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Original Research Article

Synthesis and biological activities of novel spiroquinazoline derivatives for Alzheimer's disease

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Abstract

Purpose: *To synthesize novel spiroquinazoline derivatives and determine their biological properties as acetylcholinesterase (AChE) inhibitors and antioxidant agents.*
Methods: *Several quinazoline derivatives (3-5) were synthesized using N-methylisatoic anhydride (1) as starting material. Synthesized compounds were fully characterized using thin-layer chromatography (TLC), mass spectroscopy (MS) and nuclear magnetic resonance (NMR) techniques. Furthermore, in vitro anticholinesterase and antioxidant activities of compound 5 were determined using Ellman's and 1,1-diphenyl 2-picrylhydrazyl (DPPH) assays, respectively.*
Results: *The synthesis resulted in a good yield of compound 5 (71 %), which demonstrated potent inhibitory effect on acetylcholinesterase enzyme (IC_{50} = 11.89 µmol/mL) and significant antioxidant activity (IC_{50} = 143.7 µmol/mL).*
Conclusion: *The synthesized spiroquinazoline derivative 5 exhibits very good anti-AChE anticholinesterase and antioxidant activities. Thus, it has potential for further development for use in Alzheimer's disease (AD) therapy. Future studies are required to elucidate whether compound 5 has direct effects on Tau aggregation or Aβ production and to investigate its full therapeutic potential in the context of these predominant AD hypotheses.*

Keywords: *Alzheimer's disease, Quinazoline scaffold, Ellman's assay, Spiro compounds, Cholinesterase enzyme, Antioxidant*

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INTRODUCTION

Alzheimer's disease (AD) is the leading cause of dementia and it is one of the major healthcare challenges of our time. The disease is a specific pattern of cognitive and functional decline that occurs with aging and is associated with particular neuropathogenesis which is often accompanied by behavioral disorders such as apathy, aggressiveness and depression [1]. The prevalence of AD is currently estimated at approximately 47 million individuals. Due to the expected increase in life expectancy in the coming decades, the prevalence of dementia is expected to increase rapidly with more than 130 million cases predicted by 2050 [2]. Several hypotheses have been put forward to explain this multifactorial disorder, based on various causative factors [3]. The hypotheses comprise the cholinergic hypothesis [4], Aβ hypothesis [5], tau hypothesis and inflammation hypothesis [6]. These hypotheses provide insights into the

intricate mechanisms underlying the disease.

The cholinergic system has a crucial role in AD due to its involvement in cognitive function, memory and learning. The cholinergic hypothesis of AD proposes that deterioration of cholinergic neurotransmission in CNS contributes markedly to cognitive decline associated with this disorder. Most drugs currently used in the treatment of AD are cholinesterase inhibitors, which, unfortunately, are symptomatic drugs [7]. A wide variety of commercially available drugs and lead compounds contain heterocyclic nuclei which provide privileged physicochemical features [8,9]. For example, the heteroaromatic quinazoline structure is known for its association with various pharmaceutical properties such as anticancer [10], antibacterial [11], anti-acetylcholinesterase [12,13], anti-inflammatory [14], blood pressure-lowering [15] and anti-allergic activities [16].

Quinazoline scaffold is undoubtedly one of the most important key templates in the discovery of new drug candidates. Its inherent affinity for various drug targets makes it a valuable substructure for designing compound libraries aimed at different drug targets. The versatile biological functions of the quinazoline nucleus have attracted the attention of medicinal chemists keen on investigating the potential of this scaffold for designing drugs for several diseases [17]. Many quinazoline derivatives have been reported in the literature as promising compounds for the treatment of AD [18]. Given the growing interest in the quinazoline scaffold as ChE inhibitors, the present study was aimed at synthesizing a novel spiroquinazoline derivative (compound 5) through coupling reaction with antioxidant *trans*-ferulic acid as shown in Scheme 1. The perspective is to have a biologically active compound with dual activities as an AChE inhibitor and antioxidant, to develop a potential lead compound to slow the progression of AD.

