Synthesis, characterization and enzyme inhibition study of *O*-substituted derivatives of chlorinated coumarin

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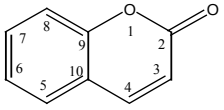
Abstract: Coumarins have much importance in dyes, drugs, perfumes and pesticides. In the demonstrated research work, a benignant series of chlorinated coumarins was synthesized and screened against different enzymes. First, 6-Chloro-7-hydroxy-4-methyl-2*H*-chromen-2-one (**3**) was geared up by the reaction of 4-chlororesorcinol (**1**) and ethyl acetoacetate (**2**) in the presence of concentrated H_2SO_4. Second, various *O*-substituted derivatives of chlorinated coumarins, **5a-j**, were set up by pairing different alkyl/aralkyl halides, **4a-j**, with **3** in the presence of NaH in DMF as solvent. The structures of all the synthesized compounds were clarified through spectral analysis using EI-MS, IR and ^{1}H-NMR. The different enzymes used for the evaluation of bioactivity of all the synthesized compounds were acetyl cholinesterase (AChE), butyryl cholinesterase (BChE) and lipoxygenase (LOX). The most proficient activity was shown against both cholinesterase enzymes.

Keywords: Ethyl acetoactate, 4-chlororesorcinol, chlorinated coumarin, enzyme inhibition.

INTRODUCTION

Coumarin is naturally occurring heterocyclic compound, first isolated from tonka bean in 1820 (Smyth *et al.*, 2009). It is also present in different plants like vanilla grass woodruff, licorice, strawberries, sweet clover etc. (Kinza *et al.*, 2010). General structure for coumarin is



Antibacterial activities of coumarin were first accounted in 1945 (Smyth *et al.*, 2009). By the ancient Egypt literature, it acts as therapeutic agent and widely used as medicine in aboriginal culture (Dekic *et al.*, 2010). Coumarin also acts as anticoagulant agent and its derivatives are reported to be used as anti HIV agents (Dekic *et al.*, 2010 and Sinhamahapatra *et al.*, 2011). Coumarin is ministrant in curing cancer and presents a vital role in anti-inflammatory and antioxidant activities (Sinhamahapatra *et al.*, 2011 and Upadhyay *et al.*, 2008). There are thousands of derivatives of coumarin which have different uses in biological and agrochemical fields (Dekic *et al.*, 2010 and Sinhamahapatra *et al.*, 2011). Cigarettes, alcoholic beverages and laser dyes contain coumarin. These molecules are used as food additive and pesticide (Smyth *et al.*, 2009). Coumarin gives specific features to different perfumes and cosmetics and also used to remove the unpleasant smell of rubber, sprays, paints and objects made up of plastic material (Lake, 1999).

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Acetyl cholinesterase (AChE, EC 3.1.1.7) and Butyryl cholinesterase (BChE, EC 3.1.1.8) constitute a group of enzymes including serine hydrolases. The particularities for substrates and inhibitors regarding these enzymes are because of the divergences in residues of amino acid of active sites possessed by AChE and BChE. Both of the enzymes are creditworthy for the terminus of acetylcholine at cholinergic synapses and are key components of cholinergic brain synapses and neuromuscular junctions (Tougu *et al.*, 2001).

Butyryl cholinesterase inhibition is an efficient one for the ailment of AD and associated dementedness. Much eminent quantities of BChE are detected in Alzheimer's plaques instead of plaques related to normal age non-demented brains. Blood circulation is enriched by BChE and is present in animals and plants (Ahmad *et al.*, 2005; Cyglar *et al.*, 1993; Ellman *et al.*, 1961; Tougu *et al.*, 2001 and Ye *et al.*, 2002).

Lipoxygenase (LOX, EC 1.13.11.12) relates to dioxygenases bearing iron but without haem and possessed by animals and plants. LOX shows a vital role in metabolism of arachidonic acid, in genesis of many biologically active lipids presenting role in inflammation, in thrombosis & tumor angiogenesis, in establishment of fresh vessels relating capillary from already present and in corroboration of many physiologic actions along with the maturation in pathological situations. These are probably targets in drug designing and also of inhibitors regarding the ailment of great disorders (Abbasi *et al.*, 2005; Alitonou *et al.*, 2006; Byrum *et al.*, 1997; Clapp *et al.*,

1985; Jensen *et al.*, 1992; Kemal *et al.*, 1987 and Steinhilber *et al.*, 1999).

This research work is a productive endeavor to inaugurate pharmacologically significant ether derivatives of coumarin having antimicrobial and antioxidant potential (Shi and Zhou, 2011; Chimenti *et al*., 2004 and Zhang *et al*., 2011). In continuation of our last work of *O*-substituted derivatives (Aziz-ur-Rehman *et al.*, 2012), the synthesis of the chlorinated coumarin and its derivatives was performed with an aim to prepare new competitors of drug having prominent activity for the remedy of numerous diseases.

MATERIALS AND METHODS

General

4-Chlororesorcinol, ethyl acetoacetate, alkyl/aralkyl halides (Merck and Alfa Aeser) and other solvents of analytical grade were purchased through local suppliers and applied without further purification. Pureness of the synthesized compounds was evaluated by thin layer chromatography (TLC) using a mixture of EtOAc and *n*-C_6H_{14} as solvent system. TLC plates (purchased from local supplier) were visualized *via* UV at 254 nm and also by spraying ceric sulfate solution. The I.R. spectra were put down in potassium bromide pellet method on a Jasco-320-A spectrophotometer with wave number in cm^{-1}. Melting points of all the synthesized compounds were recorded on a Griffin-George melting point apparatus by open capillary tube. ^{1}H-NMR and ^{13}C-NMR spectra were recorded in $CDCl_3$ on a Bruker spectrometer operating at 300 and 125 MHz respectively. The chemical shift values are reported in ppm (δ) units taking TMS as reference, and the coupling constants (*J*) are in Hz. Mass spectra (EI-MS) were recorded on a JMS-HX-110 spectrometer.

