4H, Ar-H), 7.27–7.18 (m, 5H, Ar-H), 5.05 (s, 2H,

Table 1. AChE and BchE the enzyme inhibition efficiency of the target compounds (**1–10**).

Compound No.	AChE IC_{50} ±SEM[a] (μM)	BChE IC_{50} ±SEM[a] (μM)
1	0.12 ± 0.02	3.10 ± 0.30
2	0.05 ± 0.01	0.09 ± 0.02
3	1.95 ± 0.15	3.25 ± 0.21
4	10.02 ± 0.78	27.11 ± 0.35
5	0.07 ± 0.02	2.13 ± 0.08
6	2.85 ± 0.35	9.25 ± 0.90
7	1.52 ± 0.15	14.37 ± 0.65
8	1.01 ± 0.01	3.14 ± 0.70
9	11.60 ± 0.03	12.23 ± 0.74
10	0.08 ± 0.02	1.50 ± 0.10
Donepezil[St]	0.09 ± 0.01	0.13 ± 0.04

[a]IC_{50} values (mean ± standard error of mean); [St]Standard inhibitor for cholinesterase enzymes.

CH_2-Ph); ^{13}C NMR (75 MHz, $CDCl_3$): δ 175.3, 155.2, 145.0, 136.1, 131.8, 131.7, 130.3, 129.1, 129.0, 128.7, 128.5, 128.2, 128.0, 127.5, 127.1, 125.7, 125.5, 123.6, 118.6, 74.2; accurate mass (ESI, AcCN, +ve) of $[M+H]^+$: Calcd. for $C_{20}H_{15}O_4$ 319.09703; found 319.09698.

3-(Benzyloxy)-2-(4-(dimethylamino)phenyl)-4H-chromen-4-one (10)

Dark-red solid; Yield: 85%; m.p. 75–78 °C; R_f (Ethyl acetate: *n*-hexane) = 0.8; UV λ_{max} = 318 nm (EtOAc); FTIR (cm^{-1}): 3021, 2750, 1710, 1650, 1603, 1203, 1047; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.21 (d, *J* = 3.0, 9.0 Hz, 1H, Ar-H), 7.87 (m, 2H, Ar-H), 7.67–7.57 (m, 1H, Ar-H), 7.47–7.42 (m, 1H, Ar-H), 7.40–7.30 (m, 3H, Ar-H), 7.24–7.14 (m, 5H, Ar-H), 5.07 (s, 2H, CH_2-Ph), 2.85 ($N(CH_3)_2$; ^{13}C NMR (75 MHz, $CDCl_3$): δ 174.8, 156.2, 139.0, 137.2, 136.8, 133.4, 129.6, 128.7, 128.5, 128.6, 128.4, 128.0, 127.7, 125.5, 124.6, 123.6, 122.8, 118.3, 74.0, 40.3, the other carbons are isochronous; accurate mass (ESI, AcCN, +ve) of $[M+H]^+$: Calcd. for $C_{24}H_{22}NO_3$ 372.15996; found 372.15985.

In vitro cholinesterase inhibition activity

Continuing our efforts to study the enzyme inhibition (Mughal, Sadiq, Khan, et al.2017; Mughal, Sadiq, Murtaza, et al., 2017; Mughal et al., 2018; Mughal et al., 2019), all the 3-benzyloxyflavone derivatives (**1–10**) have been examined, *in vitro*, for their inhibitory activity against commercially accessible electric eel AChE and horse serum BChE enzymes.

Donepezil was used as a standard for comparing results, and estimates of the half-maximum inhibitory concentration (IC_{50}) were calculated and are given in Table 1. From the data in Table 1, several conclusions can be drawn concerning the structure-activity relationship and the impact of the substituent in the phenyl ring (Ring B) of flavone moiety.

