

In vitro Antioxidant, Acetylcholinesterase, and Amyloid-beta Inhibitory Effects of Captopril, An Angiotensin-Converting Enzyme (ACE) Inhibitor

Vantipalli Raga Sai Harshitha¹, Royapuram Parthasarathy Parameswari², Raghul Murugan¹ and Anitha Roy¹

¹Department of Pharmacology, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, Tamil Nadu, India; ²Department of Biochemistry, Saveetha Medical College and Hospitals, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Thandalam, Chennai, Tamil Nadu, India

Correspondence:

Anitha Roy, PhD,
Department of Pharmacology,
Saveetha Dental College and
Hospitals, Saveetha Institute of
Medical and Technical Sciences,
Chennai, Tamil Nadu, India.
E-mail: anitharoy2015@gmail.
com

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ABSTRACT

OBJECTIVE This study aims to investigate the potential of captopril, an angiotensin-converting enzyme (ACE) inhibitor, to reduce reactive oxygen species (ROS) production and address critical factors associated with Alzheimer's disease (AD) pathology, including acetylcholinesterase (AChE) activity and amyloid-beta (A β) peptide aggregation. The broader goal was to explore captopril's neuroprotective properties and its potential as a therapeutic agent for AD.

METHODS The antioxidant effects of captopril were evaluated using DPPH and ABTS assays, as well as xanthine oxidase inhibition tests. The impact of captopril on AChE activity and A β aggregation was assessed through *in-vitro* experiments. Molecular docking studies were conducted to analyze captopril's binding affinity with essential AD-related proteins.

RESULTS Captopril demonstrated notable antioxidant activity with IC₅₀ values of 71.99 $\pm$ 0.40 μ M (DPPH) and 106.3 $\pm$ 0.28 μ M (ABTS), comparable to ascorbic acid (92.32 $\pm$ 0.26 μ M and 110.1 $\pm$ 0.24 μ M). Significant inhibition of xanthine oxidase activity was observed; however, the IC₅₀ value exceeded the maximum tested concentration of 320 μ M for both captopril and ascorbic acid. Captopril showed AChE inhibitory activity with an IC₅₀ of 208.2 $\pm$ 0.36 μ M, whereas donepezil exhibited an IC₅₀ of 79.05 $\pm$ 0.25 μ g/mL. In the A β aggregation assay, captopril had an IC₅₀ of 87.69 $\pm$ 0.25 μ M, while donepezil also displayed significant inhibition, with an IC₅₀ above 320 μ M. Molecular docking analyses revealed favourable binding interactions of captopril with AChE and A β -related targets, supporting its multi-target mechanism.

CONCLUSIONS Captopril demonstrated notable neuroprotective effects, including antioxidant activity and the inhibition of AChE activity and A β aggregation. These findings indicate that captopril holds promise as a therapeutic agent for AD, warranting further research and clinical trials to evaluate its efficacy in combating neurodegeneration.

KEYWORDS health, diseases, antioxidant, neuroprotection, captopril, acetylcholinesterase, amyloid peptide

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INTRODUCTION

The renin-angiotensin system (RAS) is essential for maintaining circulatory homeostasis in the periphery, but its role in brain function has only recently been recognized (1). This localised brain RAS is expressed separately and has a substantial impact on numerous brain activities, as well as contributing to neurological illnesses such as Alzheimer's disease (AD), dementia, Parkinson's disease, and Huntington's disease (2). Furthermore, angiotensin-converting enzyme (ACE) and other RAS components are present in the brain, and elevated ACE levels contribute to the development of AD (3). Elevated ACE activity has been discovered in the cerebrospinal fluid of both Alzheimer's and Parkinson's patients, which could be a sign of brain inflammation. Angiotensin II (Ang II) binds to AT1 receptors, leading to oxidative stress, inflammation, amyloid- β accumulation, and tau protein phosphorylation. This, in turn, leads to cognitive impairment and the onset of AD (4).

