Design, synthesis and biological evaluation of substituted pyrazoles endowed with brominated 4-methyl 7-hydroxy coumarin as new scaffolds against Alzheimer’s disease

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Abstract

Background: The study aimed to design, synthesize and evaluate various brominated derivatives of 7-hydroxy coumarin as a new scaffold against Alzheimer’s disease by in vivo and in vitro models. A group of three novel pyrazoles endowed with brominated 7-hydroxy 4-methyl coumarin derivatives were designed. Among the designed compounds, a single entity (D1) was selected based on the docking score, which could be considered mainly for the treatment of Alzheimer’s disease. Three novel pyrazoles endowed with brominated 7-hydroxy 4-methyl coumarin derivatives were designed and docking studies of these compounds were carried out using Argus lab4.0.1 version. According to the docking score, a single entity of compound (D1) was selected for further study.

The structure of the compound (D1) was explored by spectral analysis. The anti-Alzheimer’s activity was evaluated by in vivo and in vitro methods. All results were compared statistically by one-way ANOVA using GraphPad Prism.

Results: Molecular docking studies revealed that the compound D1 was able to bind simultaneously to the amino acid and in the active sites of the acetylcholine esterase enzyme. In acetylcholine esterase inhibition assay, the compound shows a significant increase in acetylcholine esterase level. The MAO inhibitory activities were in the nanomole range (human MAO-A IC50 = 3.9, human MAO-B IC50 = 4.4). DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) assay showed that the compound shows a promising antioxidant property. In the evaluation of learning and memory of compound D1 using elevated plus maze, the compound D1-pretreated group showed a significant increase in memory and learning when compared with donepezil.

Conclusions: Among the designed series of pyrazole endowed with brominated 7-hydroxyl 4-methyl coumarin derivatives, compound D1 showed good antioxidant property and acetylcholine esterase and MAO inhibitory activity; based on this property, the synthesized compound D1 can be considered a new scaffold on Alzheimer’s disease.

Keywords: Acetylcholine esterase inhibition, MAO inhibition, Alzheimer’s disease, Coumarin derivatives
Background
Alzheimer’s disease is a neurodegenerative disorder characterized by the degeneration of distinct neurons in a specific region of the brain manifested by cognitive and behavioural impairment [1]. The main cause of Alzheimer’s disease is dementia [2]. A patient affected by Alzheimer’s disease shows abnormal deposits of beta-amyloid peptides and spiral filaments in neurons, a low level of acetylcholine and increased oxidative stress [3].

The major treatment strategies for Alzheimer’s disease are based on acetylcholine esterase inhibition and thus improve cholinergic neuron transmission in the brain [4]. Drugs designed to slow down the disease progression are available; however, these medicines have central and peripheral side effects which are the main drawback of current therapy. Thus, the demand for safe and newer acetylcholine esterase inhibition has become important.

Coumarin, a heterocyclic compound that belongs to the benzopyrone chemical class consists of a benzene ring connected to a pyrone moiety [5]. It is well documented that various 3-, 4-, 7-substituted coumarins possess potent acetylcholine esterase inhibition activity. 7-hydroxy coumarins and 4-hydroxy coumarins as well as pyrazole have been used as a backbone to attain chemically and biologically diverse agents. In this study, we have incorporated 7-hydroxy coumarins and five-membered heterocyclic compound pyrazole with two nitrogen atoms at 1,2 position [6]. Various substituted pyrazoles are recognized as potent, selective and reversible MAO-A and MAO-B inhibitors. The substitution of pyrazole on coumarins may show alteration in anti-Alzheimer activity [7, 8].

Methods
Three novel coumarinyl pyrazoles (D1, D2, D3) ( Table 1) were designed as selective and reversible MAO-A and MAO-B inhibitors (Fig. 1). The chemicals and solvents used for experimental work were commercially procured from Merck, India. The melting point was determined in Thiele’s tube. All biological studies using animal models were conducted after ethical clearance obtained from the Institutional Animal Ethics Committee (IAEC) constituted as per CPCSEA guidelines.