Synthesis of 6-Chloro-7-hydroxy-4-methyl-2H-chromen-2-one (3)

4-Chlororesorcinol (0.020 moles, **1**) was thoroughly dissolved in ethyl acetoacetate (0.020 moles, **2**) in a 500 mL iodine flask. 10 mL of concentrated H_2SO_4 was added drop wise with continuous shaking at 10 ^oC and allowed to stand for 12-14 hours. The precipitates of product were formed after the addition of ice cold water or crushed ice to the reaction mixture. The formed precipitates of the required product **3** were collected by filtration, washing with cold distilled water and drying. Purity was checked by observing single spot on TLC plate. Recrystallization was carried out by using methanol.

General procedure for synthesis of 6-Chloro-7-alkoxy/ aralkoxy-4-methyl-2H-chromen-2-one (5a-j)

The compound **3** (0.5 g, 0.0023 moles) was made soluble in 5.0 mL of DMF in a 50 mL RB flask and 0.05 g NaH (0.0023 moles) was added. The reaction mixture was allowed to stir for 10 minutes. After that the alkyl/aralkyl halides, **4a-j**, were added to reaction mixture and the stirring was continued for 3-4 hours. Completion of reaction was monitored by TLC. After single spot on TLC, the cold distilled water along with 10% aqueous NaOH solution was added. The formed precipitates were collected by filtration, washed with cold distilled water and finally dried to obtain the synthesized products, **5a-j**. Further recrystallization of the synthesized products was not performed.

BIOLOGICAL ACTIVITY

Cholinesterase assays

The AChE and BChE inhibition activity were carried out almost by the reported method (Ellman *et al.*, 1961). Reaction mixture of volume 100×10^{-6} L containing 60×10^{-6} L Na_2HPO_4 buffer (50×10^{-3} M & pH 7.7), 10×10^{-6} L test compound (0.5×10^{-3} M well^{-1}) and 10×10^{-6} L (0.005 unit well^{-1} AChE or 0.5 unit well^{-1} BChE) enzyme was prepared. The pre-incubation of contents was performed for 10 min at 37 ºC after mixing and pre-reading at 405 nm. The reaction was set up by 10×10^{-6} L (0.5×10^{-3} M well^{-1}) substrate (acetylthiocholine iodide for AChE or butyrylthiocholine chloride for BChE) and 10×10^{-6} L DTNB (0.5×10^{-3} M well^{-1}). After incubation for 15 min at 37 ºC, the measurement of absorbance was performed at 405 nm using 96-well plate reader (Synergy HT, Biotek, USA). All the experiments were executed in three-folds. As a positive control and reference standard, eserine (0.005 M well^{-1}) was employed. The %age inhibition was computed through the given equation.

$$\text{Inhibition}(\%) = \frac{\text{Control - Test}}{\text{Control}} \times 100$$

Where Control=Total enzyme activity without inhibitor
Test =Activity in the presence of synthesized compound
IC_{50} values were accounted utilizing EZ-Fit Enzyme Kinetics software (Perrella Scientific Inc. Amherst, USA). IC_{50} values were calculated from the graph by a serial dilution of compounds to different concentrations. The IC_{50} values were expressed as average of three values obtained *via* three different observations.

Lipoxygenase assay

Lipoxygenase (LOX) activity was attempted almost by the mentioned procedure (Baylac *et al.*, 2003; Bertaccini *et al.*, 1982; Clapp *et al.*, 1985 and Kemal *et al.*, 1987). Total 200×10^{-6} L volume containing 150×10^{-6} L Na_3PO_4 buffer (100 mM and pH 8.0), 10×10^{-6} L test compound (0.5 mM well^{-1}) and 15×10^{-6} L (600 units well^{-1}) enzyme was developed. This mixture was passed through pre-reading at 234 nm and then pre-incubation for 0.17 hours at 25°C. 25×10^{-6}L substrate solution was applied as reaction initiator. The absorbance change was detected after 0.1 hours at 234 nm using 96-well plate reader (Synergy HT, Biotek, USA). All the experiments were

executed in three-folds. As a positive control and reference standard, baicalein (0.005 M well^{-1}) was applied. The %age inhibition was computed by the same method as described above for cholinesterase assays. IC_{50} values were calculated employing the same procedure as mentioned for acetyl cholinesterase and butyryl cholinesterase enzyme.

STATISTICAL ANALYSIS

All the measurements were accomplished in three-folds and the analysis was executed by Microsoft Excel 2010. The outcomes are offered as mean ± sem.

SPECTRAL CHARACTERIZATION OF THE SYNTHESIZED COMPOUNDS

6-Chloro-7-hydroxy-4-methyl-2H-chromen-2-one (3)