Structure-activity relationship

The synthesized compounds (**1–10**) were screened to confirm their importance as potent inhibitors of the cholinesterase enzymes. It is interesting to note that all synthesized compounds (**1–10**) showed inhibition against both enzymes (AChE and BChE). However, they are relatively more active against acetylcholinesterase than butyrylcholinesterase. Noteworthy, the compound **2** was recognised as the most active inhibitor of both AChE (IC_{50} = 0.05 ± 0.01 μM) and BChE (IC_{50} = 0.09 ± 0.02 μM), and thus exhibited highest inhibition against both enzymes among the series, even more than the standard Donepezil (IC_{50}= 0.09 ± 0.01 AChE and 0.13 ± 0.04 μM BChE). This lead compound of the series was roughly two times more active than standard regarding *in vitro* AChE-inhibitory activity and one and half times more potent against *in vitro* BChE-inhibitory activity. The presence of diphenylamino group at 4′ position of ring B enables the compound **2** to show the highest inhibition activity. Owing to the bulky size and hydrophobic nature of the diphenylamino substituent, this derivative interacts strongly with active pockets of both enzymes.

Furthermore, the compound **5** (IC_{50} = 0.07 ± 0.02 for AChE and IC_{50} = 2.13 ± 0.08 μM for BChE) was the second potent selective inhibitor of AChE. The presence of only one chloro group at *para*-position (Ring B) in that derivative markedly enhance the inhibitory activity against AChE than BChE, because this nicely fits in the active pocket of former enzyme than latter.

In addition, the next most potent selective inhibitor of AChE (IC_{50} = 0.08 ± 0.02 μM) was found 3-benzyloxyflavone **10**, analogous to the derivative **2,** relative to the standard Donepezil (IC_{50} = 0.09 ± 0.01 μM). The presence of a dimethylamino substitution at 4′ position of the phenyl ring (ring B) and non-polar and electron-rich structural core of this compound are accountable to develop strong intermolecular interactions with the electrophilic active site of the AChE enzyme.

The compounds bearing electron-donating groups (-OCH_3, -CH_3 etc.) such as **3** (IC_{50} = 1.95 ± 0.15 for AChE and IC_{50} = 3.25 ± 0.21 μM for BChE) and **8** also displayed better inhibitory activity against both enzymes. This might be attributed to positions of those groups and effective interactions with the pockets of enzymes. However, introducing strongly electron-withdrawing groups (-NO_2) at the same position of ring B distinctly resulted in decreased inhibitory activity, for example, in case of the flavones **6** and **7**. Interestingly, the meta nitro substituted compound **6** (IC_{50} = 2.85 ± 0.35 for AChE and IC_{50} = 9.25 ± 0.90 μM for BChE) disclosed decreased activity relative to its para analogue **7** (IC_{50} = 1.52 ± 0.15 for AChE and IC_{50} = 14.37 ± 0.65 μM for BChE). Perhaps, the former compound offers steric hindrance to fit in the active site of cholinesterase enzymes. Moreover, replacing aryl ring B with other heterocyclic rings such as thiophene and furan in compound **4** (IC_{50} = 10.02 ± 0.78 for AChE and IC_{50} = 27.11 ± 0.35 μM for BuChE) and **9** (IC_{50} = 11.60 ± 0.03 for AChE and IC_{50} = 12.23 ± 0.74 μM for BChE) respectively resulted in significantly decreased inhibitory activity as compared to the standard.

Overall, it was noted that all the compounds (**1–10**) are potent inhibitors against AChE in comparison to BChE. These findings showed that the nature and substitution pattern at rings B & C improves the inhibitory activity of these compounds against both suggested enzymes relative to unsubstituted 3-benzyloxyflavone **1** (IC_{50} = 0.12 ± 0.02 for AChE and IC_{50} = 3.10 ± 0.30 μM for BChE). Although all the structural