Molecular docking [9]
To reveal the possible intermolecular interaction behind the inhibitory activities of novel 4-methyl-substituted coumarinyl pyrazole derivatives, a molecular modelling study was carried out using the docking program Argus lab 4.0.1 version. All designed compounds were docked into the binding site of the enzyme acetylcholine esterase and recorded the possible docking score. Based on the docking score, a single compound was selected from the series with better binding affinity and binding mode.

Docking protocol
Argus lab 4.0.1 version was employed in the docking between protein and ligand using an Argus dock with a fast and simplified potential of mean force (PMF). For the preparation of the receptor, the crystal structure of acetylcholine esterase (PDB entry: 5HF6) was taken from the RCSB protein data bank (http://www.rcsb.org). The ligand 5HF6 was extracted from the crystal structure. The active site of acetylcholine esterase inhibitors (PDB entry: 4EY7) was identified by using scfbio-iit.res.in: an energy-based method for the prediction of the protein ligand-binding site. The predicted amino acid residue was selected and saved as the binding site for the docking study of final pyrazole endowed with substituted brominated coumarin derivatives. All the final synthesized derivatives were built by using ACD LABS Chem sketch 2015 version software. The structure save in mol format and can be imported to Argus lab 4.0.1 version and geometry optimization was done, then the hybridization level and valency of atom in the ligand was checked to make sure of the exact hybridization pattern
of the molecule. The energy minimized structures were saved in PDB format for further studies.

The starting material 7-hydroxy 4-methyl coumarin was prepared according to the standard literature procedure [10].

**Synthesis of 3-bromo-7-hydroxy-4-methyl-2H-chromen-2-one [11]**

4-Methyl-7-hydroxy coumarin was added to acetic acid and stirred for 40 min. Potassium bromate was added to the reaction mixture stirred for 90 min to dissolve completely. Potassium bromide was charged and further stirred for 2 h. The reaction mixture was poured into cold water and stirred for half an hour, filtered and washed with water and recrystallized using ethanol.

**Synthesis of (3-bromo-4-methyl-2-oxo-2H-cromen-7-yl) oxy acetate (Scheme 3) [5]**

4-Methyl-7-hydroxy coumarin and potassium carbonate were dissolved in dimethylformamide. The solution was stirred at room temperature for several minutes and then ethyl chloroacetate was added dropwise to the mixture. The solution was then heated to 90 °C for 5 h. After completion of the reaction, the mixture was cooled to room temperature and diluted with cold water and the precipitate was filtered and washed. The was solution dried and recrystallized from ethanol.

**Synthesis of (7-hydroxyl-2-oxo-2H-cromen-7-yl) oxy acetic acid hydrazide**

In a 500-ml round bottom flask, the required quantity of ethanol was added to 7 g of 7-hydroxy-2-oxo-2H-cromen-7-yl acetic acid and 1.3 ml of hydrazine hydrate. The resultant reaction mixture was reflexed for 5–6 h. After completion of the reaction, the reaction mixture was added into ice-cold water and the solid separated which was filtered, dried and recrystallized from ethanol.

**In vitro studies**

**Effect of compound D1 on brain acetylcholine esterase level [12, 13]**

Brain acetylcholinesterase activity was measured by using an auto-analyser (Fig. 8). The working reagent was mixed with 50μl of the brain supernatant; incubate the reaction mixture at 37 °C for 30 s. Measure the change in absorbance per 30 s for 90 s. Cholinesterase activity was calculated by the following equation.

\[
\text{Cholinesterase activity} = \frac{\Delta \text{OD}_{60}}{22653}
\]

where \(\Delta \text{OD}\) is the changes in optical density.