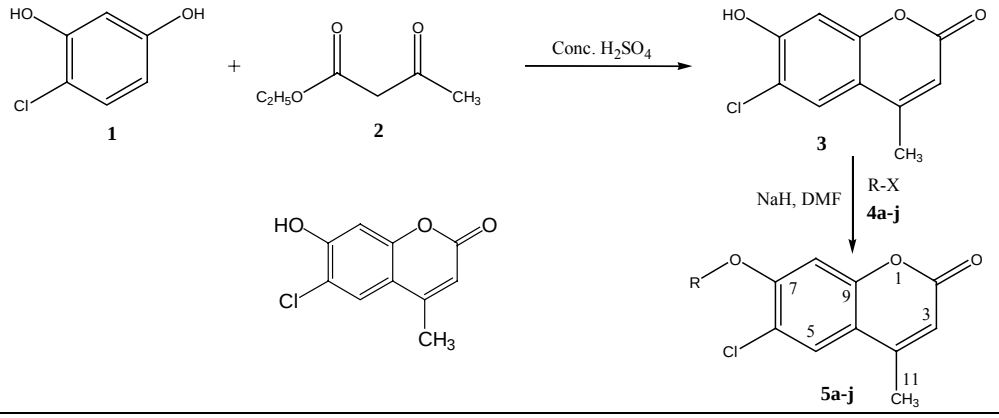
Light brown amorphous solid; Yield: 78%; M. P. 262-264 ^{o}C; Mol. formula: $C_{10}H_7ClO_3$; Mol. weight: 210 gmol^{-1}; IR (KBr): ν_{max} (cm^{-1}): 3410 (stretching of O-H), 3056 (C-H aromatic stretching), 1720 (stretching of α,β-unsaturated C=O), 1625 (C=C stretching of aromatic ring); ^{1}H-NMR (300 MHz, $CDCl_3$, ppm): δ 7.54 (s, 1H, H-5), 6.98 (s, 1H, H-8), 6.17 (s, 1H, H-3), 2.37 (s, 3H, CH_3-11); ^{13}C-NMR (125 MHz, $CDCl_3$, ppm): δ 160.5 (C-2), 157.7 (C-7), 153.4 (C-4), 151.5 (C-9), 125.4 (C-5), 118.7 (C-6), 113.6 (C-10), 112.7 (C-3), 100.3 (C-8), 18.5 (C-11); EIMS (*m/z*): 212 [M+2]$^+$, 210 [M]$^+$, 193 [M-OH]$^+$, 182 [M-CO]$^+$, 175 [M-Cl]$^+$, 166 [M-CO_2]$^+$, 143 [M-CH_4ClO]$^+$, 134 [M-C_2HClO]$^+$.

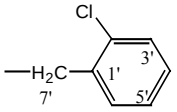
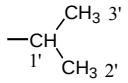
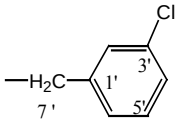
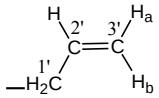
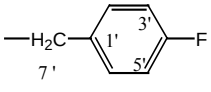
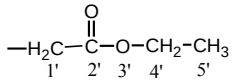
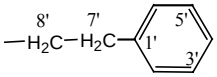
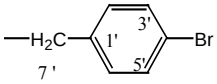
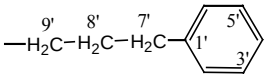
6-Chloro-7-ethoxy-4-methyl-2H-chromen-2-one (5a)

Buff amorphous solid; Yield: 75%; M. P. 150-152 ^{o}C; Mol. formula: $C_{12}H_{11}ClO_3$; Mol. weight: 238 gmol^{-1}; IR (KBr): ν_{max} (cm^{-1}): 3054 (C-H aromatic stretching), 1726 (stretching of α,β-unsaturated C=O), 1628 (C=C stretching of aromatic ring); ^{1}H-NMR (300 MHz, $CDCl_3$, ppm): δ 7.55 (s, 1H, H-5), 6.82 (s, 1H, H-8), 6.15 (s, 1H, H-3), 4.14 (q, J = 6.9 Hz, 2H, H-1'), 2.37 (s, 3H, CH_3-11), 1.25 (t, J = 7.2 Hz, 3H, CH_3-2'); EIMS (*m/z*): 240 [M+2]$^+$, 238 [M]$^+$, 210 [M-C_2H_4]$^+$, 203 [M-Cl]$^+$, 194 [M-CO_2]$^+$, 193 [M-OC_2H_5]$^+$, 143 [M-C_3H_8ClO]$^+$, 134 [M-C_4H_5ClO]$^+$.

6-Chloro-7-(propan-2-yloxy)-4-methyl-2H-chromen-2-one (5b)

Peach amorphous solid; Yield: 58%; M. P. 118-120 ^{o}C; Mol. formula: $C_{13}H_{13}ClO_3$; Mol. weight: 252 gmol^{-1}; IR



Compd. No.	-R	Compd. No.	-R
5a	—CH_2—CH_3 (1' 2')	5f	
5b		5g	
5c		5h	
5d		5i	
5e		5j	

Scheme 1: Outline for the synthesis of various derivatives of chlorinated coumarin (**3**)

(KBr): $\square_{max}$ (cm^{-1}): 3059 (C-H aromatic stretching), 1724 (stretching of α,β-unsaturated C=O), 1621 (C=C stretching of aromatic ring); ^{1}H-NMR (300 MHz, $CDCl_3$, ppm): δ 7.55 (s, 1H, H-5), 6.83 (s, 1H, H-8), 6.14 (s, 1H, H-3), 4.59-4.63 (m, 1H, H-1'), 2.36 (s, 3H, CH_3-11), 1.41 (d, J=6.9 Hz, 6H, CH_3-2', CH_3-3'); EIMS (*m/z*): 254 $[M+2]^+$, 252 $[M]^+$, 224 $[M-CO]^+$, 217 $[M-Cl]^+$, 210 $[M-C_3H_6]^+$, 193 $[M-OC_3H_7]^+$, 188 $[M-CO_2]^+$, 143 $[M-C_4H_{10}ClO]^+$, 134 $[M-C_5H_7ClO]^+$.