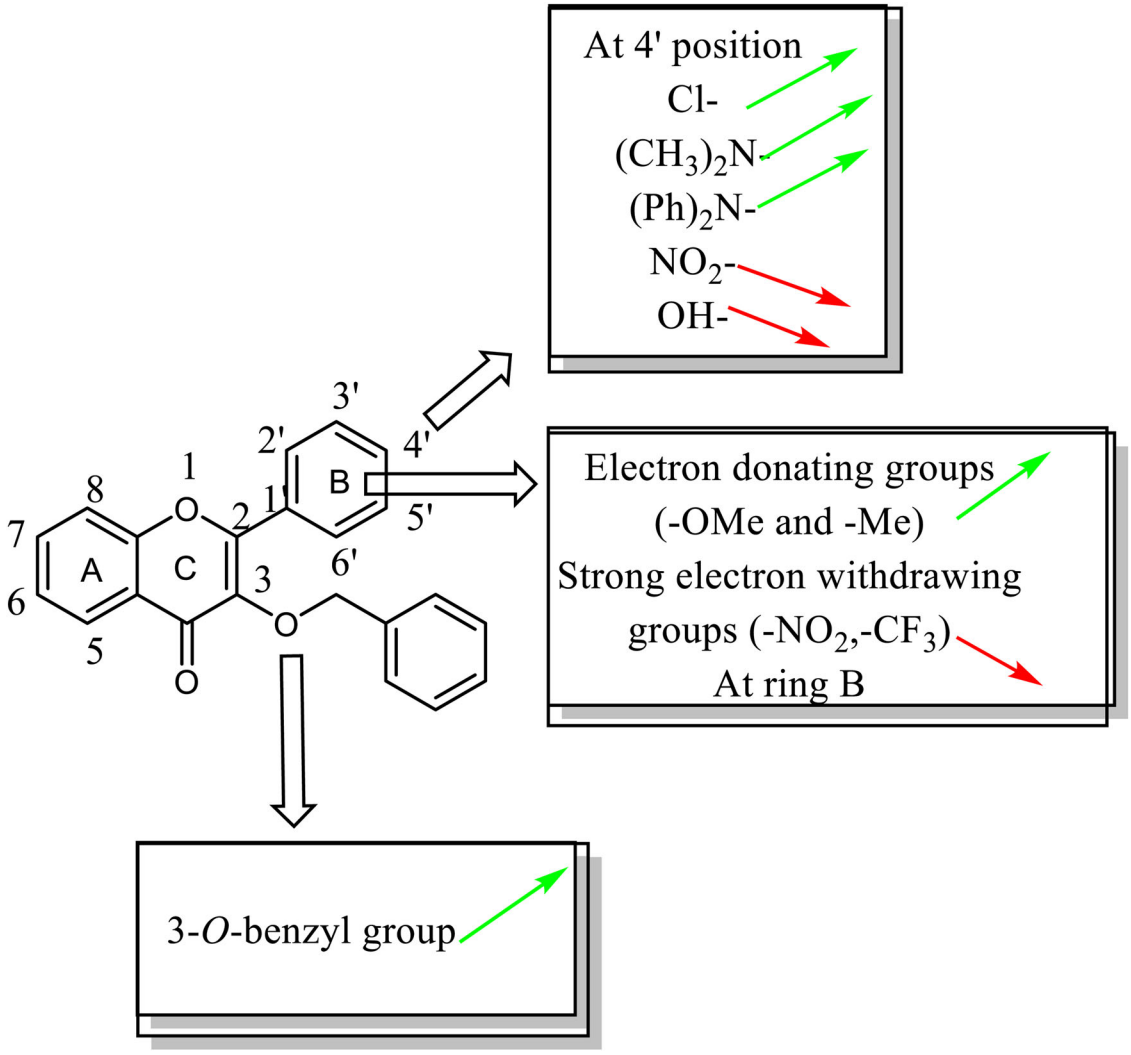


Figure 2. Structure-activityrelationship for cholinesterase enzymes activity of 3-*O*-benzylflavonol derivatives. Downward red arrows indicate a decrease in activity; upward green arrows indicate an increase in activity.

Table 2. Lowest binding energies of the compounds (**1–10**) against various selective modes.