### Table 2 Docking score and molecular properties of the designed compound

| Sl. no. | Compound | Binding energy (Kcal/Mol) | Log p | Molecular weight | Hydrogen bond acceptor | Hydrogen bond donor | No. of variation |
|---------|----------|--------------------------|-------|------------------|------------------------|---------------------|-----------------|
| 1       | D1       | −8.93                    | 4.93  | 576.21           | 9                      | 1                   | 1               |
| 2       | D2       | −7.53                    | 4.96  | 567.21           | 9                      | 1                   | 1               |
| 3       | D3       | −7.91                    | 4.98  | 567.20           | 9                      | 1                   | 1               |

Fig. 2 Hydrogen bonding of D1 at **a** Arginine 513 position and **b** Glutamine 515 position
**MAO inhibition studies [14–16]**

In vitro monoamine oxidase inhibitory activity of compound D1 was evaluated by using a MAO assay kit based on the colourimetric assay method. Monoamine oxidase with its substrate generated hydrogen peroxide; in the presence of horseradish peroxide (HRP), the colourimetric probe reacts with hydrogen peroxide to form a red/pink colour. Prepare and mix all reagents before use. Add 50 μl of each sample into an individual microtiter well. Add 5 μl of the 100 μM MAO inhibitor to the appropriate well. Add 5 μl buffer to hydrogen peroxide standard and sample without inhibitor. Mix well and incubate for 30 min at room temperature. Add 50 μl of working solution to each well and mix thoroughly and allow to incubate 45–60 min at room temperature protected from light. Read the plate spectrophotometric microplate reader in the 540–570-nm range as per Table 4 and is represented graphically in Fig. 9.

\[
\text{MAO Activity (Units/L) } = \frac{\text{Hydrogen peroxide generated}}{\text{Reaction Time (minutes)}} \times \text{sample dilution}
\]

**Evaluation of antioxidant activity by DPPH radical scavenging assay [17–19]**

The solution of DPPH in ethanol (0.1Mm) was prepared. This solution of 1 ml was added to 3 ml of different concentrations of the compound D1 (12.5, 25, 50, 100, 200 μg/ml). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min; then, absorbance was measured at 517 nm by using a spectrophotometer[19]. The reference standard compound being used was ascorbic acid and the experiment was carried out in triplicate. The IC₅₀ value of the sample was calculated. DPPH scavenging effect was calculated by using the following equation [20].

\[
\text{DPPH scavenging effect (\% or Percent inhibition) } = \frac{\text{Absorbance of control (\% or Percent inh)}}{\text{Absorbance of control}} \times 100
\]

**In vivo studies**

Male albino mice weighing between (15–25 g) were used for this study. They were procured from Animal House, College of Pharmaceutical Sciences, Govt. Medical College, Kannur, Kerala, India with registration number 1097/PO/Re/S/07/CPCSEA under Ministry of Environment, Forest and Climate Change, Government of India. Ethical clearance was obtained from Institutional Animal Ethics Committee (IAEC) constituted as per CPCSEA guidelines with the clearance number CPCSEA/IAEC-18/19-10 - 1097/PO/Re/S/07/ CPCSEA. Animals were randomly assigned to the control and experimental group consisting of 6 mice each. In vivo pharmacological screening of anti-Alzheimer’s disease activity done by evaluation of learning and memory by elevated plus maze apparatus. Finally, the histopathological study was done to observe the pathological changes in the brain.

