In Vitro Acetylcholinesterase Inhibition and Cytotoxic Effects of Selected Phenolic Compounds on Sh-Sy5y Cells

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Abstract. This study investigated the *in vitro* acetylcholinesterase (AChE) inhibitory activities and cytotoxic effects of twelve naturally derived phenolic compounds isolated from *Scorzonera ketzkhowelii* Sosn. ex Grossh. AChE inhibition was assessed by the Ellman colorimetric method, using galantamine as a reference inhibitor, while cytotoxicity was evaluated by the MTT assay at six serial dilutions ranging from 100 µM. Among the tested compounds, 3,5-di-*O*-caffeoylquinic acid and quercetin 3-*O*-*α*-arabinopyranoside exhibited notable AChE inhibitory activities, with IC_{50} values of 0.56 µM and 0.89 µM, respectively, closely approaching the potency of galantamine (0.34 µM). Additionally, hydrangeic acid 4'-*O*-*β*-D-glucopyranoside, hydrangenol, and thunberginol F 7-*O*-*β*-D-glucopyranoside displayed moderate inhibitory activities, with IC_{50} values ranging between 1.69 and 2.57 µM. In the cytotoxicity assays, hydrangeic acid 4'-*O*-*β*-D-glucopyranoside demonstrated the strongest effect on SH-SY5Y cells, yielding an IC_{50} of 1.47 µM. This was followed by 3,5-di-*O*-caffeoylquinic acid, p-hydroxybenzaldehyde, and esculin, each showing IC_{50} values below 3 µM, indicating considerable cytotoxic potential. Overall, these findings highlight the dual pharmacological promise of phenolic compounds from *S. ketzkhowelii*, which not only act as effective AChE inhibitors relevant to neurodegenerative disorders but also exhibit significant cytotoxic effects against neuroblastoma cells. This study provides a valuable basis for future investigations aimed at elucidating the molecular mechanisms underlying these activities and supports the continued exploration of phenolic structures from edible and medicinal plants as

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promising multitarget agents in both neurological and oncological therapeutic research.

Keywords: Phenolic compounds, Acetylcholinesterase inhibition, SH-SY5Y cytotoxicity.

1 Introduction

Neurodegenerative disorders, particularly Alzheimer's disease (AD), are among the leading causes of disability and mortality worldwide, posing an increasing public health challenge as global populations age. AD is characterized by progressive neuronal loss, oxidative stress, neuroinflammation, and cholinergic dysfunction. Acetylcholinesterase (AChE), the enzyme responsible for hydrolyzing acetylcholine in synaptic clefts, plays a central role in the pathophysiology of AD. Inhibiting AChE enhances cholinergic neurotransmission, thereby alleviating cognitive symptoms. However, currently used AChE inhibitors such as donepezil, rivastigmine, and galantamine are associated with limited efficacy and adverse side effects, highlighting the need for safer and more effective natural alternatives[1,2].

Phenolic compounds, a diverse class of plant-derived secondary metabolites, are well known for their antioxidant, anti-inflammatory, neuroprotective, and anticancer activities. Recent studies have shown that phenolic compounds, particularly phenolic acids and flavonoids, can inhibit acetylcholinesterase (AChE) and modulate multiple pathways related to neurodegenerative diseases. [3,4]. Flavonoids interact with both the catalytic active site and the peripheral anionic site of AChE through non-covalent interactions such as aromatic and hydrogen bonding, suggesting potential multitarget mechanisms [5]. These properties make phenolic compounds promising leads in developing multifunctional agents capable of addressing oxidative stress and cholinergic deficits simultaneously.

The genus Scorzonera (Asteraceae) includes around 160 species distributed mainly in Eurasia and is known for its rich phytochemical diversity, encompassing phenolic acids, flavonoids, sesquiterpene lactones, and triterpenoids. Previous investigations have revealed that Scorzonera species exhibit significant antioxidant, anti-inflammatory, and enzyme-inhibitory activities [6–8].