According to studies, angiotensin-converting enzyme inhibitors (ACEIs) may be neuroprotective, lowering the chance of acquiring neurodegenerative illnesses. ACE inhibitors such as captopril, lisinopril, enalapril, ramipril, quinapril, perindopril, and trandolapril have been shown to ameliorate neurodegenerative diseases in animals and humans. Numerous clinical trials have confirmed the therapeutic efficacy of ACE inhibitors in treating these disorders (5). Captopril and perindopril, both ACE inhibitors, lower Ang II levels and can cross the blood-brain barrier (6). Perindopril and captopril were previously shown in studies to reduce or reverse cognitive deterioration in people with late-onset AD. Additionally, captopril therapy was found to be related to a decrease in amyloidogenic processing of the amyloid precursor protein (APP) and a reduction in reactive oxygen species (ROS) levels in the hippocampus of Tg2576 AD mice, as previously described (7). It also acts as a potent anti-inflammatory drug, alleviating cognitive impairment by reducing A β production and increasing microglial activation and phagocytosis (8). In addition, as a sulfhydryl (SH) group containing ACEI, captopril has been shown to have a considerable antioxidant impact (9).

Experimental data suggest that a brain-penetrating ACE inhibitor can prevent cognitive

decline, although its impact on A β deposition and the mechanism of action remain unknown (10). In this study, we evaluated the effects of captopril, a first-generation ACE inhibitor, on ROS products, in vitro acetylcholinesterase (AChE) activity, and amyloid beta peptide formation, using in vitro assays and molecular docking.

METHODS

Chemicals and reagents

2,2'-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS), xanthine, acetylthiocholine iodide, acetylcholine enzyme (0.3 U/mL) were procured from Sigma-Aldrich, St. Louis, Missouri, USA. Ascorbic acid was purchased from Tokyo Chemical Industry, Chennai, Chennai, India. donepezil hydrochloride was purchased as a tablet from a local pharmacy. All other chemicals, reagents and solvents used were of analytical grade and purchased from Sisco Research Laboratories Pvt. Ltd, Mumbai, India.

Preparation of captopril, standard ascorbic acid and standard donepezil solutions

Stock solutions of captopril and donepezil hydrochloride were freshly prepared before the assay. Captopril and standard ascorbic acid were dissolved in distilled water to obtain a 10 mM stock solution. In contrast, donepezil hydrochloride was dissolved in 0.1 M phosphate buffer (pH 7.4) to get a 1 mg/mL stock solution. Working solutions were prepared by serial dilution of the respective stock solutions to yield final concentrations of 10, 20, 40, 80, 160, and 320 μ M for captopril and ascorbic acid, and 10, 20, 40, 80, 160, and 320 μ g/mL for donepezil. All solutions were prepared under subdued light conditions to minimise degradation and were stored on ice until use.

In vitro antioxidant activity

DPPH (2,2'-diphenyl-1-picrylhydrazyl) free radical scavenging assay

The DPPH assay was performed to evaluate the free radical scavenging activity of the extract following previously described methods (11). A 0.004% DPPH solution was prepared in 100 mL of methanol. 10 μ L of captopril at concentrations of 10 μ M, 20 μ M, 40 μ M, 80 μ M, 160 μ M, 320 μ M was added to 190 μ L of DPPH solution. After vortexing,

the mixture was incubated for 20 mins at 37°C. The control blank contained solvent without the test compound/standard. The decrease in absorbance of the test mixture (due to quenching of DPPH free radicals) was measured at 517 nm. The IC₅₀ value was determined as the concentration of the test mixture that resulted in a 50% reduction in absorbance relative to a control blank. The experiments were repeated in triplicate, and the percentage inhibition was calculated. Ascorbic acid was used as a reference standard. The DPPH radical scavenging activity of the extract was calculated using the equation below.