**Acute toxicity studies**

Acute toxicity studies were carried out for the synthesized derivative D1 as per OECD guideline 423 in female Swiss albino female mice weighing 15–25 g by administering a dose of 2000 mg/kg orally. The animals were almost continuously observed for mortality and behavioural changes during the first 24 h. and then daily for 14 days. Animals were well tolerated. So D1 was found to be safe up to a dose of 2000 mg/kg in mice and it was considered a cut-off dose. From this cut-off dose, 1/10th and 1/5th doses were selected to study in vivo anti-Alzheimer’s disease activity.
Experimental design
As per OECD guidelines, No. 423, animals were randomly assigned to two groups (n = 3). A limit test at one dose level of 2000 mg/kg of compound D1 was given orally. Albino mice fasted (food but not water should be withheld overnight) overnight before dosing. Following the period of fasting, the animals were weighed and the test substance was administered. After the test dose administration, food was withheld for a further 3–4 h. The volume used for administration is 1 ml/100 g of body weight. The doses were prepared before administration to keep the stability. The test substance was administered in a single dose by gavage using a stomach tube or suitable intubation cannula. Before dosing, they were kept in their cages for 5 days for acclimatization. The temperature in the experimental animal room should be 22 ± 3 °C. Relative humidity should be maintained between 30 and 70%. The lighting provided is artificial, the sequence being 12 h light and 12 h dark. Animals were observed individually after dosing, at least once during the first 30 min, with special attention given during the first 4 h and thereafter for a total of 14 days. Changes in skin, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behavioural pattern were noted. Attention should be directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. Animals were fed with conventional laboratory diets with

Scheme 1 Bromination of 7-hydroxy 4-methyl coumarin

Scheme 2 Esterification followed by coupling with hydrazide of brominated coumarin
an unlimited supply of drinking water. The effect of compound D1 was assessed. If toxic signs or lethality were not observed, then 1/5th and 1/10th of the limit test dose were considered test doses for the present investigation.

**Evaluation of learning and memory—elevated plus maze test [21, 22]**

Elevated plus maze is the exteroceptive behavioural model to evaluate learning and memory. The apparatus consists of a pair of a close arm and an open arm. The arms were extended to a central platform. Swiss albino mice weighing 15–25 g were used for the study. Five groups of six mice were grouped as control (1% w/v CMC), toxic control scopolamine (0.4mg/kg), standard (donepezil hydrochloride (5mg/kg)) and test group (lower dose—scopolamine + compoundD1 (200 mg/Kg), higher dose (scopolamine + compound D1(400mg/kg)) (Fig. 11). Scopolamine was used as a positive control and donepezil used as a standard drug. Mice were treated with the test, standard, and positive control and the transfer of latency was measured. On the 8th day, after the last treatment, observe the transfer latency and evaluate learning and memory.

**Histopathological investigation and biochemical estimation [23]**

After completion of experiments, all animals were sacrificed and brains were isolated and biochemical estimation of brain acetylcholine esterase, malondialdehyde (MDA), brain-reduced glutathione (GSH), vitamin C, protein, superoxide dismutase (SOD), catalase (CAT) and nitrite using a biochemical analyser with the help of diagnostic kits was done.

**Statistical analysis**

The data were represented as the mean ± SEM of six observations for in vivo studies. The results obtained were statistically analysed by one-way ANOVA followed by Dunnett’s multiple comparison tests. A value $p < 0.01$ was taken as a criterion of significance.

| Table 3 Effect of compound D1 on brain acetylcholine esterase level |
|---|---|---|
| Sl no. | Group | Mean AchE level in μ/ML |
| 1 | Negative control (1% w/v CMC) | 0.3843 ± 0.03237*** |
| 2 | Toxic control (scopolamine (0.4mg/kg)) | 0.7002 ± 0.01101 |
| 3 | Standard (donepezil hydrochloride 5mg/kg) | 0.5003 ± 0.002883*** |
| 4 | Lower dose (scopolamine + compound D1(200mg/kg)) | 0.5943 ± 0.01065 |
| 5 | Higher dose (scopolamine + compound D1(400mg/kg)) | 0.5250 ± 0.006851 |

Values are the mean ± SEM, $n = 6$, ***significant at $p < 0.001$, **significant at $p < 0.01$ compared to toxic control. Data were analysed by one-way ANOVA followed by Dunnett’s multiple comparison test. |
Results
The 3 designed compounds (Table 1) were subjected to docking via the Argus Lab docking software. The docking results showed that all derivatives have an almost similar binding mode with different docking scores. The computational study suggested that D1 showed better binding energy than other analogues with a binding score of \(-8.01\text{Kcal/mol}\) (Table 2). In D1, the coumarinyl ring and phenyl ring contributed all hydrogen bonding with a bond distance between arginine (ARG) 513 and glutamine (GLY) 515 in the active site of the enzyme residue (Fig. 2) and other interactions like hydrophobic (Fig. 3) and steric hindrances (Fig. 4).