Our recent study [9] identified and characterized twelve phenolic compounds isolated from S. ketzkhowelii, including caffeoylquinic acid derivatives, flavonoid glycosides, and hydrangeic acid glucosides. These compounds showed notable antioxidant and molecular docking properties, suggesting their potential neuroprotective relevance. Building on these findings, the current study aimed to evaluate the in vitro AChE inhibitory and cytotoxic activities of these phenolic compounds using the SH-SY5Y human neuroblastoma cell line. To the best of our knowledge, this is the first study to comprehensively evaluate the acetylcholinesterase-inhibitory and cytotoxic activities of individual phenolic compounds isolated from S. ketzkhowelii. In our previous work, only the extract-level activities of this species could be assessed experimentally, and the bioactivities of the isolated phenolics were investigated solely in silico using molecular docking simulations. Here, we advance that research by experimentally confirming, for the first time, the in vitro enzyme-inhibitory and cytotoxic effects of these

individual compounds. This approach bridges the gap between computational prediction and functional validation, revealing the dual role of these natural molecules in both neuroprotective and antiproliferative contexts. By combining enzymatic and cell-based assays, we provide novel insights into the dual neuroactive and cytotoxic potential of S. ketzkhowelii-derived phenolics.

2 Material and Method

2.1 Phenolic compounds

Phenolic compounds were obtained in our previous study [9] where twelve major constituents were isolated and structurally characterized from the aerial parts of Scorzonera ketzkhowelii using chromatographic and spectroscopic techniques. The isolated phenolics were hydrangenol, p-hydroxybenzaldehyde, luteolin, esculin, 3-O-caffeoylquinic acid ethyl ester, 3-O-caffeoylquinic acid methyl ester, kaempferol 3-O-β-glucopyranoside, quercetin 3-O-α-arabinopyranoside, 3,5-di-O-caffeoylquinic acid, thunberginol F 7-O-β-D-glucopyranoside, hydrangeic acid 4′-O-β-D-glucopyranoside, and 3-O-caffeoylquinic acid.

2.2 Acetylcholinesterase Inhibition Assay

A spectrophotometric method developed by [10] was used to indicate the acetylcholinesterase inhibitory activities. Aliquots of 150 µL of 100 mM sodium phosphate buffer (pH 8.0), 10 µL of sample solution, and 20 µL of AChE solution were stirred and incubated for 15 min at 25 ºC; then, DTNB (10 µL) was added to the mixture. In the next step, the reaction was initiated by adding acetylthiocholine iodide (10 µL). At the end, the final concentration of the tested solutions was 200 µg/mL. BioTek Power Wave XS at 412 nm was used to monitor the hydrolysis of these substrates.

2.3 Cell Culture

SH-SY5Y (neuroblastoma cell line, HTB-11) cells were purchased from the American Type Culture Collection (ATCC) and were cultivated in Dulbecco's Modified Eagle's Medium F12 (DMEM-F12) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin antibiotics. Cells were maintained in a humidified incubator at 37 °C with 5% CO_2.

2.4 Cell Viability Assay

The cytotoxic effects of the compounds were evaluated using the MTT assay [11]. SH-SY5Y cells (1×10^4 cells/well in a 96-well plate) were treated with various concentrations (100 µM to 3.125 µM, prepared by 1:2 serial dilutions) of twelve naturally derived phenolic compounds from S.ketzkhowelii for 24 h. After 24h incubation, MTT

solution (5 mg/mL in PBS) was added to each well and further incubated at 37 °C with 5% CO2 for 4 h in the dark. The cell culture media was aspirated from the wells, and afterward, 100 µL of dimethyl sulfoxide (DMSO) was added into each well to dissolve the formazan crystals formed. The absorbance values were measured at 540 nm using an ELISA microplate reader. The experiments were conducted in triplicate, and the results were presented as the mean ± standard deviation. This experiment was conducted to identify compounds exhibiting cytotoxic effects on SH-SY5Y cells and to calculate their IC_{50} values.

2.5 Statistical Analysis

IC_{50} values were calculated by nonlinear regression analysis using the GraphPad Prism version 10 software (GraphPad Software Inc., USA). Dose–response curves were fitted to a variable slope model of "[inhibitor] vs. normalized response," and IC_{50} values were expressed with their 95% confidence intervals (CI).

For the acetylcholinesterase inhibition assay, statistical differences among compounds were evaluated using one-way analysis of variance (one-way ANOVA) followed by Tukey's post hoc test, with $p \leq 0.001$ considered statistically significant.

For the cytotoxicity assays, comparisons among multiple compounds and concentrations were performed using two-way ANOVA, and results with $p < 0.05$ were regarded as statistically significant.

All data were obtained from at least three independent experiments and are presented as the mean ± standard deviation (SD).

3 Results

3.1 Anticholinesterase activity

The IC_{50} values of the tested compounds against acetylcholinesterase (AChE), determined by the Ellman method, revealed a range of inhibitory activities (Table 1). Among the tested molecules, galantamine, a known AChE inhibitor used as a reference compound, showed the most potent activity, with an IC_{50} of 0.34 µM.