$$\% \text{ inhibition} = \frac{\text{absorbance of control} - \text{absorbance of test}}{\text{absorbance of control}} \times 100$$

ABTS assay (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) radical scavenging

ABTS radical scavenging activity of captopril was determined according to a method described by Gonzalez-Palma et al., 2016. 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was dissolved in water and diluted to 7 mM. ABTS radical cation (ABTS⁺) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in a dark at room temperature for 12–16 h before use. The ABTS⁺ solution was diluted with water to an absorbance of 0.70 (± 0.02) at 734 nm. The reaction mixture consisted of 0.07 mL of the captopril at different concentrations (10–320 µM) and 3 mL of the ABTS radical. After incubation for 6 min, absorbance was measured at 734 nm in a spectrophotometer. Ascorbic acid was used as a reference standard. The antioxidant activity was calculated using the following equation (12).

$$\% \text{ inhibition} = \frac{\text{absorbance of control} - \text{absorbance of test}}{\text{absorbance of control}} \times 100$$

Xanthine oxidase inhibitory activity

The Xanthine oxidase (XO) inhibitory activity was assayed spectrophotometrically under aerobic conditions, based on the procedure reported earlier (13). The substrate and the enzyme solutions were freshly prepared. The assay mixture, consisting of 50 µL of different concentrations of captopril (10–320 µM), various concentrations of ascorbic acid (10–320 µM), 35 µL of 0.1 mM phosphate buffer (pH = 7.5) and 30 µL of enzyme solu-

tion (0.01 units/mL of XO in 0.1 mM phosphate buffer, pH = 7.5), was prepared immediately before use. After 30 mins of incubation at 25°C, the reaction was initiated by the addition of 60 µL of substrate solution (150 mM Xanthine in 0.1 mM phosphate buffer). The absorption at 295 nm, indicating the formation of uric acid at 25°C, was monitored and the initial rate was calculated. A blank was prepared in the same manner. One unit of XO was defined as the amount of enzyme required to produce 1 mmol of uric acid/minute at 25°C. XO inhibitory activity is expressed as the percentage inhibition of XO in the above system, calculated as (1-B/A) × 100, where A and B are the activities of the enzyme without and with different concentrations of captopril and ascorbic acid. IC₅₀ values were calculated from the mean values of data from three determinations. Ascorbic acid was used as a reference standard.

In vitro AChE inhibition assay

The inhibitory activity of captopril and donepezil hydrochloride against AChE was determined using a modified Ellman's method suitable for microplate analysis (14). The assay was performed in 96-well plates with a final reaction volume of 200 µL. Each well contained 140 µL of 0.1 M phosphate buffer (pH 7.4), 20 µL of 3 mM DTNB, 20 µL of AChE enzyme solution, and 20 µL of test samples or standard at concentrations of 10, 20, 40, 80, 160, and 320 µM for captopril or 10, 20, 40, 80, 160, and 320 µg/mL for donepezil. For the compound interference control, the enzyme was replaced with buffer while maintaining the test compound and substrate. A blank control lacking both enzyme and test compound was included in each run. The plates were pre-incubated for 15 mins at room temperature in the dark to allow enzyme-inhibitor interaction. The reaction was initiated by adding 20 µL of 5 mM acetylthiocholine iodide (ATChI). Immediately after substrate addition, the absorbance was measured at 412 nm for 5 min using a microplate reader. The rate of increase in absorbance (ΔA/min) was obtained from the linear portion of the kinetic curve. Non-enzymatic hydrolysis and compound interference were corrected by subtracting the respective blank or compound control values. The percentage inhibition of AChE activity was calculated using the formula:

$$\% \text{ inhibition} = 1 - \frac{\text{Rate sample} - \text{Rate blank}}{\text{Rate control} - \text{Rate blank}} \times 100$$

Preparation of A β solution

The A β solution was prepared by dissolving the synthetic A β 1–42 peptide in a 0.1% ammonia solution to a final concentration of 250 μ M. To prevent pre-aggregation, the solution was sonicated in an ice bath for 5 mins, divided into 5 cycles of 1 min each. The A β solution was diluted to 25 μ M in a buffer containing 50 mM phosphate (pH 7.5) and 100 mM NaCl.