Synthesis of compound D1
Based on the docking score, compound D1 showed a strong affinity towards the target enzyme acetylcholine esterase. Compound D1 (see Fig. 1) was synthesized by the reaction of 7-hydroxy-4-methyl coumarin with potassium bromide and potassium bromate solution to form 3-bromo-7-hydroxy-4-methyl-2H-chromen-2-one (Scheme 1) is converted to ethyl[(3-bromo-4-methyl-2-oxo-2H-chromen-7-yl)oxy]acetate in the presence of ethyl chloroacetate (Scheme 2), which reacts with hydrazine hydrate in ethanol afforded 2-(4-methyl-2-oxo-2H chromen-7-yl)oxy]acetohydrazide (Scheme 3). The reaction of this hydrazide with pyrazolone leads to the formation of 4-methyl-substituted coumarinyl pyrazole 5-one.

Yellow-coloured crystalline powder of compound D1 with a percentage yield of 75%, molecular weight 576.2101 and melting point 179–182 °C was synthesized. Rf value = 0.64 (10% methanol: chloroform = 1:9).

\[^1\text{H NMR}\ (500 \text{MHz, DMSO-d6, ppm}): \delta 2.24 (3\text{H, s}), 2.49 (3\text{H, s}), 5.00 (2\text{H, s}), 6.74 (1\text{H, dd, } J = 1.5, 0.4 \text{ Hz}),\]

| Sl. no. | Groups | Concentration (\(\mu\text{g/ml}\)) | Absorbance (mean ± SD) | % Scavenging IC\(_{50}\) (\(\mu\text{g/ml}\)) |
|---------|--------|--------------------------------|------------------------|-------------------------------|
| 1       | Control| 0.139 ± 0.0004                  |                        |                               |
| 2       | Standard (ascorbic acid) | 12.5 | 0.058 ± 0.0004 | 42.24 | 23 |
| 2       | 25     | 0.071 ± 0.0002                  | 51.56                  |                               |
| 3       | 50     | 0.095 ± 0.0008                  | 69.82                  |                               |
| 4       | 100    | 0.112 ± 0.0005                  | 82.05                  |                               |
| 5       | 200    | 0.127 ± 0.0005                  | 92.41                  |                               |
| 3       | Test compound D1 | 12.5 | 0.005 ± 0.0001 | 39.56 | 29.2 |
| 6       | 25     | 0.065 ± 0.0045                  | 47.26                  |                               |
| 7       | 50     | 0.092 ± 0.0011                  | 67.89                  |                               |
| 8       | 100    | 0.104 ± 0.0036                  | 78.45                  |                               |
| 9       | 200    | 0.123 ± 0.0025                  | 88.92                  |                               |

Table 4 IC\(_{50}\) value for the inhibition of MAO-A and MAO-B by the compound D1 and reference inhibitor

| Treatment      | Maximum % inhibition | IC\(_{50}\) |
|----------------|----------------------|-------------|
|                | MAO-A | MAO-B | MAO-A | MAO-B |
| Compound D1    | 61%   | 65%   | 2.4    | 3.5    |
| Reference inhibitor | 60%   | 65%   | 3.9    | 4.4    |
6.83 (1H, dd, J = 8.5, 1.5 Hz), 7.01 (1H, ddd, J = 7.9, 1.2, 0.5 Hz), 7.13–7.26 (2H), 7.18 (ddd, J = 8.1, 7.5, 1.2 Hz), 7.21 (ddd, J = 7.9, 7.5, 1.6 Hz)), 7.49 (1H, ddd, J = 8.1, 1.6, 0.5 Hz), 8.06 (1H, dd, J = 8.5, 0.4 Hz). 13C NMR (500 MHz, DMSO-d6, ppm) 169.7, 167.3, 162, 161.6, 153.8, 153.8, 151.4, 140.5, 132.6, 129.2, 128.2, 127.7, 126.7, 117.3, 115, 114.6, 112.7, 110.4, 99.6, 68.9, 18.0, 15.3 ESI-MS: [M + H]+ calculated 576.2101 found 573.9487