EXPERIMENTAL

Materials

All chemicals and solvents used in this study were obtained from commercial sources and were of analytical grade.

Determination of AhCE inhibitory activity using Ellman's assay

Ellman's test is a colorimetric assay that operates on the principle that AhCE hydrolyzes acetylthiocholine to produce acetic acid (CH_3CO_2H) and thiocholine. Then, the thiocholine reacts with Ellman's reagent, i.e., 5,5-dithiobis (2-nitrobenzoic acid; DTNB) at pH 7 to yield a yellow-colored anion, 5-thio-2-nitrobenzoic acid (TNB), the concentration of which is read spectrophotometrically at 412 nm. In the microplate assay carried out as subscribed previously [19], 2000 µL of the reaction mixture was dispensed in wells. The mixture was prepared in test tubes by adding 1700 µL of 50 mM Tris-HCl buffer (pH 7), 250 µL of different concentrations (400 to 6.25 µg/mL) of tested compound 5 in Tris-HCl buffer: ethanol (9:1 v:v) solution, 20 µL of 10 mM DTNB (Molekula, UK), and 10 µL of a 6.66 U/mL AChE (Sigma Aldrich, UK). The control was galantamine prepared in a way similar to test samples by dissolving it in 50 mM Tris-HCl solution (pH 7.0). For the blank, the test compound 5 was substituted with an equivalent volume of Tris-HCl buffer. The enzymatic reaction was triggered by the addition of 10 µL of 75 mM acetylthiocholine iodide (Sigma Aldrich, UK) to the wells after the prior incubation period for 15 minutes at 37 ^oC. The plate was shaken for 2 seconds and absorbance was read using a microplate spectrometer at λ = 412 nm, every 10 seconds for 3 minutes. The percentage inhibition of AChE (%) was determined by calculating the rate of change (Δ) in absorbance over time ($v = Abs/\Delta t$) as shown in Eq 1.

$$\text{Inhibition (\%)} = \{100-(\Delta \text{ absorbance}_{\text{sample}} / \Delta \text{ absorbance}_{\text{blank}})\}100 \quad \ldots\ldots\ldots\ldots\ldots (1)$$

The assay was done in triplicates and a regression analysis was performed between the % inhibition concentrations of compound 5. This was used for the determination of concentrations at which the test sample inhibited acetylcholine hydrolysis by 50 % (i.e., IC_{50}).

Determination of antioxidant activity using DPPH assay

The DPPH scavenging assay is a quick, precise and straightforward test used to assess antioxidant activity by tracking changes in the absorbance of DPPH radical at 517 nm. Free radical (purple) reacts with scavenger, producing faintly yellow product. The antioxidant activity of compound 5 was performed using DPPH assay according to a previously reported method [20]. Standard solutions of compound 5 in ethanol (62.5 - 1000 µL) were prepared and added separately to 100 µL of DPPH$^{\cdot}$ solution in ethanol. The mixtures were vigorously shaken and kept at room temperature in the dark. After 30 min, the absorbance of each solution was read at 517 nm using a spectrophotometer (Multiscan^R GO). Ascorbic acid (vitamin C) was

used as a positive control and a DPPH solution without compound 5 served as control. The percentage of DPPH radical scavenging activity was calculated using Eq 2.

Scavenging (%) = {($A_0 - A$)/ A_0}100 …….... (2)

where A_0 is the absorbance of the control sample and A is the absorbance of the test sample.

Assay was conducted in triplicates and the IC_{50} values (the concentrations of the test sample that resulted in 50 % inhibition of the DPPH free radical) were determined from the linear region of a calibration curve obtained by plotting the logarithm of concentration against scavenging activity. A low absorbance value of test samples indicates high free radical activity.