Thioflavin T fluorescence assay

The Thioflavin (ThT) fluorescence assay was conducted to monitor the rate of fibril formation. A mixture of A β solution (8 μ L) and varying concentrations of captopril (10–320 μ M) or donepezil (10–320 μ g/mL) was added to a ThT solution (1.6 mL) containing 55 μ M ThT and 50 mM NaOH-glycine buffer (pH 8.5). The samples were incubated at 37°C, and the fluorescence levels of the samples were measured using a Biotek Synergy H4 hybrid multimode reader with excitation and emission wavelengths of 446 nm and 490 nm, respectively (15).

Molecular docking analysis

Molecular docking analysis was conducted to explore the binding characteristics of captopril with protein targets linked to AD. The 3D structure of captopril (CID: 44093) was sourced from the PubChem database. Similarly, the structural models of crucial protein targets, including β -amyloid (PDB ID: 8EZD), AChE (PDB ID: 4PQE), and tau protein (p-tau) (PDB ID: 2MZ7), were obtained from the Protein Data Bank in the RCSB. Proteins

were processed by removing water molecules, adding hydrogen bonds, and other charges. After protein and ligand processing, the grid box was placed over the macromolecules at dimensions X, Y, and Z of 203.0, 213.5, and 232.0, respectively. Finally, docking was performed using the Lamarckian genetic algorithm, and outputs were generated (16). Following the docking process, the optimal conformation of captopril for each protein target was identified, and the molecular interactions were visualised using a PYMOL molecular viewer (17).

Statistical analysis

Data analysis was carried out using GraphPad Prism (version 7.0). Based on triplicate experiments, results were reported as Mean $\pm$ SEM, and IC₅₀ values were calculated using linear regression. Differences among means were evaluated using a two-way ANOVA, with a significance threshold of $p < 0.001$. Comparisons with control groups were performed using the Holm-Sidak Test.

RESULTS

DPPH radical scavenging assay

Captopril's free radical scavenging activity was carried out using DPPH radical scavenging assay, ABTS radical scavenging assay, and xanthine oxidase inhibitory assay (Table 1). The results of the free radical scavenging assays exhibited significant ($p < 0.001$) inhibition of the free radical formation at the highest concentration of 320 μ M. A maximum inhibition of $> 75\%$ was observed in all three assays, compared with standard ascor-

Table 1. In vitro antioxidant and neuroprotective activities of captopril compared with standard reference compounds

Parameters	Captopril (IC ₅₀)	Standards (IC ₅₀)
DPPH free radical scavenging activity	71.99 $\pm$ 0.40 μ M	92.32 $\pm$ 0.26 μ M (ascorbic acid)
ABTS radical scavenging activity	106.3 $\pm$ 0.28 μ M	110.1 $\pm$ 0.24 μ M (ascorbic acid)
Xanthine oxidase inhibitory activity	> 320 μ M	> 320 μ M (ascorbic acid)
Acetylcholinesterase inhibitory activity	208.2 $\pm$ 0.36 μ M	79.05 $\pm$ 0.25 μ g/mL (donepezil)
β -Amyloid aggregation inhibition assay	87.69 $\pm$ 0.25 μ M	> 320 μ M (donepezil)

The table presents IC₅₀ values (Mean $\pm$ SEM) for DPPH and ABTS radical scavenging, xanthine oxidase inhibition, acetylcholinesterase inhibition, and β -amyloid aggregation inhibition assays. DPPH, diphenyl-1-picrylhydrazyl; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