6-Chloro-7-(2-propen-1-yloxy)-4-methyl-2H-chromen-2-one (5c)

Milky white amorphous solid; Yield: 67%; M. P. 116-118 ^oC; Mol. formula: $C_{13}H_{11}ClO_3$; Mol. weight: 250 $gmol^{-1}$; IR (KBr): $\square_{max}$ (cm^{-1}): 3054 (C-H aromatic stretching), 1723 (stretching of α,β-unsaturated C=O), 1626 (C=C stretching of aromatic ring); ^{1}H-NMR (300 MHz, $CDCl_3$, ppm): δ 7.56 (s, 1H, H-5), 6.84 (s, 1H, H-8), 6.16 (s, 1H, H-3), 5.98-6.09 (m, 1H, H-2'), 5.48 (dd, J = 15.9, 1.2 Hz, 1H, H_b-3'), 5.36 (dd, J = 9.3, 1.2 Hz, 1H, H_a-3'), 4.66 (d, J = 6.9 Hz, 2H, H-1'), 2.37 (s, 3H, CH_3-11); EIMS (*m/z*): 252 $[M+2]^+$, 250 $[M]^+$, 222 $[M-CO]^+$, 215 $[M-Cl]^+$, 209 $[M-C_3H_5]^+$, 206 $[M-CO_2]^+$, 193 $[M-OC_3H_5]^+$, 143 $[M-C_4H_8ClO]^+$, 134 $[M-C_5H_5ClO]^+$.

6-Chloro-7-((ethoxycabonyl)methoxy)-4-methyl-2H-chromen-2-one (5d)

White amorphous solid; Yield: 54%; M. P. 184-186 ^oC; Mol. formula: $C_{14}H_{13}ClO_5$; Mol. weight: 296 $gmol^{-1}$; IR (KBr): $\square_{max}$ (cm^{-1}): 3054 (C-H aromatic stretching), 1727 (stretching of α,β-unsaturated C=O), 1627 (C=C stretching of aromatic ring); ^{1}H-NMR (300 MHz, $CDCl_3$, ppm): δ 7.59 (s, 1H, H-5), 6.71 (s, 1H, H-8), 6.18 (s, 1H, H-3), 4.74 (s, 2H, H-1'), 4.27 (q, J = 7.2 Hz, 2H, H-4'), 2.37 (s, 3H, CH_3-11), 1.30 (t, J = 6.9 Hz, 3H, CH_3-5'); EIMS (*m/z*): 298 $[M+2]^+$, 296 $[M]^+$, 268 $[M-CO]^+$, 267 $[M-C_2H_5]^+$, 261 $[M-Cl]^+$, 252 $[M-CO_2]^+$, 209 $[M-C_4H_7O_2]^+$, 193 $[M-OC_4H_7O_2]^+$, 143 $[M-C_5H_{10}ClO_3]^+$, 134 $[M-C_6H_7ClO_3]^+$.

6-Chloro-7-((4-bromophenyl)methoxy)-4-methyl-2H-chromen-2-one (5e)

Dark tea pink amorphous solid; Yield: 58%; M. P. 134-136 ^oC; Mol. formula: $C_{17}H_{12}BrClO_3$; Mol. weight: 379 $gmol^{-1}$; IR (KBr): $\square_{max}$ (cm^{-1}): 3057 (C-H aromatic stretching), 1725 (stretching of α,β-unsaturated C=O), 1630 (C=C stretching of aromatic ring), 667 (stretching of C-Br); ^{1}H-NMR (300 MHz, $CDCl_3$, ppm): δ 7.58 (s, 1H, H-5), 7.52 (d, J = 9.6 Hz, 2H, H-3', H-5'), 7.32 (d, J = 8.1 Hz, 2H, H-2', H-6'), 6.85 (s, 1H, H-8), 6.16 (s, 1H, H-3), 5.14 (s, 2H, H-7'), 2.37 (s, 3H, CH_3-11); EIMS (*m/z*): 383 $[M+4]^+$, 381 $[M+2]^+$, 379 $[M]^+$, 351 $[M-CO]^+$, 344 $[M-Cl]^+$, 335 $[M-CO_2]^+$, 210 $[M-C_7H_5Br]^+$, 193 $[M-OC_7H_6Br]^+$, 155 $[C_6H_4Br]^+$, 143 $[C_5H_4Br]^+$, 134 $[M-C_9H_6BrClO]^+$, 65 $[C_5H_5]^+$, 51 $[C_4H_3]^+$.

6-Chloro-7-((2-chlorophenyl)methoxy)-4-methyl-2H-chromen-2-one (5f)

Tea pink amorphous solid; Yield: 57%; M. P. 204-206 ^oC; Mol. formula: $C_{17}H_{12}Cl_2O_3$; Mol. weight: 335 $gmol^{-1}$; IR (KBr): $\square_{max}$ (cm^{-1}): 3052 (C-H aromatic stretching), 1722 (stretching of α,β-unsaturated C=O), 1624 (C=C stretching of aromatic ring), 703 (stretching of C-Cl); ^{1}H-NMR (300 MHz, $CDCl_3$, ppm): δ 7.59 (s, 1H, H-5), 7.57 (br.s, 1H, H-3'), 7.40 (t, J = 6.9 Hz, 1H, H-5'), 7.33 (br.s, 1H, H-6'), 7.29 (ddd, J = 6.9, 1.5 Hz, 1H, H-4'), 6.90 (s, 1H, H-8), 6.17 (s, 1H, H-3), 5.28 (s, 2H, H-7'), 2.38 (s, 3H, CH_3-11); EIMS (*m/z*): 339 $[M+4]^+$, 337 $[M+2]^+$, 335 $[M]^+$, 307 $[M-CO]^+$, 300 $[M-Cl]^+$, 291 $[M-CO_2]^+$, 210 $[M-C_7H_5Cl]^+$, 193 $[M-OC_7H_6Cl]^+$, 143 $[M-C_8H_9Cl_2O]^+$, 134 $[M-C_9H_6Cl_2O]^+$, 111 $[C_6H_4Cl]^+$, 99 $[C_5H_4Cl]^+$, 65 $[C_5H_5]^+$, 51 $[C_4H_3]^+$.