Compound No.	*h* AChE Lowest binding energy Δ*G* (kcal/mol)	*h* BChE Lowest binding energy Δ*G* (kcal/mol)
1	−10.76	−9.32
2	−12.80	−12.37
3	−11.01	−9.60
4	−9.86	−9.42
5	−11.02	−9.85
6	−10.23	−9.65
7	−10.42	−9.41
8	−10.75	−9.35
9	−9.85	−9.01
10	−10.65	−9.45
Standard	−10.00 **(HUW)**	−6.83 **(THA)**

features are actively involved in inhibitory behaviour. However, the differentiation of various groups on the key structural motif was in fact responsible for altering the inhibitory activity. Since, all synthesized compounds have a common skeleton of 3-*O*-benzylflavonol in their structures, the activity was mainly due to different functional groups attached to the main framework of 3-benzyloxyflavone.

Based on above-mentioned findings, these multi-functional 3-benzyloxyflavone derivatives (**1–10**) could use as lead compounds for the designing and development of new inhibitors against cholinesterase enzymes. The conclusions of the SAR studies are summarized in Figure 2.

Molecular docking simulations

In an attempt to understand the enzyme inhibition activity of the synthesized derivatives, molecular docking study was carried out to determine the binding modes of synthesized ligands with cholinesterase enzymes. The human X-ray structures of AChE (PDB ID: 4BDT) and BChE (PDB ID: 4BDS) were chosen as models for this analysis. In due course, for each conformer with best (minimum) docking score was saved in preferences. The binding energies are listed in Table 2. The ligand developing most stable drug-receptor complex is the one which is having minimum docking score. The drug-receptor complexes of some potent ligands were analysed for various types of interactions such as hydrogen bonding, hydrophobic type interactions and van der Waals interactions etc.

