

Antioxidant and Cholinesterase Inhibitory Activities of Zinc Oxide Nanoparticles Biosynthesized from Neem Leaf Extract

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ABSTRACT

Green metal oxide nanoparticles has emerged as bioactive nanomaterials with broad therapeutic potential. In this study, zinc oxide nanoparticles biosynthesised using *Azadirachta indica* (neem) leaf extract (AIZnONPs) were evaluated for their in vitro antioxidant capacity and cholinesterase inhibitory activity. X-ray diffraction analysis of AIZnONPs revealed a hexagonal wurtzite crystal structure and an average crystallite size of 16.8 nm. Dynamic Light Scattering results showed a gradual decrease in particle size of highly monodisperse particles with a polydispersity index of less than 0.3. The surface area of smaller particles was found to be relatively high from the Brunauer- Emmett-Teller analysis. The complete preparation and characterization report of AIZnONPs is currently under review and not detailed here. Antioxidant activity was assessed using DPPH, ABTS, ferric reducing antioxidant power (FRAP), nitric oxide (NO) scavenging, lipid peroxidation (LPO) inhibition, iron chelation, and copper chelation assays, while neuroprotective potential was evaluated through inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). The nanoparticles demonstrated concentration-dependent activity across all assays. The strongest antioxidant responses were recorded for ABTS ($IC_{50} = 210.21 \mu\text{g/mL}$), DPPH ($IC_{50} = 290.96 \mu\text{g/mL}$), and copper chelation ($IC_{50} = 168.76 \mu\text{g/mL}$), while FRAP analysis revealed a dose-dependent increase in reducing power (absorbance = 0.6099 ± 0.005 at $100 \mu\text{g/mL}$). Enzyme inhibition assays showed notable AChE inhibitory activity ($IC_{50} = 245.70 \mu\text{g/mL}$) compared to BChE ($IC_{50} = 1160.85 \mu\text{g/mL}$). All activities were significantly lower than reference standards, indicating moderate but measurable bioactivity attributable to the phytochemical composition of *A. indica* extract and the nanoscale properties of the particles. These findings suggest that AIZnONPs hold fair promise as green-derived nanomaterials with dual antioxidant and neuroprotective properties, warranting further in vivo investigation.

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INTRODUCTION

Oxidative stress, arising from an imbalance between the generation of reactive oxygen species (ROS) and the capacity of biological systems to detoxify them, is a central pathophysiological mechanism underlying a wide spectrum of chronic and degenerative diseases, including cancer, diabetes, cardiovascular disorders, and neurodegenerative conditions (Jomova et al., 2023). ROS such as superoxide anions, hydroxyl radicals, and hydrogen peroxide can induce cellular damage through lipid peroxidation, protein oxidation, and DNA strand breakage, ultimately leading to cell dysfunction or apoptosis (Ansari et al., 2025). Among neurological consequences of oxidative stress, the progressive decline in cholinergic neurotransmission associated with Alzheimer's disease and related dementias has attracted particular attention, given the established role of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) in the breakdown of the neurotransmitter acetylcholine (Chen et al., 2022). Inhibition of these enzymes represents one of the primary pharmacological strategies for managing cognitive decline, making the identification of

novel cholinesterase inhibitors with favourable safety profiles a research priority (Petrov et al., 2026).

In recent decades, the development of nanomaterials from biological sources has opened a compelling new avenue for the discovery of bioactive compounds. Green synthesis, which exploits the reducing and capping potential of plant-derived phytochemicals including phenolics, flavonoids, alkaloids, and terpenoids to fabricate metal and metal oxide nanoparticles without reliance on toxic chemical reducing agents, has emerged as a particularly sustainable approach (Akinsipo et al., 2026). The resulting nanoparticles are understood to inherit, and in many cases amplify, the inherent biological activities of the parent plant material, while the nanoscale dimensions confer enhanced surface area, reactivity, and cellular interaction capacity relative to bulk counterparts (Akinsipo et al., 2024). Nanoparticles synthesised through green routes have consequently been reported to exhibit significant antioxidant, antimicrobial, anti-inflammatory, and enzyme inhibitory activities across a range of *in vitro* systems (Akinsipo et al., 2024).

Among metal oxide nanoparticles, zinc oxide (ZnO) has attracted considerable research interest owing to its unique

physicochemical properties, biocompatibility, low toxicity, and broad spectrum of biological activities (Akinsipo et al., 2026; Mohammed et al., 2025). ZnO nanoparticles have been reported to scavenge free radicals, chelate transition metals implicated in Fenton-type oxidative reactions, and inhibit enzymes relevant to metabolic and neurological disease (Fayed & Reda, 2026; Jiang et al., 2022). The biological potency of ZnO nanoparticles is strongly influenced by their synthetic route, and plant-mediated synthesis using phytochemically rich extracts has been shown to yield particles with superior bioactivity compared to those produced by conventional chemical methods (Akinsipo et al., 2026). *Azadirachta indica* (neem), a member of the Meliaceae family, is one of the most extensively characterised medicinal plants in traditional systems of medicine and is known to contain a diverse array of secondary metabolites including nimbidin, nimbin, azadirachtin, quercetin, and rutin, among others, which confer potent antioxidant, anti-inflammatory, and neuroprotective properties (Alzohairy, 2016). The use of *A. indica* leaf extract as a biological matrix for nanoparticle synthesis is therefore strategically advantageous, as the capping phytochemicals retained on the nanoparticle surface are expected to contribute directly to the observed bioactivity. Despite the growing body of literature on green-synthesised ZnO nanoparticles, comprehensive biochemical characterisation of nanoparticles fabricated specifically using *A. indica* leaf extract remains limited. Most existing studies report on single or dual assay systems, and a systematic evaluation of both the radical scavenging capacity and the cholinesterase inhibitory potential of neem-derived ZnO nanoparticles within the same study, alongside metal chelation and lipid peroxidation data has not been fully addressed. Such a multi-assay approach is important for establishing the translational relevance of these materials, particularly given the co-occurrence of oxidative stress and cholinergic dysfunction in neurodegenerative pathology. The present study therefore aimed to evaluate the *in vitro* antioxidant and neuroprotective properties of zinc oxide nanoparticles biosynthesised using *Azadirachta indica* leaf extract (AIZnONPs). Antioxidant activity was assessed through DPPH, ABTS, ferric reducing antioxidant power (FRAP), nitric oxide scavenging, lipid peroxidation inhibition, iron chelation, and copper chelation assays, while neuroprotective potential was evaluated through inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Standard reference compounds were employed across all assays to provide comparative benchmarks for the observed activities.