Synthesis of compounds 2 – 5

The synthesis of compound 5 was processed as shown in Figure 1. Compound 2 (2-methylaminobenzamide) was synthesized as previously reported and ^{1}H-NMR spectroscopy was in accordance with that reported in literature [21]. Compound 3 (1'-methyl-1H'-spiro(cyclopentane-1, 2'-quinazolin)-4'(3'H)-one) was also synthesized as previously reported utilizing 2-methylaminobenzamide (2) as a starting material and ^{1}H-NMR spectroscopy also conformed with that reported in the literature [22]. Compound 4 was synthesized by reducing the amide group present in compound 3. In this reaction, the amide group was first activated using TMSCl, followed by the addition of the strong reducing agent $LiAlH_4$. Briefly, a solution of compound 3 (2.00 g/ 9.25 mmol) was treated with 5.9 mL (46.29 mmol) of TMSCl in dry THF (25 mL) and stirred at room temperature for 15 minutes. Then, $LiAlH_4$ (4.47 g/ 118 mmol) was subsequently added to the reaction mixture and the progress of the reaction was monitored using TLC. After 3 h, the starting material was fully consumed and the reaction was quenched by the addition of 3.5 mL of deionized water and, 3.5 mL of NaOH (10 %). Finally, 10 mL of deionized water was added, with continuous stirring for 15 minutes. The mobile phase used for TLC was dichloromethane: methanol (9:1, v:v).

Compound 5 ((E)-3-(4-hydroxy-3-methoxyphenyl)-1-(1'-methyl-1', 4'-dihydro-3'H spiro (cyclopentane-1, 2'-quinazolin) -3'-yl) prop-2-en-1-one) was synthesized by dissolving 2.28 g (11.76 mmol) of *trans*-ferulic acid in dry tetrahydrofuran (THF). Subsequently, triethylamine (4.09 mL, 29.41 mmol) was added, and the mixture was stirred while being cooled using an ice bath. Then, thionyl chloride (0.85 mL/ 11.76 mmol) was added to the mixture. Following a 5-min interval, the reaction mixture was subsequently treated with a solution of compound 4 (1.0 g/4.9 mmol) in dry THF and the reaction was stirred overnight at room temperature It was then quenched with 10 mL of deionized water and the mixture was extracted with ethyl acetate. The organic layer was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. Then, the resultant crude product was purified utilizing column chromatography with chloroform: methanol (9:1) as mobile phase.

Statistical analysis

Data was expressed as mean ± standard error of the mean (SEM). The IC_{50} of the test drug was calculated using Microsoft Excel software. Statistical analysis was done with a two-way analysis of variance (ANOVA) using GraphPad Prism version 8. Two-group comparisons were done with *t*-test. Differences were deemed statistically significant at $p \leq 0.05$.

RESULTS

Synthesis of compounds 2 – 5

The synthesis of compounds 2 – 5 is depicted in Figure 1. For compound 4, a pure brownish oily residue weighing 1.71 g was obtained after ethyl acetate extraction and organic solvent evaporation (yield = 87.7 %). The R_f value of compound 3 was 0.5 and the MS showed a molecular ion peak at m/z 204.

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 1.3 - 1.9 (m, 8H, cyclopent), 2.86 (s, 3H), 3.12 (m, NH), 3.78 (s, 2H), 6.62 - 6.65 (m, 2H, arom.), 7.02-7.23 (m,2 H, arom.).

Finally, compound 5 was obtained as a slightly yellow solid crystal (1.32 g) at a yield of 71.35 %. The mobile phase used for TLC was chloroform: methanol (9:1, v:v) and its R_f value was 0.3. Furthermore, MS showed a molecular ion peak at m/z 380.

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 1.51-2.05 (m, 8H, cyclopentyl), 2.83 (s, 3H, NCH_3), 3.54 (s, 3H, OCH_3), 3.98 (s, 2H, CH_2), 6.76 (d, 1H, *J* = 16 Hz, *trans*-CH=CH), 6.86 (m, 1H, aroma), 6.93 (d, 1H, aroma), 7.05 (s, 1H, aroma), 7.37 (d, 1H, aroma), 7.39 (m, 1H, aroma), 7.70 (d, 1H, aroma), 7.94 (d, 1H, *J* = 16 Hz, *trans*-CH=CH), 9.42 (s, 1H, OH).