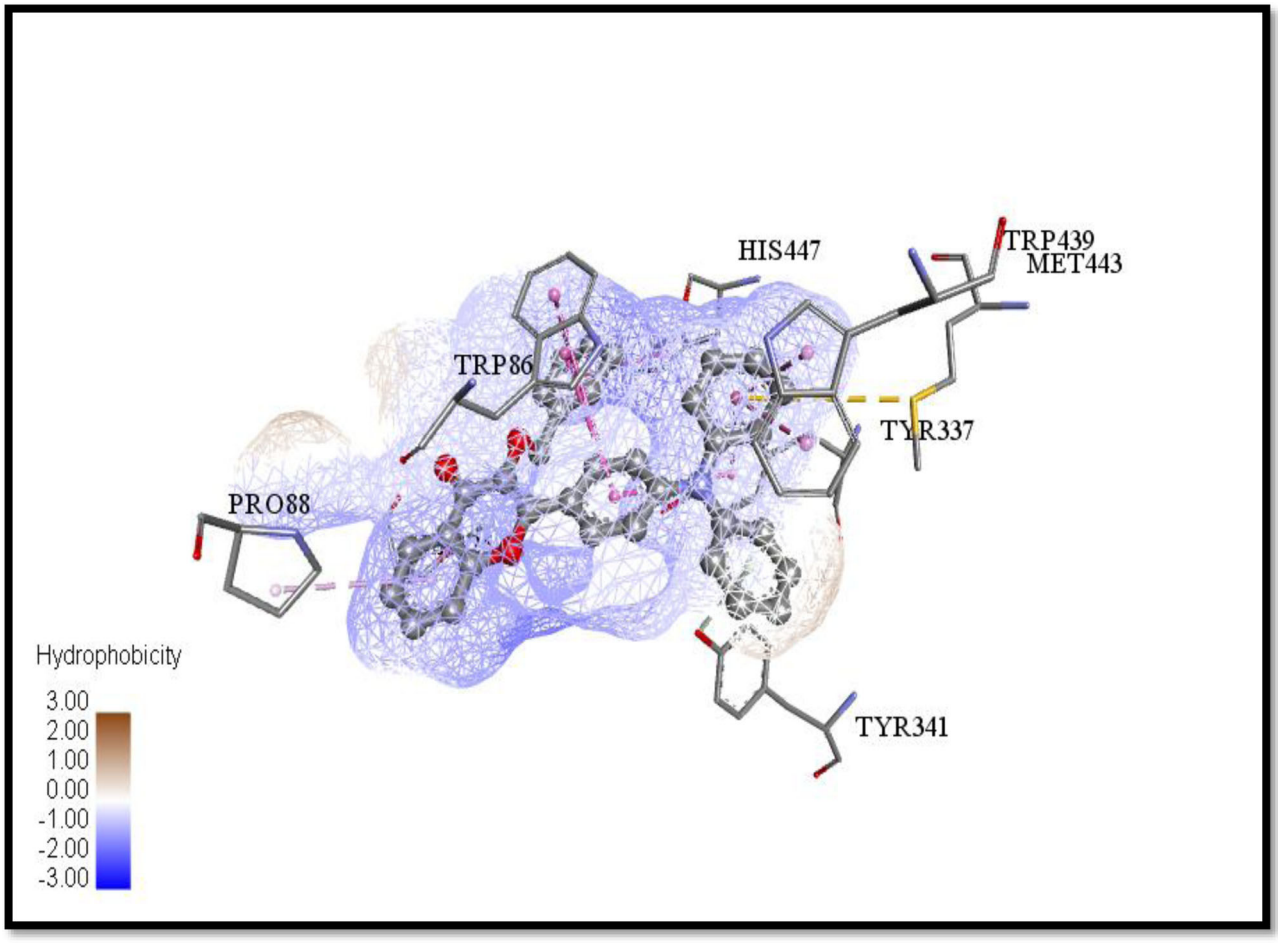


Figure 3. Putative binding interactions between ligand **2** and AChE.

For example, one of the under-study the most potent compound **2** with binding energy (-12.80 kcal mol^{-1}) expresses the ability to block the active sites of AChE. It develops the hydrophobic $\pi-\pi$ stacked type associations with Trp86 and Tyr337 of catalytic triad amino acid residues. Trp439, Met443, Tyr341, Ser125, Pro88 and His447 amino acid residues of active pockets of acetylcholinesterase develop hydrophobic $\pi-\pi$ stacked, π-sulphur, hydrogen bond and hydrophobic π-alkyl type interactions, as shown in Figures 3 and 4.

Additionally, the derivative **2** reveals its inhibitory potential against BChE by forming fruitful types of electrostatic interactions. This compound builds up hydrophobic π-alkyl type interaction Leu286 of acyl binding pocket inside the BChE. Trp82 of peripheral anionic site (PAS) develops the hydrophobic $\pi-\pi$ stacked type associations inside the active pocket of butyrylcholinesterase. This ligand also exhibits π-lone pair, hydrophobic $\pi-\pi$ T-Shaped and hydrophobic π-alkyl type associations with Pro285, Phe329 and Ala328 amino acid residues inside the pockets butyrylcholinesterase as shown in Figures 5 and 6.

Conclusions

In conclusion, we have demonstrated the synthesis, characterization and biological assessment of 3-benzyloxyflavone derivatives. The desired 3-benzyloxyflavones (**1–10**) were synthesized using precedent methodologies starting from 2′-hydroxyacetophenone and different substituted aromatic aldehydes over three steps. All the synthesized 3-benzyloxyflavones (**1–10**) were evaluated against cholinesterase inhibitory potential. All analogues were found the active dual inhibitors of acetylcholinesterase and butyrylcholinesterase. However, the enzyme inhibition study disclosed that most of the compounds are comparatively more active against AChE relative to BChE. Noteworthy, among the series, the compound **2** displayed potent dual inhibitory activity ($IC_{50} = 0.05 \pm 0.01$ for AChE and $IC_{50} = 0.09 \pm 0.02\,\mu M$ for BChE), demonstrating that **2** was about threefold more effective AChE inhibitor and fourfold more effective BChE inhibitor as compared to the reference compound, Donepezil ($IC_{50} = 0.09 \pm 0.01$ for AChE and $IC_{50} = 0.13 \pm 0.04\,\mu M$ for BChE). *In silico* computational studies of all compounds also augmented the *in vitro* analysis, whereby these molecules exhibited strong interactions with the target protein and formed stable complexes with AChE and BChE. The structure-activity relationship analysis discovered that cholinesterase inhibition of these derivatives is strongly depending on the nature and positions of the functional group on the aromatic ring B. In this way, a number of exciting lead compounds have been explored as cholinesterase inhibitors and thus enabling further development of novel drugs for the treatment of Alzheimer's disease.