6-Chloro-7-((3-chlorophenyl)methoxy)-4-methyl-2H-chromen-2-one (5g)

Brown sticky solid; Yield: 58%; M. P. 224-226 ^oC; Mol. formula: $C_{17}H_{12}Cl_2O_3$; Mol. weight: 335 $gmol^{-1}$; IR (KBr): $\square_{max}$ (cm^{-1}): 3060 (C-H aromatic stretching), 1730 (stretching of α,β-unsaturated C=O), 1631 (C=C stretching of aromatic ring); ^{1}H-NMR (300 MHz, $CDCl_3$, ppm): δ 7.59 (s, 1H, H-5), 7.35-7.45 (m, 2H, H-4', H-5'), 7.33 (br.s, 1H, H-6'), 7.31 (br.s, 1H, H-2'), 6.85 (s, 1H, H-8), 6.16 (s, 1H, H-3), 5.17 (s, 2H, H-7'), 2.37 (s, 3H, CH_3-11); EIMS (*m/z*): 339 $[M+4]^+$, 337 $[M+2]^+$, 335 $[M]^+$, 307 $[M-CO]^+$, 300 $[M-Cl]^+$, 291 $[M-CO_2]^+$, 210 $[M-C_7H_5Cl]^+$, 193 $[M-OC_7H_6Cl]^+$, 143 $[M-C_8H_9Cl_2O]^+$, 134 $[M-C_9H_6Cl_2O]^+$, 111 $[C_6H_4Cl]^+$, 99 $[C_5H_4Cl]^+$, 65 $[C_5H_5]^+$, 51 $[C_4H_3]^+$.

6-Chloro-7-((4-flourophenyl)methoxy)-4-methyl-2H-chromen-2-one (5h)

Light tea pink amorphous solid; Yield: 53%; M. P. 174-176 ^oC; Mol. formula: $C_{17}H_{12}ClFO_3$; Mol. weight: 318 $gmol^{-1}$; IR (KBr): $\square_{max}$ (cm^{-1}): 3062 (C-H aromatic stretching), 1731 (stretching of α,β-unsaturated C=O), 1621 (C=C stretching of aromatic ring), 1035 (stretching of C-F); ^{1}H-NMR (300 MHz, $CDCl_3$, ppm): δ 7.58 (s, 1H, H-5), 7.43 (d, J = 8.7 Hz, 2H, H-3', H-5'), 7.08 (d, J = 8.7 Hz, 2H, H-2', H-6'), 6.87 (s, 1H, H-8), 6.16 (s, 1H, H-3), 5.15 (s, 2H, H-7'), 2.37 (s, 3H, CH_3-11); EIMS (*m/z*): 320 $[M+2]^+$, 318 $[M]^+$, 290 $[M-CO]^+$, 283 $[M-Cl]^+$, 274 $[M-CO_2]^+$, 210 $[M-C_7H_5F]^+$, 193 $[M-OC_7H_6F]^+$, 143 $[M-C_8H_9ClFO]^+$, 134 $[M-C_9H_6ClFO]^+$, 95 $[C_6H_4F]^+$, 83 $[C_5H_4F]^+$, 65 $[C_5H_5]^+$, 51 $[C_4H_3]^+$.

6-Chloro-7-(2-phenylethan-1-yloxy)-4-methyl-2H-chromen-2-one (5i)

Light brown amorphous solid; Yield: 55%; M. P. 112-114 ^oC; Mol. formula: $C_{18}H_{15}ClO_3$; Mol. weight: 314 $gmol^{-1}$; IR (KBr): $\square_{max}$ (cm^{-1}): 3064 (C-H aromatic stretching), 1717 (stretching of α,β-unsaturated C=O), 1617 (C=C stretching of aromatic ring); ^{1}H-NMR (300 MHz, $CDCl_3$, ppm): δ 7.54 (s, 1H, H-5), 7.25-7.33 (m, 5H, H-2' to H-6'),

Table 1: Enzyme inhibition studies of various derivatives of chlorinated coumarin

Compound	AChE		BChE		LOX	
	Inhibition (%) Conc./well (0.5 mM)	IC_{50} µM	Inhibition (%) Conc./well (0.5 mM)	IC_{50} µM	Inhibition (%) Conc./well (0.5 mM)	IC_{50} µM
3	76.98±0.78	169.41±0.09	71.28±0.31	125.61±0.15	93.22±0.21	57.31±0.19
5a	64.41±0.11	211.52±0.06	59.28±0.13	231.52±0.06	53.39±0.33	>400
5b	61.91±0.69	245.36±0.14	78.72±0.27	121.21±0.15	60.47±0.24	289.11±0.63
5c	70.99±0.35	152.91±0.35	64.74±0.11	201.31±0.14	44.67±0.15	127.31±0.14
5d	67.71±0.15	212.71±0.12	67.15±0.13	217.51±0.12	41.38±0.81	-
5e	91.88±0.62	59.11±0.15	87.36±0.33	63.91±0.08	52.03±0.31	>400
5f	66.56±0.18	226.36±0.11	69.38±0.15	206.11±0.18	53.68±0.81	>400
5g	65.57±0.33	71.25±0.04	90.53±0.87	24.51±0.04	41.86±0.81	>400
5h	74.27±0.31	152.31±0.22	86.28±0.22	68.11±0.21	39.73±0.27	-
5i	53.38±0.11	>400	68.87±0.18	63.91±0.17	44.19±0.15	-
5j	77.56±0.55	144.91±0.14	97.01±0.21	34.71±0.14	65.89±0.21	199.51±0.14
Control	Eserine 91.29±1.17	0.04±0.001	Eserine 82.82±1.09	0.85±0.0001	Baicalein 93.79±1.27	22.4±1.3

Note: IC_{50} values (concentration at which there is 50% enzyme inhibition) of compounds were calculated using EZ–Fit Enzyme kinetics software (Perella Scientific Inc. Amherst, USA).
AChE = Acetyl cholinesterase. BChE = Butyryl cholinesterase. LOX = Lipoxygenase.