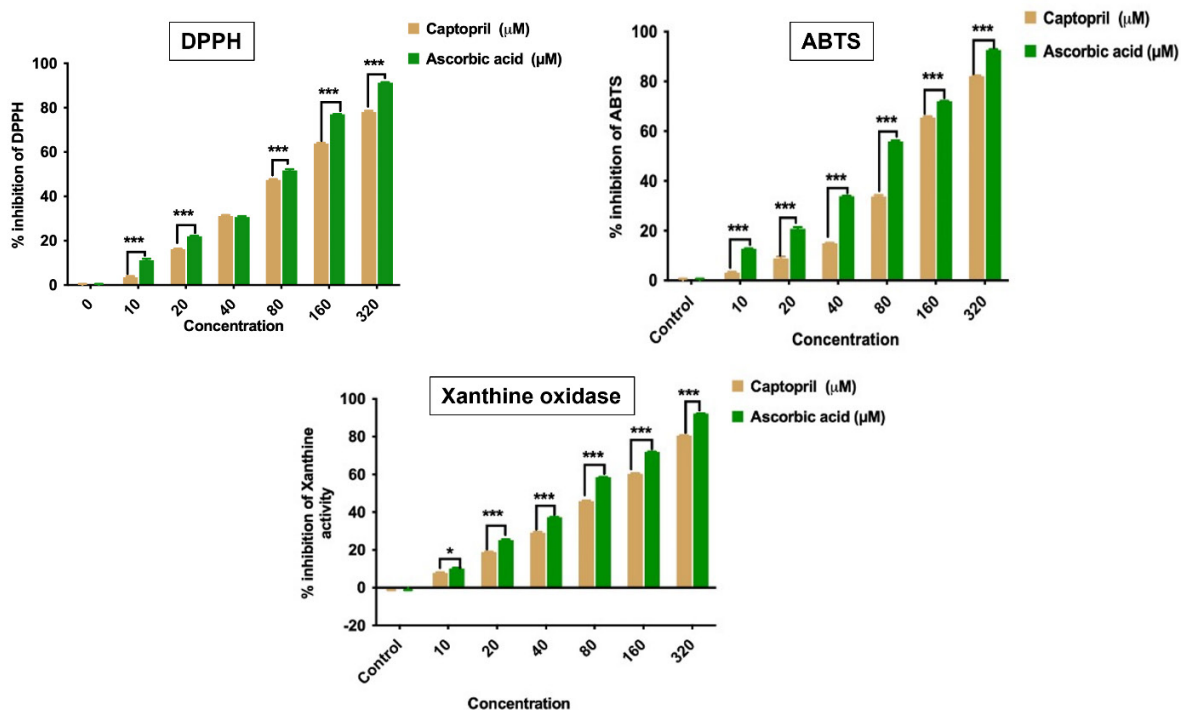


Figure 1. Free radical scavenging effect of captopril at different concentrations compared with standard ascorbic acid. Data are expressed as Mean $\pm$ SEM of triplicate values. Two-way ANOVA was used to assess differences between means at *** $p < 0.001$, and * $p < 0.05$ levels of significance vs. standard ascorbic acid. The means were compared with the standard group using the Holm-Sidak Test.

bic acid, which demonstrated $> 90\%$ inhibition at a similar concentration. The % inhibition of free radical formation exhibited by captopril was concentration-dependent (Figure 1).

In vitro AChE activity

To assess the impact of captopril on AChE activity, various concentrations (10–320 μM) of captopril and the standard donepezil hydrochloride were combined with acetylthiocholine iodide, DTNB, and AChE enzyme. Substantial inhibition of AChE activity was evident across all tested concentrations for captopril and donepezil (Figure 2). Captopril had significantly lower inhibitory activity than donepezil at concentrations ranging from 40 to 320 μM ($p < 0.001$). The inhibitory activity of captopril ranged from 6.98% to 87.03% across the concentration range of 10–320 μM , while donepezil exhibited inhibitory activity in the range of 6.73% to 91.00%.