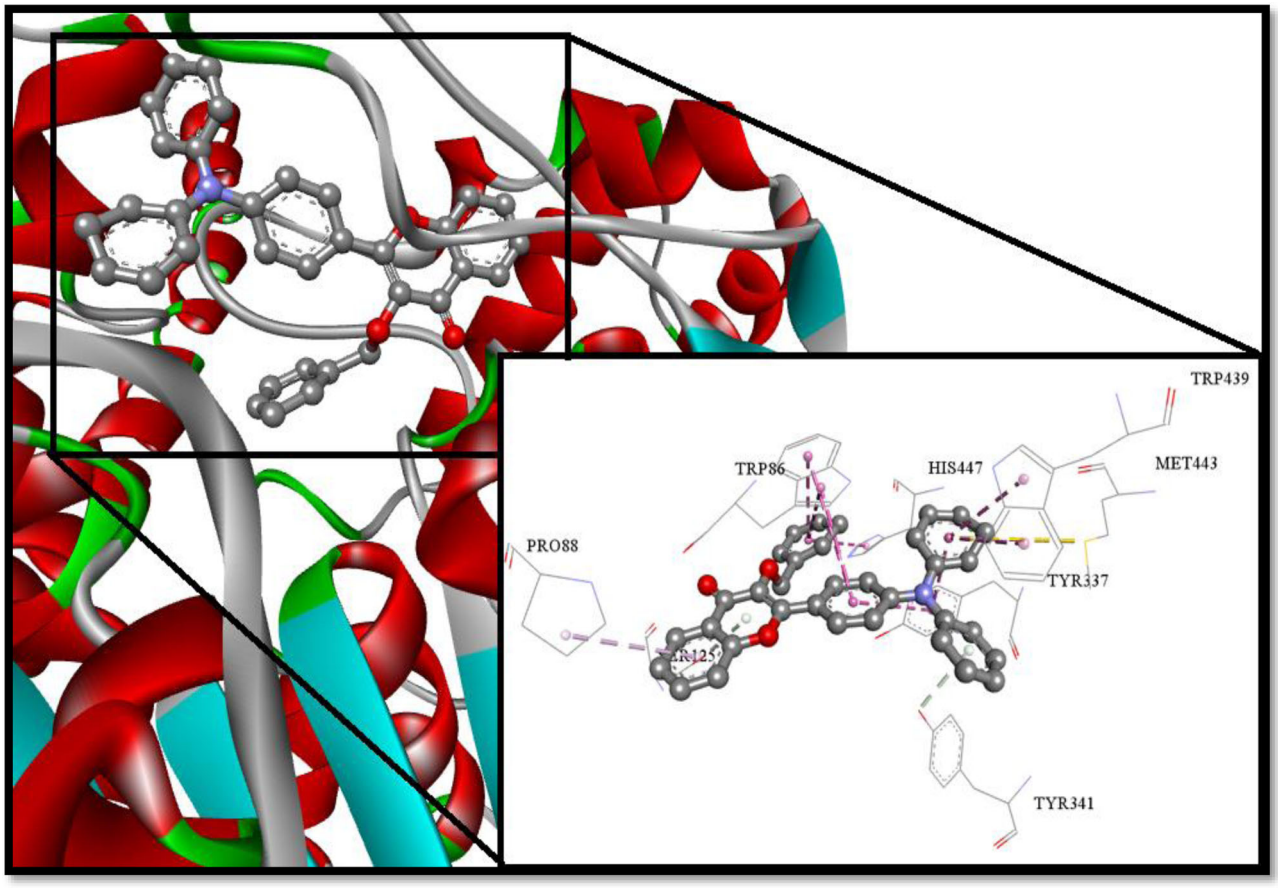


Figure 4. Compound **2** interactions with acetylcholinesterase in 3 D orientation. Interactions with different amino acid residues are depicted in the box. The 3 D ribbon along with the interaction