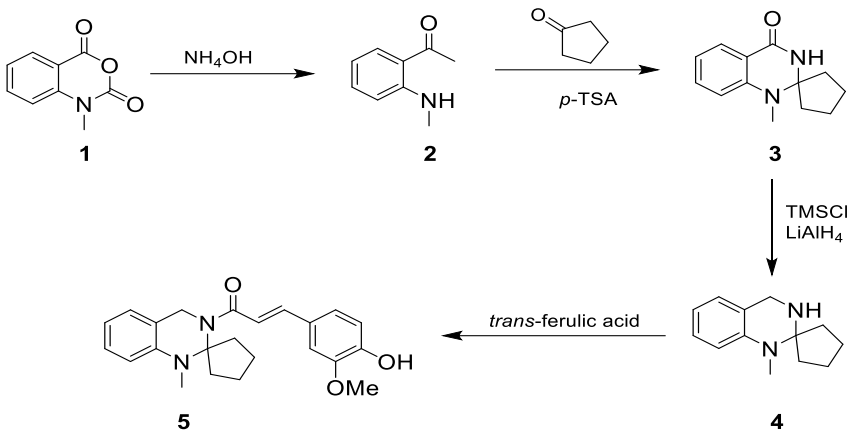


Figure 1: Synthetic route of compound **5**

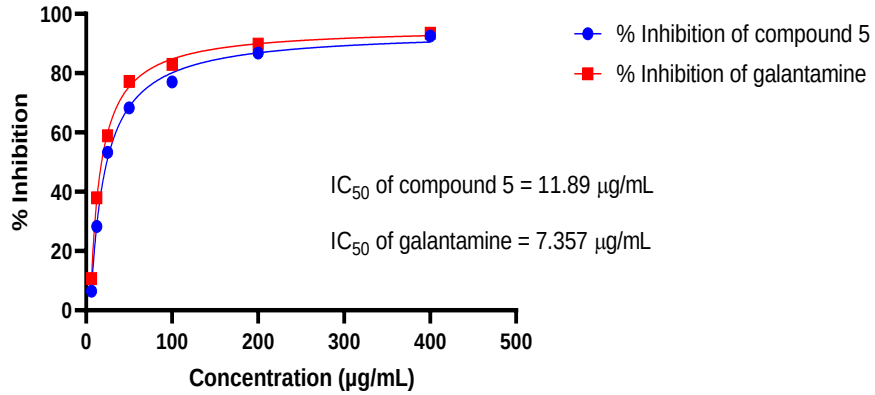


Figure 2: The IC_{50} value of galantamine versus compound 5 against AChE

^{13}C-NMR (100.61 MHz, $CDCl_3$): δ = 23.28 (2C), 32.68, 36.65 (2C), 42.48, 56.78, 98.78, 107.82, 110.13, 114.07, 117.56, 118.58, 123.30, 124.22, 127.42, 128.32, 134.01, 141.12, 146.78, 147.53, 149.35, 164.94 (C=O).

In vitro inhibition of AChE

The inhibitory potential of ferulic acid-hybrid compound 5 on AChE was tested using Ellman's assay. The results are presented in Table 1 as % inhibition relative to galantamine, a standard drug. It was found that compound 5 inhibited the activity of AChE in a concentration-dependent manner ($p < 0.05$). At a concentration of 400 µg/mL, compound **5** produced very potent inhibitory activity of 92.537 %, which was comparable to that of galantamine (93.466 %).

The concentration needed to inhibit 50 % of the initial AChE (IC_{50}) and the AChE inhibition activity of compound 5 were determined. Compound 5 showed significant AChE inhibitory activity, with an IC_{50} value of 11.89 µg/mL compared to control, galantamine, which had an IC_{50} value of 7.36 µg/mL. These data are presented in Figure 2.