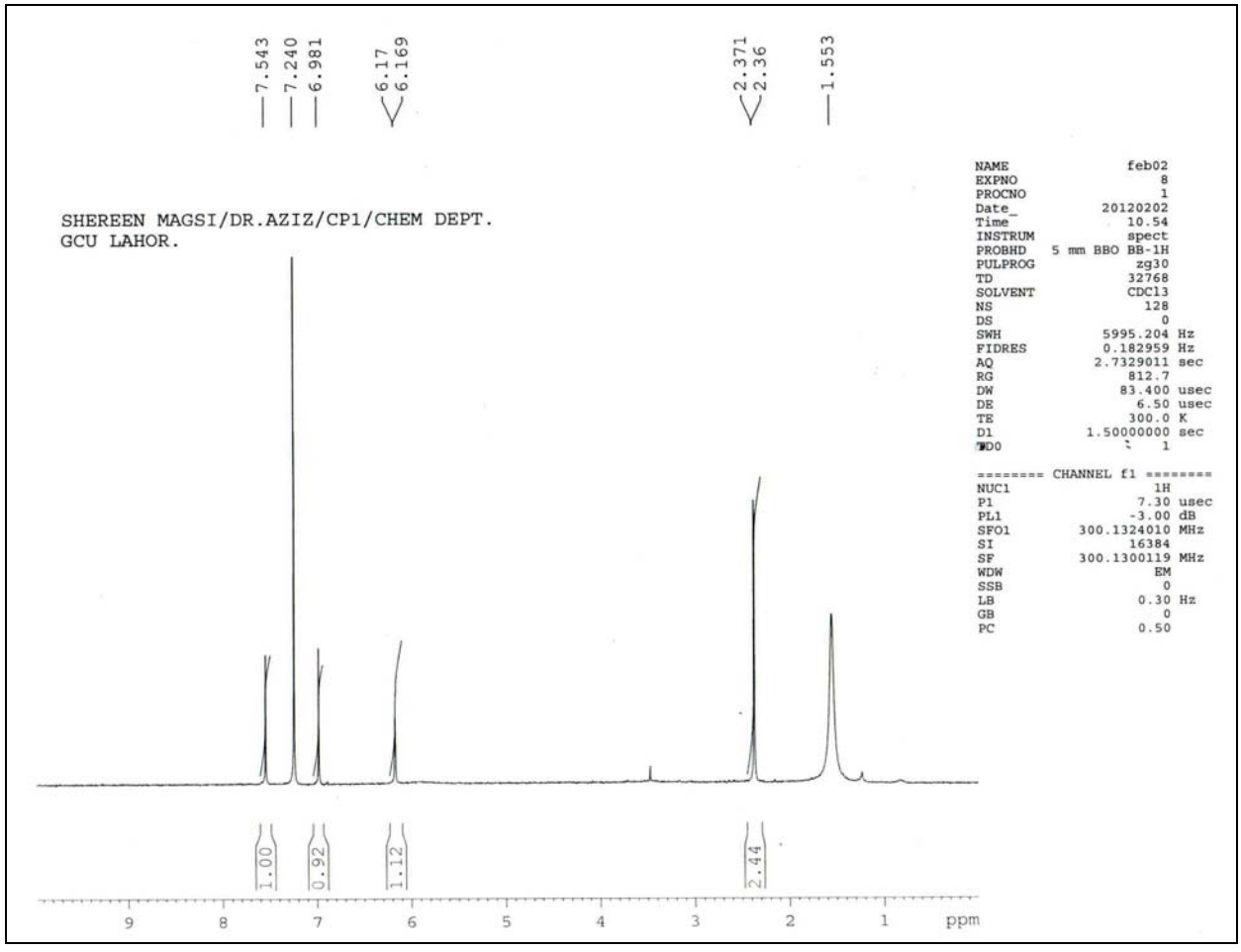


Fig. 1: ^{1}H-NMR spectrum of 6-Chloro-7-hydroxy-4-methyl-2*H*-chromen-2-one (**3**)