of AChE amino acids represents an enzyme-stick configuration with the lowest energy content of the inhibitor **2**.

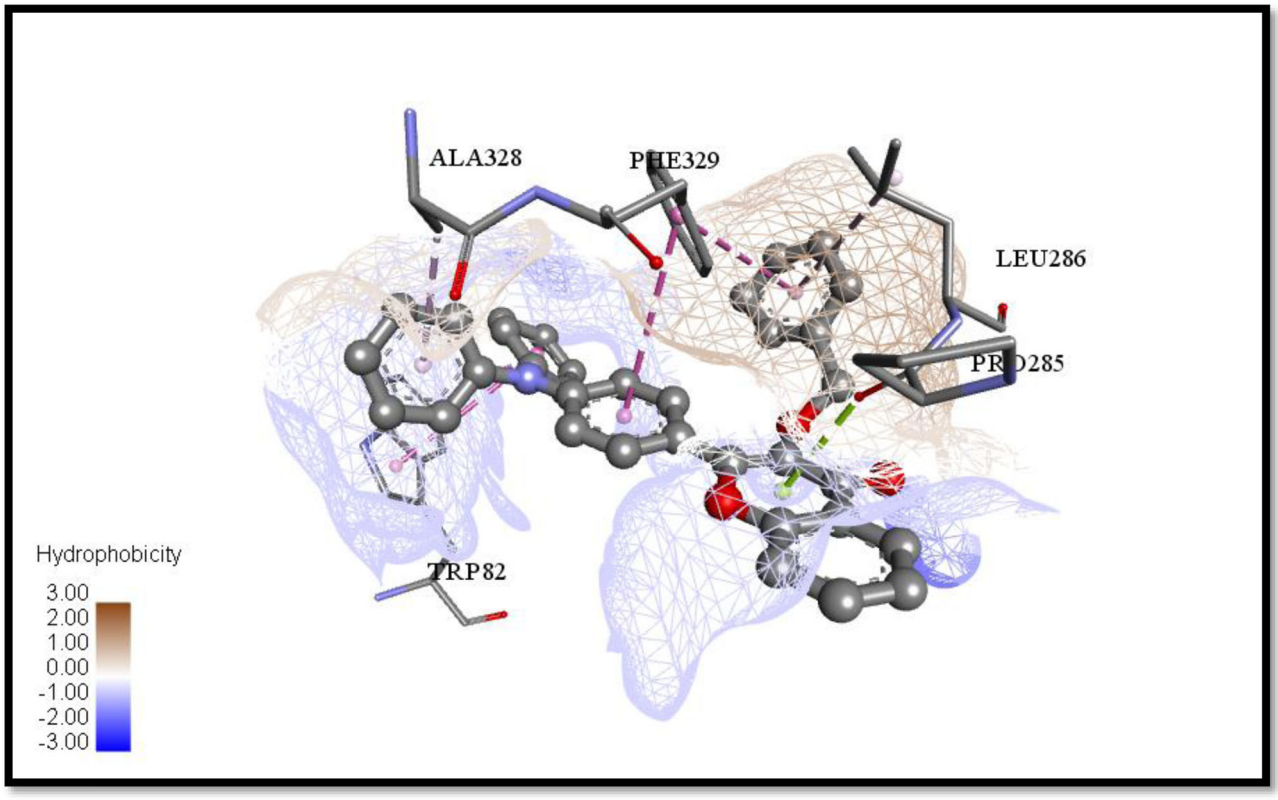


Figure 5. Putative binding interactions between ligand **2** and BchE.