Table 1: Concentration-dependent inhibition of AChE by compound 5 and galantamine

Concentration (µg/mL)	Inhibition of compound 5 (%)	Inhibition of galantamine (%)
400	92.537±0.28	93.466±0.68
200	86.755±0.15	89.801±0.37
100	77.031±0.41	82.957±0.75
50	68.328±0.13	77.177±0.28
25	53.320±0.57	58.874±0.41
12.5	28.251±0.78	37.985±0.36
6.25	6.477±0.24	10.707±0.62

DPPH free radicals scavenging assay

Antioxidant activity of synthesized compound 5 was evaluated using 1,1-diphenyl 2-picrylhydrazyl (DPPH) and 2,2-azinobis(3-ethylbenzthiazoine)-6-sulfonic acid (ABTS) as free radicals (Figure 3). Results of DPPH radical scavenging assay are shown in Table 2. Compound 5 produced significant antioxidant activity, with an IC_{50} value of 143.7 µg/mL, as illustrated in Figure 4.

Table 2: Percentage of free radical scavenging activity of compound 5

Concentration (µg/mL)	% inhibition by compound 5	% Inhibition by ascorbic acid
1000	93.021±0.27	96.409±0.58
500	83.152±0.63	92.477±0.89
250	75.828±0.87	87.624±0.23
125	41.075±0.34	73.613±0.75
62.5	22.983±0.61	60.208±0.38

DISCUSSION

In this study, the mechanism involved in the reduction reaction or the synthesis of compound 4 is proposed as shown in Scheme 1. The activation of the amide group by TMSCl is thought to facilitate the subsequent reduction by $LiAlH_4$, which may explain the high yield observed. This approach highlights the importance of the activation step in achieving efficient reduction, which is consistent with the results reported in the literature.

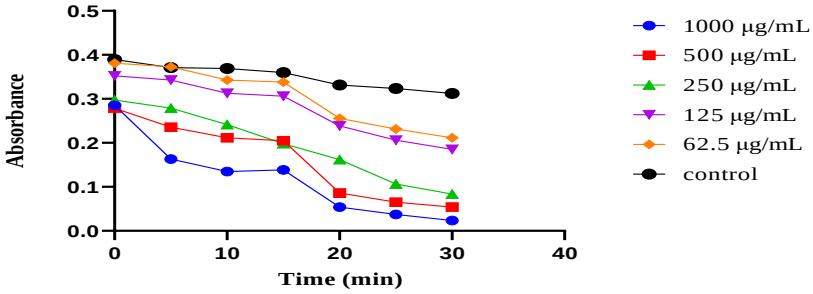


Figure 3: Effects of varied concentrations of compound 5 and galantamine on the time course of DPPH oxidation

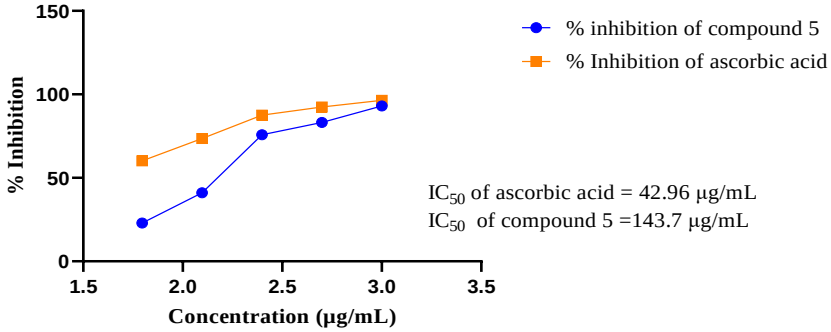
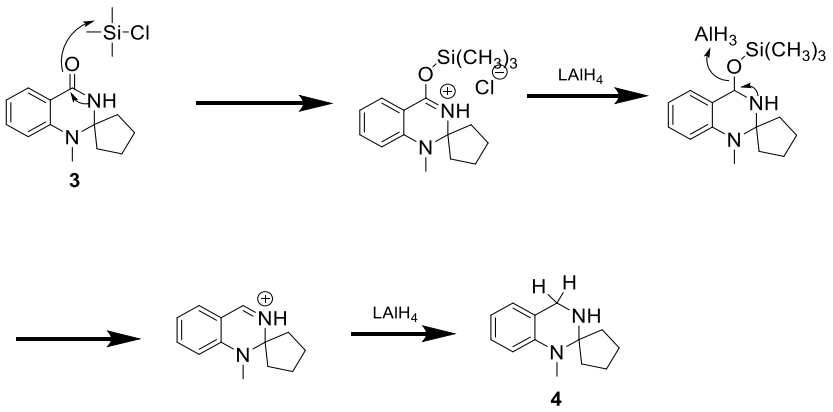