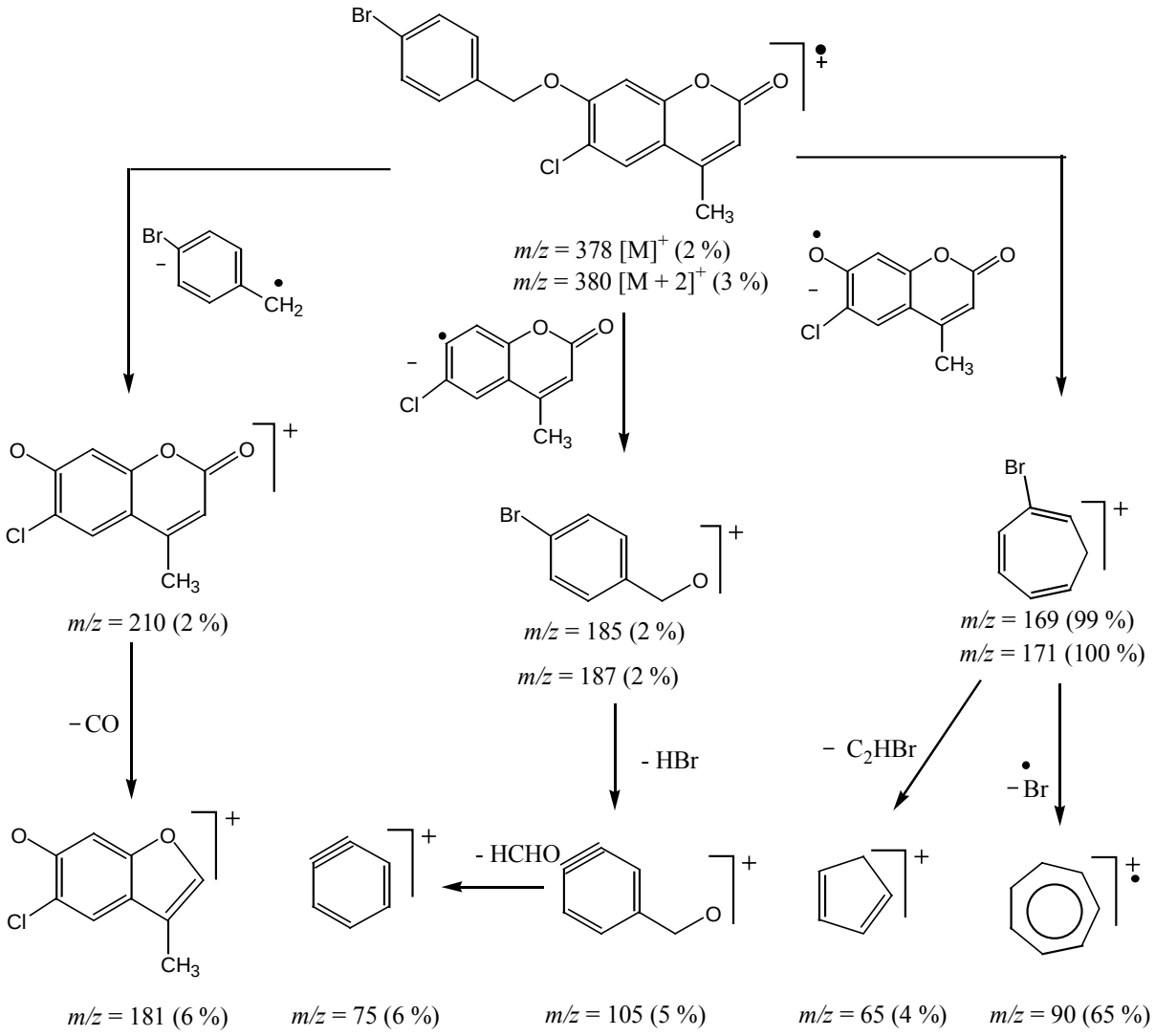


Fig. 2: Mass Fragmentation pattern of 6-Chloro-7-((4-bromophenyl)methoxy)-4-methyl-2*H*-chromen-2-one (**5e**)

6.80 (s, 1H, H-8), 6.14 (s, 1H, H-3), 4.25 (t, *J* = 6.6 Hz, 2H, H-8'), 3.17 (t, *J* = 6.6 Hz, 2H, H-7'), 2.36 (s, 3H, CH_3-11); EIMS (*m/z*): 316 $[M+2]^+$, 314 $[M]^+$, 286 $[M-CO]^+$, 279 $[M-Cl]^+$, 270 $[M-CO_2]^+$, 209 $[M-C_8H_9]^+$, 193 $[M-OC_8H_9]^+$, 143 $[M-C_9H_{12}ClO]^+$, 134 $[M-C_{10}H_9ClO]^+$, 91 $[C_7H_7]^+$, 77 $[C_6H_5]^+$, 65 $[C_5H_5]^+$, 51 $[C_4H_3]^+$.

6-Chloro-7-(3-phenylpropan-1-yloxy)-4-methyl-2H-chromen-2-one (5j)

Brown crystalline solid; Yield: 83%; M. P. 126-128 °C; Mol. formula: $C_{19}H_{17}ClO_3$; Mol. weight: 328 $gmol^{-1}$; IR (KBr): $\square_{max}$ (cm^{-1}): 3057 (C-H aromatic stretching), 1728 (stretching of *α, β*-unsaturated C=O), 1629 (C=C stretching of aromatic ring); ^{1}H-NMR (300 MHz, $CDCl_3$, ppm): *δ* 7.56 (s, 1H, H-5), 7.18-7.30 (m, 5H, H-2' to H-6'), 6.78 (s, 1H, H-8), 6.15 (s, 1H, H-3), 4.04 (t, *J* = 6.3 Hz, 2H, H-9'), 2.85 (t, *J* = 7.2 Hz, 2H, H-7'), 2.37 (s, 3H, CH_3-11), 2.18 (qui, *J* = 6.9 Hz, 2H, H-8'); EIMS (*m/z*): 330 $[M+2]^+$, 328 $[M]^+$, 300 $[M-CO]^+$, 293 $[M-Cl]^+$, 284 $[M-CO_2]^+$, 209 $[M-C_9H_{11}]^+$, 193 $[M-OC_9H_{11}]^+$, 143 $[M-C_{10}H_{14}ClO]^+$, 134 $[M-C_{11}H_{11}ClO]^+$, 105 $[C_8H_9]^+$, 91 $[C_7H_7]^+$, 77 $[C_6H_5]^+$, 65 $[C_5H_5]^+$, 51 $[C_4H_3]^+$.