Table 1. Acetylcholinesterase inhibitory activities (IC_{50} values) of phenolic compounds.

Compound	IC_{50}
Hydrangenol	2 53±0 01
p-Hydroxybenzaldehyde	12.75±0.01
Luteolin	5.41±0.01
Esculin	4.36±0.01
3-*O*-caffeoylquinic acid ethyl ester	11.52±0.03
3-*O*-caffeoylquinic acid methyl ester	2.81±0.02
Kaempferol 3-*O*-*β*-glucopyranoside	2.87±0.02
Quercetin 3-*O*-*α*-arabinopyranoside	**0.89±0.02**
3,5-di-*O*-Caffeoylquinic acid	**0.56±0.01**

Thunberginol F 7-*O*-*β*-D-glucopyranoside	2.57±0.01
Hydrangeic acid 4′-*O*-*β*-D-glucopyranoside	**1.69±0.03**
3-*O*-caffeoylquinic acid	3.13±0.02
Galantamine	0.34±0.01

Values are expressed as mean ± standard deviation (SD) of three parallel measurements. Galantamine was used as the reference compound. Statistical significance was assessed by one-way ANOVA followed by Tukey's post hoc test, $p \leq 0.001$.

Notably, 3,5-di-O-caffeoylquinic acid (0.56 μM) and quercetin 3-O-α-arabinopyranoside (0.89 μM) also exhibited strong AChE inhibitory effects, demonstrating comparable potency to galantamine. Other compounds with relatively low IC_{50} values included hydrangeic acid 4'-O-β-D-glucopyranoside (1.69 μM), thunberginol F 7-O-β-D-glucopyranoside (2.57 μM), hydrangenol (2.53 μM), 3-O-caffeoylquinic acid methyl ester (2.81 μM), and kaempferol 3-O-β-glucopyranoside (2.87 μM), suggesting moderate inhibitory activity.

Compounds such as 3-O-caffeoylquinic acid (3.13 μM) and esculin (4.36 μM) showed weaker activity, while luteolin (5.41 μM), (5) 3-O-caffeoylquinic acid ethyl ester (11.52 μM), and p-hydroxybenzaldehyde (12.75 μM) displayed relatively low inhibitory potency, indicating less favorable interactions with the AChE active site.

Overall, these results suggest that several derivatives—especially 3,5-di-O-caffeoylquinic acid and quercetin 3-O-α-arabinopyranoside are promising AChE inhibitors and may warrant further investigation as potential candidates for neurodegenerative disease treatment.

3.2 Cytotoxic effects on SH-SY5Y cell line

Among the twelve tested phenolic compounds, 4-hydroxybenzaldehyde, esculin, 3,5-di-O-caffeoylquinic acid, and hydrangeic acid 4′-O-β-D-glucopyranoside exhibited the lowest IC_{50} values, measured as 2.422 μM, 2.910 μM, 2.412 μM, and 1.472 μM, respectively (Table 2). Notably, hydrangeic acid 4′-O-β-D-glucopyranoside exhibited the lowest IC_{50} value, being almost two-fold lower than those of 4-hydroxybenzaldehyde and esculin. These results indicate that compounds 4-hydroxybenzaldehyde, esculin, 3,5-di-O-caffeoylquinic acid ethyl ester and hydrangeic acid 4′-O-β-D-glucopyranoside represent the most potent cytotoxic agents among the tested phenolics. A two-way ANOVA revealed a statistically significant difference in cell viability among the four compounds ($p = 0.0004$), indicating that their cytotoxic potencies differed significantly within the tested concentration range.

Table 2. Cytotoxic activities (IC_{50} values) of phenolic compounds.

Compound	IC_{50}
Hydrangenol	>100 μM
p-Hydroxybenzaldehyde	**2.422 ± 0.110**
Luteolin	97.12 ± 0.058
Esculin	**2.910 ± 0.05**
3-*O*-caffeoylquinic acid ethyl ester	>100 μM

3-*O*-caffeoylquinic acid methyl ester	>100 μM
Kaempferol 3-*O*-*β*-glucopyranoside	>100 μM
Quercetin 3-*O*-*α*-arabinopyranoside	>100 μM
3,5-di-*O*-caffeoylquinic acid	**2.412± 0.058**
Thunberginol F 7-*O*-*β*-D-glucopyranoside	>100 μM
Hydrangeic acid 4′-*O*-*β*-D-glucopyranoside	**1.471 ± 0.225**
3-*O*-caffeoylquinic acid	>100 μM

Cell viability was expressed as a percentage relative (%) to untreated cells (control).