Figure 4: DPPH scavenging activities and IC_{50} values of compound 5 and ascorbic acid



Scheme 1: Proposed reduction reaction mechanism underlying the synthesis of quinazoline derivative 4

Compound 5 was synthesized through a coupling reaction between *trans*-ferulic acid and compound 4. First, the carboxylic acid in *trans*-ferulic acid was activated by converting it to the

corresponding acid chloride, utilizing thionyl chloride ($SOCl_2$) as the chlorinating agent, while TEA was used to speed up the reaction. Then, compound 4 was added, resulting in the formation of compound 5 at a good yield of 71 %. The target compound 5 was tested for its capacity to inhibit AChE. This enzyme isoform shares high (88 %) amino acid sequence homology with the human AChE. Since the amount of ACh neurotransmitter diminishes during the progression of AD, the inhibition of AChE activity has received great interest for the application in the development of drugs effective in the management of dementia. During Ellman's assay, compound 5 exhibited strong inhibitory activity against AChE when compared to the commercially available reversible inhibitor drug, galantamine. Results from the free radical scavenging assays showed that the oxidation process was blocked in the presence of the ethanol solution of compound 5. An increase in the antioxidant activity of compound 5 was associated with increasing concentrations of the compound compared to ascorbic acid, which was used as a standard antioxidant. It is important to note that while compound 5 produced strong inhibitory activity against AChE, as well as strong antioxidant activity in biochemical assays, these results may not necessarily translate to similar outcomes in cellular assays or *in vivo* experiments. Therefore, further studies are required for the evaluation of the efficacy and safety of compound ***5*** in more complex biological systems. While the primary focus of this study was on the investigation of dual activity of the spiroquinazoline derivative 5 as an AChE inhibitor and antioxidant, it is important to consider its potential relevance to Tau and Amyloid-Beta (Aβ) hypotheses, which are central to the pathogenesis of AD. The Aβ hypothesis posits that deposition of Aβ peptides leads to plaque formation, contributing to neurodegeneration, while the Tau hypothesis emphasizes the role of hyperphosphorylated Tau protein in forming neurofibrillary tangles. Although compound 5 was not specifically designed to target these pathways, its antioxidant properties may help mitigate oxidative stress, a known factor that exacerbates both Aβ toxicity and Tau phosphorylation. Additionally, as an AChE inhibitor, compound 5 may alleviate symptoms related to cholinergic deficits, thereby indirectly influencing these pathways by preserving neuronal function. Given the multi-factorial nature of AD, dual activity of compound 5 may be beneficial to a multi-target strategy, which addresses multiple targets associated with disease. Further studies will be necessary to elucidate whether compound 5 has direct effects on Tau aggregation or Aβ production and to investigate its full therapeutic potential in the context of these predominant AD hypotheses.

CONCLUSION

A novel spiroquinazoline derivative (compound 5) was synthesized with a good yield and its biological evaluation shows promising dual activities as an AChE inhibitor and antioxidant. This finding represents a significant advancement in the search for new therapeutic agents against AD. Given the multi-factorial nature of AD which presents several possible therapeutic targets, a multi-target approach may be effective for addressing multiple associated with the pathogenesis of AD. In this context, these findings suggest that this novel quinazoline-based template has the potential for use in the design and development of new anti-AD drugs. However, *in vitro* and *in vivo* studies are needed to reveal the possible mechanism of action and therapeutic potential of this compound. These findings pave the way for more studies aimed at investigating the interaction of the compound with other key AD-related targets, such as Tau and Amyloid-Beta (Aβ).

DECLARATIONS

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Funding

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities

pertaining to claims relating to the content of this article will be borne by them.

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