Effect of captopril on amyloid ($\text{A}\beta$ 1, 42) peptide aggregation

The assessment of captopril's impact on $\text{A}\beta$ (1–42) aggregation revealed a notable inhibition

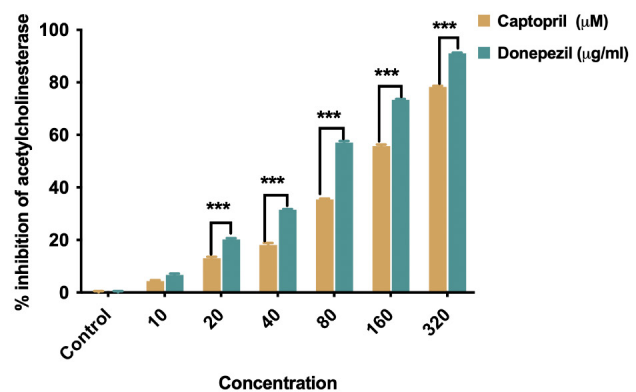


Figure 2. In vitro AChE inhibitory effect of captopril at different concentrations compared with standard donepezil hydrochloride. Data are expressed as mean $\pm$ SEM of triplicate values. Two-way ANOVA was used to assess differences between means at *** $p < 0.001$, levels of significance vs. donepezil. The means were compared with the standard group using the Holm-Sidak Test.

compared with the standard donepezil. At 320 μM concentration, captopril significantly suppressed $\text{A}\beta$ (1, 42) aggregation by 14.45%, while donepezil, at a similar concentration, exhibited 12.22% aggregation. This indicates a substantial reduction in amyloid aggregation by captopril. Concentra-

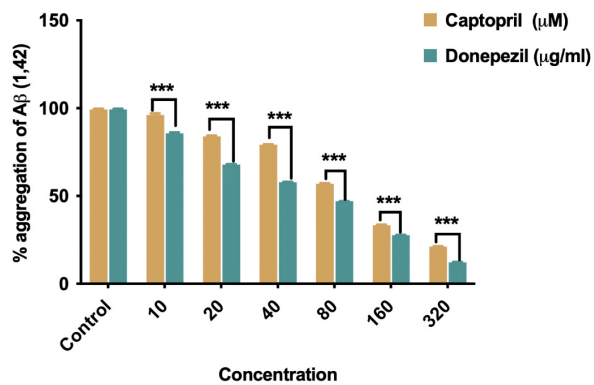


Figure 3. In vitro amyloid A β (1,42) inhibitory effect of captopril at different concentrations compared with standard donepezil hydrochloride. Data are expressed as mean $\pm$ SEM of triplicate values. Two-way ANOVA was used to assess differences between means at *** $p < 0.001$, levels of significance vs. donepezil. The means were compared with the standard group using the Holm-Sidak Test.

tions ranging from 10 to 320 μ M showed a significant ($p < 0.001$) difference in inhibitory activity between captopril and donepezil (Figure 3). Despite this, captopril's inhibitory effect on amyloid aggregate formation was comparable to that of donepezil.

Molecular docking

The docking analysis of captopril with various target proteins revealed differing binding energies and inhibition constants (Figure 4). For beta-amyloid, the captopril formed hydrogen bonds at the binding site at ALA-21 and PHE-20, with a binding energy of -3.73 kcal/mol and an inhibition constant (K_i) of 1.86 mM. In the case of AChE, captopril formed a h-bond at PHE-295 and ARG-296 with a notably lower binding energy of -6.11 kcal/mol and a significantly higher K_i of 33.01 μ M. For phosphorylated p-tau, captopril binds to LYS-267 with a binding energy of -3.83 kcal/mol and a K_i of 1.55 mM. The docking analysis results are shown in Table 2.