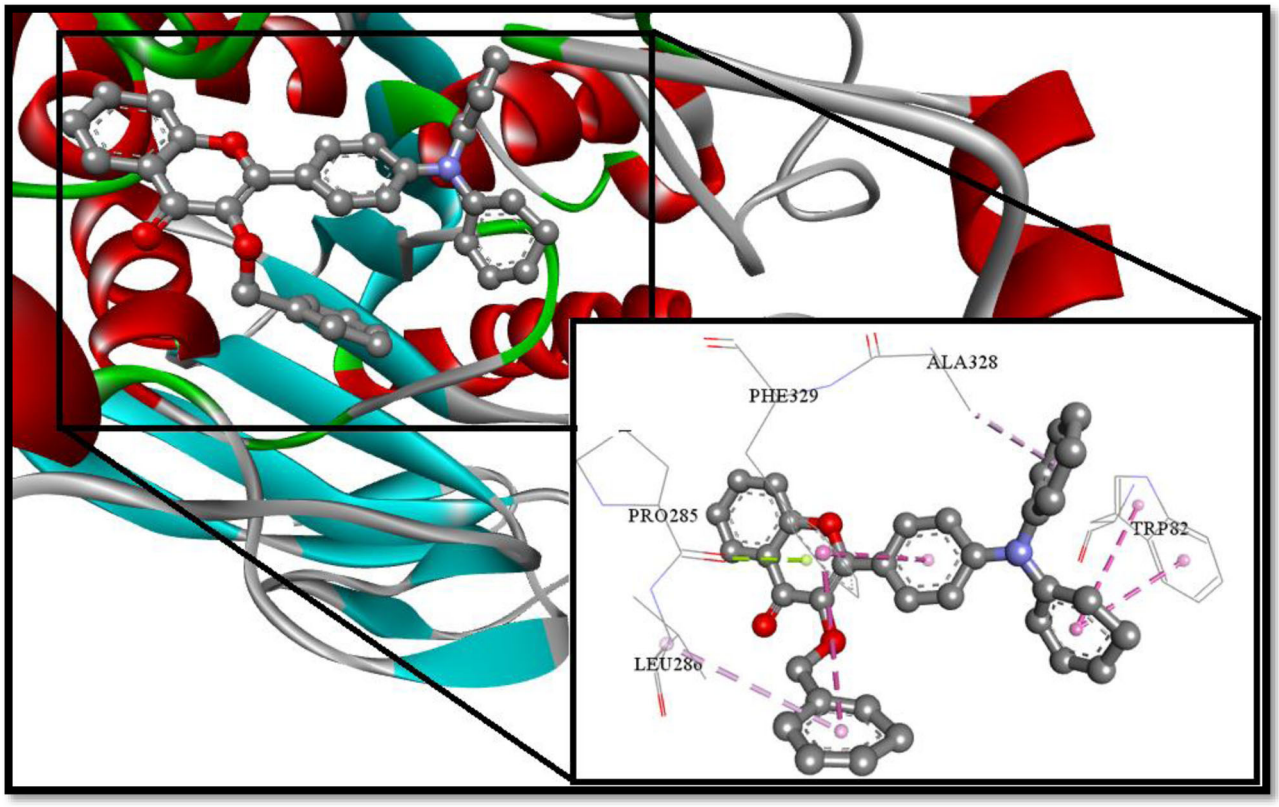


Figure 6. Compound **2** interactions with butyrylcholinesterase in 3 D orientation. Interactions with different amino acid residues are depicted in the box. The 3 D ribbon along with the interaction of BChE amino acids represents an enzyme-stick configuration with the lowest energy content of the inhibitor **2**.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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