RESULTS

In the presented research work, a series of compounds was derived from chloro substituted coumarin. The research work comprised a two step synthesis. First, the chlorinated coumarin (**3**) was synthesized in a highly acidic medium by the intermixing of 4-chlororesorcinol (**1**) with ethyl acetoacetate (**2**) in the presence of concentrated sulphuric acid as catalyst in 12-14 hours. The high %age yield of product, **3**, was collected by filtration after adding ice cold water and washing was processed with cold distilled water. **3** was further treated with different alkyl/aralkyl halides as electrophiles, **4a-j**, to yield different substituted products, **5a-j**, in the presence of NaH acting as base and aprotic polar solvent, DMF. The

products were collected as precipitates by filtration after the addition of ice cold distilled water and 10% aqueous NaOH. The sodium hydroxide was utilized to get rid of non-reacted chlorinated coumarin by getting it soluble in the form of salt. All the synthesized compounds were screened against acetyl cholinesterase (AChE), butyryl cholinesterase (BChE) and lipoxygenase (LOX) enzymes and their structures were clarified through spectral data.

DISCUSSION

Chemistry

The compound **3** was synthesized as light brown amorphous solid having yield of 78% and melting point of 262-264 ^{o}C. The mol. formula $C_{10}H_7ClO_3$ was launched by EI-MS pointing molecular ion peak at *m/z* 210 and also by counting the number of protons *via* ^{1}H-NMR spectral data. The IR spectrum depicted absorption bands at 3410 cm^{-1}, 3056 cm^{-1}, 1720 cm^{-1} and 1625 cm^{-1} because of O-H stretching of hydroxyl group, C-H aromatic stretching, stretching of α, β-unsaturated C=O and C=C stretching of aromatic ring in the molecule. The EI-MS presented two distinct peaks at *m/z* 182 and *m/z* 166 after the removal of CO and CO_2 molecules respectively. The other eminent peaks were mentioned in the spectral data. In ^{1}H-NMR spectrum, two signals appeared at δ 7.54 (s, 1H, H-5) and 6.98 (s, 1H, H-8) in aromatic section owing to two protons of aromatic ring at fifth & eighth position and one signal at δ 6.17 (s, 1H, H-3) because of one proton of six membered ring at third position. The signal appearing at δ 2.37 (s, 3H, CH_3-11) was allotted to three protons of methyl group attached to fourth position of six membered ring. The ^{1}H-NMR spectrum is given in fig. 1 for confirmation of coumarin synthesis. In the ^{13}C-NMR (BB and DEPT) spectrum, the ten signals resonated for six quaternary carbons at δ 160.5 (C-2), 157.7 (C-7), 153.4 (C-4), 151.5 (C-9), 118.7 (C-6) & 113.6 (C-10), three methine carbons at δ 125.4 (C-5), 112.7 (C-3) & 100.3 (C-8) and one methyl carbon at δ 18.5 (C-11). On the basis of all these manifests, the structure of compound **3** was identified as 6-Chloro-7-hydroxy-4-methyl-2*H*-chromen-2-one. The mass fragmentation pattern of 6-Chloro-7-(4-bromophenyl) methoxy)-4-methyl-2*H*-chromen-2-one (**5e**) was sketched in fig. 2. Similarly, the structures of other synthesized compounds, **5a-j**, were characterized by ^{1}H-NMR, IR and EI-MS as described in spectral data section.

Enzyme inhibition activity

The screening of these synthesized compounds against AChE, BChE and LOX enzymes displayed good inhibitory potential against cholinesterase enzymes as shown by their IC_{50} values (table 1). Inhibition study showed that all the compounds possessed prominent activity against AChE except 6-chloro-7-(2-phenylethan-1-yloxy)-4-methyl-2*H*-chromen-2-one (**5i**). 6-Chloro-7-((4-bromophenyl)methoxy)-4-methyl-2*H*-chromen-2-one (**5e**) and 6-chloro-7-((3-chlorophenyl)methoxy)-4-methyl-2*H*-chromen-2-one (**5g**) were the most efficient AChE inhibitors having IC_{50} values of 59.11±0.15 and 71.25±0.04 µmoles/L, respectively, with respect to eserine, a reference standard with IC_{50} value of 0.04±0.001 µmoles/L. The efficient inhibitory results of these compounds were likely due to the presence of bromine at *para* position and chlorine at *meta* position of aralkyl groups, respectively, attached to coumarin nucleus *via* oxygen.

The screening against BChE revealed that all the synthesized compounds were active but 6-chloro-7-((3-chlorophenyl)methoxy)-4-methyl-2*H*-chromen-2-one (**5g**) and 6-chloro-7-(3-phenylpropan-1-yloxy)-4-methyl-2*H*-chromen-2-one (**5j**) were found to be the most potent inhibitors having IC_{50} values of 24.51±0.04 µmoles/L and 34.71±0.14 µmoles/L, relative to eserine, a reference standard with IC_{50} value of 0.85±0.0001 µmoles/L. The beneficial inhibition activity of these compounds was likely due to the occurrence of *meta* chloro-substituted and unsubstituted aralkyl groups, respectively, attached to coumarin *via* oxygen. Compounds **5e**, **5h** and **5i** also inhibited BChE activity with low IC_{50} values. The descending order of BChE inhibition activity is as follows; **5g<5j<5e<5i<5h**.

Against LOX, most of the compounds were inactive. The parent compound, 6-chloro-7-hydroxy-4-methyl-2*H*-chromen-2-one (**3**) was the most efficient having IC_{50} value of 57.31±0.19 µmoles/L, relative to baicalein, a reference standard with IC_{50} value of 22.4±1.3 µmoles/L. However, the derivatives of **3** could not reveal good LOX inhibition activity.

CONCLUSION

The presented series was synthesized to elaborate the biological activities of the *O*-substituted derivatives of chlorinated coumarin. The synthesized compounds showed relatively better results against cholinesterase enzymes rather than lipoxygenase enzyme. The most active compounds can be further evaluated for *in vivo* study and so might be helpful for the designing of pharmacologically important new drug candidates.

ACKNOWLEDGEMENT

The authors are much thankful to the Higher Education Commission (HEC) of Pakistan for financial assistance.

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