Results of biological evaluation

The result obtained on the effect of compound D1 on the brain acetylcholine esterase level is depicted in Table 3 (Fig. 5), MAO inhibition is depicted in Table 4 (Fig. 6) and DPPH antioxidant activity is depicted in Table 5 (Fig. 7). The in vivo results are depicted in Fig. 11 and histopathological images are mentioned in Fig. 12.

Discussion

Alzheimer’s disease (AD) is the most common form of dementia, which affects 35 million people worldwide with increasing tendency. Satisfying therapies and prevention are not available. Since the first description of the fatal progressive neurodegenerative disease in 1907, however, major findings on the molecular mechanisms have been reported.

The present study was aimed to the design, synthesis, molecular docking and evaluation of the anti-Alzheimer activity of substituted pyrazole endowed with brominated-4-methyl-7-hydroxy coumarin (Figs. 8, 9, 10, 11 and 12). A group of 3 novel substituted pyrazole-endowed -4-methyl-7-hydroxy coumarins were designed. Among the designed series, a single entity (D1) was selected according to the docking study and Lipinski’s rule of 5. The selected derivative D1 was synthesized and the structure was confirmed by spectral analysis. The in vitro antioxidant activity was evaluated by DPPH assay. The derivative D1 showed good antioxidant activity comparable to that of standard ascorbic acid. An acute toxicity study was carried out according to OECD guideline 423 in female albino mice and the derivative D1 did not show any sign of toxicity up to the dose of 2000 mg/kg.
body weight, and hence, it was considered to be safe and non-toxic. Evaluation of learning and memory was performed using elevated plus maze. The results showed significant improvement in memory in mice pre-treated with synthesized derivative D1. Biochemical parameters like acetylcholine esterase in brain homogenates were measured. Histopathological investigation of mice brain confirmed the neuroprotective ability of the synthesized derivative D1 by protecting the hippocampus neuronal cells from degeneration.

**Conclusion**

A group of three novel pyrazoles endowed with brominated 4-methyl 7-hydroxy coumarin derivatives were designed and out of which a single entity (D1) was selected based on molecular docking studies and D1 showed good binding affinity towards the crystallographic structure (4EY7) of acetylcholine esterase enzyme. The result obtained from the present study proved that synthesized compound D1 possesses antioxidant, memory enhancing, and acetylcholine esterase inhibitory and monoamine oxidase enzyme inhibitory activity contributed to the overall pharmacological activity of synthesized compound D1 in the central nervous system. All these proven mechanisms make the compound effective in treating Alzheimer’s disease.
Abbreviations
AchE: Acetylcholine esterase; CMC: Carboxymethyl Cellulose; MAO: Monoamine oxidase; OECD: Organisation for Economic Co-operation and Development

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Authors’ contributions
SEN, RGR and SB designed the entire project after an extensive literature review. MM and HN carried out the design, docking and synthesis of the compounds. VCP, AM and AG drafted the manuscript. All authors have read and approved the final manuscript.

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Availability of data and materials
All data and materials is available upon request.

Declarations

Ethics approval and consent to participate
Ethical clearance was obtained from Institutional Animal Ethics Committee (IAEC) constituted as per CPCSEA guidelines with the clearance number CPCSEA/IAEC-18/19-10 with registration number 1097/PO/Re/S/07/CPCSEA under Ministry of Environment, Forest and Climate Change, Government of India.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interest.

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