4 Discussion

Among the tested phenolic compounds, 4-hydroxybenzaldehyde, esculin, 3,5-di-O-caffeoylquinic acid ethyl ester, and especially hydrangeic acid 4′-O-β-D-glucopyranoside demonstrated the most potent cytotoxic effect in SH-SY5Y cells, with IC_{50} values in the low micromolar range. Notably, compound hydrangeic acid 4′-O-β-D-glucopyranoside exhibited the strongest activity (1.472 μM). In contrast, the remaining compounds displayed IC_{50} values above 100 μM, suggesting weak or negligible cytotoxicity. These findings suggest that certain phenolic constituents of Scorzonera ketzkhowelii may exert significant effects on neuronal cell viability and could serve as lead structures for further pharmacological evaluation. However, as cytotoxicity was assessed solely via MTT assay, additional biochemical studies are warranted to clarify the underlying pathways and confirm the observed effects.

Contrary to Zhao et al., 2007) [12], who reported that esculin protects SH-SY5Y neuroblastoma cells against dopamine-induced neurotoxicity, our MTT data indicate measurable cytotoxicity of esculin under the current experimental conditions. There are no previous studies evaluating the cytotoxicity of 4-hydroxybenzaldehyde, 3,5-di-O-caffeoylquinic acid ethyl ester, or hydrangeic acid 4′-O-β-D-glucopyranoside in SH-SY5Y cells using the MTT assay. Accordingly, our findings provide the first evidence of the effects of these compounds on neuroblastoma cell viability and expand the existing literature in this field. These compounds, especially those with low micromolar IC_{50} values, are promising candidates for follow-up mechanistic studies to determine their biochemical mechanisms of action and pharmacological significance.

In addition to their cytotoxic properties, several of the tested phenolic compounds demonstrated remarkable in vitro acetylcholinesterase inhibitory effects. Among these, 3,5-di-O-caffeoylquinic acid and quercetin 3-O-α-arabinopyranoside showed IC_{50} values comparable to galantamine, confirming their strong affinity for the AChE active site. Similar results were reported by Cichon et al. (2024) [4], who highlighted the multitarget potential of flavonoids as natural AChE inhibitors capable of interacting with both the catalytic and peripheral anionic sites. Moreover, caffeoylquinic acid derivatives are known to exhibit significant AChE inhibition through π–π interactions and hydrogen bonding within the catalytic gorge, as demonstrated in other plant species [3].

The present findings also align with those of Sezer Şenol et al. (2014), who reported AChE inhibition in Scorzonera extracts containing phenolic acids and flavonoid glycosides, suggesting that these structural classes contribute substantially to the genus's neuroactive potential [6]. The high inhibitory capacity observed for 3,5-di-O-

caffeoylquinic acid may result from the presence of multiple hydroxyls and caffeoyl groups that enhance hydrogen bonding and aromatic stacking within the enzyme pocket. On the other hand, moderate inhibitory activity observed for hydrangeic acid 4′-O-β-D-glucopyranoside and thunberginol F 7-O-β-D-glucopyranoside suggests a role for glycosylation and the phenolic substitution pattern in modulating enzyme affinity.

Collectively, these data provide new experimental evidence supporting the dual pharmacological behavior of S. ketzkhowelii phenolics as both neuroactive and cytotoxic agents. Their AChE inhibition complements their previously reported antioxidant and in silico docking properties [9], reinforcing the potential of these molecules as multitarget scaffolds in the search for natural anti-Alzheimer candidates.

5 Conclusion

This study provides the first experimental evidence that phenolic compounds isolated from Scorzonera ketzkhowelii exhibit notable in vitro acetylcholinesterase inhibitory and cytotoxic activities. Among the isolated compounds, 3,5-di-O-caffeoylquinic acid and quercetin 3-O-α-arabinopyranoside displayed strong AChE inhibition, whereas hydrangeic acid 4′-O-β-D-glucopyranoside showed the highest cytotoxicity on SH-SY5Y cells. These dual effects suggest that S. ketzkhowelii phenolics act through multiple pathways, combining neuroactive and antiproliferative mechanisms.

The findings confirm that caffeoylquinic acid derivatives and flavonoid glycosides are promising natural scaffolds for multitarget drug discovery aimed at neurodegenerative diseases. Furthermore, this work bridges the gap between in silico predictions and in vitro validation, contributing valuable experimental data to the phytochemistry and pharmacology of the Scorzonera genus. Future studies should focus on in vivo confirmation and mechanistic analyses to clarify the relationship between structural characteristics and biological activities.

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