DISCUSSION

The present study's results demonstrated captopril's significant antioxidant and neuroprotective properties using an in vitro enzyme inhibition assay, suggesting its potential as a therapeutic agent for neurodegenerative conditions (18). Previous studies have demonstrated that captopril's neuroprotective ability is attributed to its free radical scavenging property (19). An earlier study showed that captopril exhibited a pro-

Table 2. Molecular docking interactions of the ligand with Alzheimer's disease-related target proteins.

Target protein	Binding interactions (h-bond)	Binding energy range	Inhibition constant
Beta-amyloid	ALA-21 (2.115 Å), PHE-20 (2.045 Å) UNL-1 (2.121 Å)	-2.59 to -3.73	1.86 mM
AChE	UNL-1 (2.191 Å) PHE-295 (2.001 Å) ARG-296 (2.199 Å)	-3.2 to -6.11	33.01 μ M
p-tau	LYS-267 (1.954 Å)	-2.68 to -3.83	1.55 mM

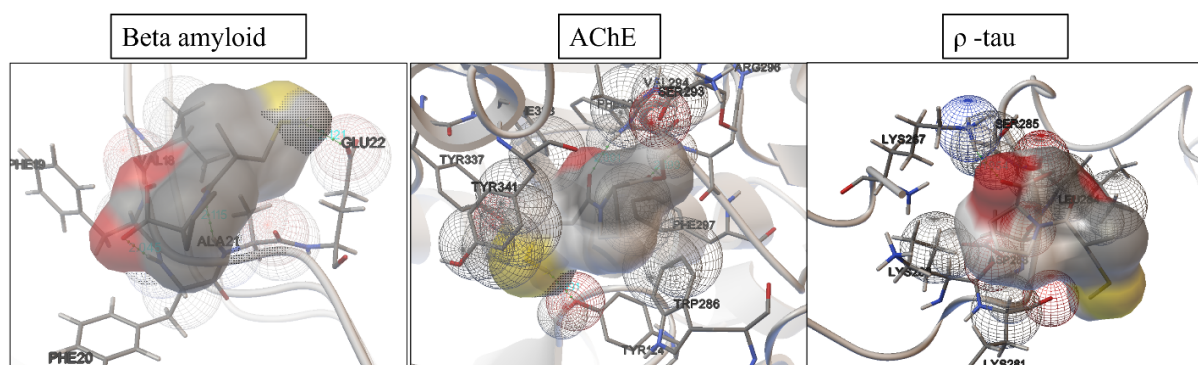


Figure 4. Representative image of molecular docking for captopril against beta-amyloid, AChE, and p- tau receptors

portional response to concentration in the FRAP assay, demonstrating specific antioxidant activity (20). Similarly, another study revealed that captopril attenuated hemolysis induced by free radicals, 2,2'-azobis(2-amidinopropane) (AAPH), and hypochlorite. However, other ACE inhibitors failed to inhibit free radical product formation, suggesting that the unique thiol group present in captopril may account for its antioxidant effect (19). In agreement with the earlier findings, the present findings also substantiate captopril's free radical scavenging capacity at all the tested DPPH, ABTS, and xanthine oxidase assay systems. The antioxidant activity of captopril has been shown to alleviate oxidative stress, thereby reducing the likelihood of the onset of a cascade of events responsible for neurodegeneration.

In AD, a prominent feature is the presence of AChE, often associated with β -amyloid plaques and neurofibrillary tangles (NFT). AChE appears to interact directly with amyloid- β , potentially enhancing its accumulation in insoluble plaques. This newfound function implies that AChE inhibitors could serve as disease-modifying agents (21). Corroborating the given hypothesis, the present study showed that captopril efficiently inhibited AChE activity, suggesting that it might arrest the progression to amyloid accumulation, tau phosphorylation, and the development of AD. Earlier studies have revealed the significant neuroprotective effect of captopril against AD. Captopril has been shown to slow down the accumulation of A β plaques in the hippocampus of mice with AD (7). Captopril treatment not only normalised the elevated levels and activity of the ACE protein associated with AD but also decreased angiotensin II accumulation in the hippocampus and reduced ROS generation. Further, treatment with captopril fully reversed hippocampal neurodegeneration (22). In line with these findings, the present study also substantiates that captopril markedly reduces amyloid peptide aggregation, thereby demonstrating its effectiveness as an Alzheimer's drug.

The binding energies and inhibition constants indicate how effectively captopril binds and potentially inhibits these target proteins implicated in AD. Negative binding energies indicate favourable interactions, with AChE showing the most energetically favourable binding among the three proteins. Additionally, captopril's lowest K_i value

against AChE suggests that comparatively very low concentrations are required for effective inhibition. However, the concentration (33.01 μ M) obtained is higher than the reported concentration of the established AChE inhibitor, donepezil, which showed an IC_{50} value of 0.71 μ M (23). Structurally, a key conserved interaction was identified among captopril, coumarin, and donepezil that forms a hydrogen bond with the backbone of the pivotal PHE-295 residue within the active site. This common binding feature suggests the involvement of a shared molecular recognition motif, likely a C=O $\cdots$ H-N hydrogen bond, which may contribute to anchoring both ligands in a favourable orientation (23). On the other hand, the high K_i values observed with p-tau and β -amyloid suggest that higher captopril concentrations may be required to inhibit these targets. These differences highlight the specificity of drug-protein interactions and indicate that captopril could have varied therapeutic potential against different pathogenic targets in AD.

Comprehending the antioxidant and neuroprotective qualities of captopril in vitro holds significance for possible therapeutic approaches in neurodegenerative conditions like Parkinson's and AD (24). Given its complex pharmacological effects, investigation of captopril as an adjuvant therapy in conjunction with conventional treatments for neurological conditions may be taken into consideration. More studies are required to clarify the precise molecular mechanisms by which captopril provides its neuroprotective and antioxidant benefits.

Although captopril may exhibit neuroprotective effects, further research is needed to determine the specificity and applicability of these effects across a range of neurological disorders and diseases. The mechanisms underlying neuroprotection are diverse and can differ among various pathologies. More preclinical and clinical research is required to determine the precise neuroprotective effects of captopril in humans.

CONCLUSIONS

In conclusion, the present study reinforces captopril's significant antioxidant and neuroprotective properties, particularly in neurodegenerative diseases such as Alzheimer's. The study's findings align with previous research, demonstrating captopril's ability to scavenge free radi-

cals, inhibit AChE activity, and reduce amyloid peptide aggregation, all of which are critical in slowing the progression of Alzheimer's. The binding energy analysis further highlights captopril's potential to target specific proteins associated with the disease, particularly p-tau, suggesting its therapeutic potential at lower concentrations. While these in vitro results are promising, further preclinical and clinical studies are needed to fully elucidate captopril's mechanisms of action and its potential as a neuroprotective agent across various neurological disorders. The potential of captopril as an adjuvant therapy alongside conventional treatments warrants additional investigation to optimise its use in clinical settings.

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CONFLICTS OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

AUTHOR CONTRIBUTIONS

V.R.S.H.: investigation, data curation, formal analysis, writing — original draft; R.P.P.: conceptualization, designing, methodology, investigation, data curation, formal analysis; R.M.: conceptualization, designing, methodology, investigation, data curation, formal analysis; A.R.: conceptualization, designing, data curation, formal analysis, resources, supervision, writing — review & editing.

All authors consent to the publication of the identifiable details of the text.

DATA AVAILABILITY STATEMENT

The original findings of this study are provided in the article and the supplementary material. For additional inquiries, please contact the corresponding authors.

INSTITUTIONAL REVIEW BOARD STATEMENT

Not applicable

INFORMED CONSENT STATEMENT

Not applicable

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