We hypothesized that the bioactivity of AIZnONPs would occur with two complementary contributions: (i) the ZnO core, as a result of surface oxygen vacancies, redox-active surface sites and partial release of Zn^{2+} that could favor metal-ion interactions and radical quenching; and (ii) the retained neem phytochemical corona (flavonoids, including quercetin and rutin, and limonoids) expected to dominate by hydrogen-atom/electron donation and interactions with cholinesterase active sites. We also predicted that this combination would yield a multi-target profile of relevance to the co-occurrence of oxidative stress, metal dyshomeostasis and cholinergic deficit in Alzheimer's disease.

MATERIALS AND METHODS

Preparation of Sample Solutions

Stock solutions of AIZnONPs (1000 µg/mL) were prepared by dispersing a weighed quantity of the nanoparticles in dimethyl sulfoxide (DMSO) with brief sonication to ensure homogeneous dispersion. Working concentrations of 6.25,

12.5, 25, 50, and 100 µg/mL were prepared by serial dilution of the stock solution using the appropriate assay buffer or solvent for each experiment. Ascorbic acid, ethylenediaminetetraacetic acid (EDTA), donepezil, and galantamine were used as positive controls and were prepared similarly at the same concentration range. Electric eel AChE (EC 3.1.1.7) and equine serum BChE (EC 3.1.1.8) were used as standard screening enzymes

Antioxidant Assays

DPPH Radical Scavenging Activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity was evaluated according to the method of Blois (Blois, 1958) with minor modifications. Briefly, 1 mL of each sample at varying concentrations was added to 2 mL of a 0.1 mM DPPH solution prepared in methanol. The reaction mixtures were incubated in the dark at room temperature for 30 minutes, after which absorbance was measured at 517 nm. The percentage radical scavenging activity was calculated as:

$$\% \text{ Inhibition} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100.$$

The IC₅₀ was determined by log-linear regression. Ascorbic acid served as the reference standard.

ABTS Radical Scavenging Activity

ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical cation decolorisation was performed as described by (Re et al., 1999). The ABTS^{•+} was generated by reacting 7 mM ABTS with 2.45 mM potassium persulfate in the dark for 12–16 hours, then diluted to an absorbance of 0.70 ± 0.02 at 734 nm. Samples were mixed with the ABTS^{•+} solution and incubated for 6 minutes, and absorbance was recorded at 734 nm. IC₅₀ and percentage inhibition were calculated as in Section 2.3.1.

Ferric Reducing Antioxidant Power (FRAP)

The reducing power of AIZnONPs was determined by the FRAP assay of Benzie and Strain (Benzie & Strain, 1996). Samples were mixed with FRAP reagent (acetate buffer pH 3.6, TPTZ in HCl, and $FeCl_3 \cdot 6H_2O$; 10:1:1 v/v/v) and incubated at 37°C for 4 minutes. Absorbance was measured at 593 nm. Higher absorbance values indicate greater reducing power. Results were expressed as mean absorbance at each concentration, with ascorbic acid as reference.

Nitric Oxide Scavenging Activity

Nitric oxide scavenging was assessed using the Griess reagent method (Marocchi et al., 1994). Sodium nitroprusside (10 mM) in phosphate-buffered saline was incubated with samples at room temperature for 150 minutes, then treated with Griess reagent, and absorbance was measured at 546 nm. Percentage inhibition and IC₅₀ were calculated relative to the control, using ascorbic acid as the positive control.

Lipid Peroxidation Inhibition

Inhibition of lipid peroxidation was assessed by the thiobarbituric acid reactive substances (TBARS) (Ohkawa et al., 1979). Egg yolk homogenate (10% w/v) served as the lipid substrate. After incubation with $FeSO_4$ -induced peroxidation and reaction with TBA reagent at 100°C, absorbance of the supernatant was read at 532 nm. Percentage inhibition was calculated relative to the uninhibited control.

Metal Chelation Assays

Iron (Fe^{2+}) Chelation

Iron chelation activity was determined by the method of Dinis et al. (Dinis et al., 1994) Samples were mixed with 0.1 mM

FeSO₄ and 0.25 mM ferrozine. After 10-minute incubation, absorbance was measured at 562 nm. Percentage inhibition of ferrozine-Fe²⁺ complex formation was calculated as described in Section 2.2.1, with EDTA as the reference.

Copper (Cu²⁺) Chelation

Copper chelation was evaluated following Gyamfi et al. (n.d.). Samples were combined with 0.1 mM CuSO₄ and 0.5 mM pyrocatechol violet in acetate buffer (pH 6.0). Absorbance was recorded at 632 nm after 10-minute incubation. EDTA served as the reference standard.

Enzyme Inhibition Assays

Acetylcholinesterase (AChE) Inhibition

AChE inhibitory activity was measured using the modified Ellman's colorimetric method (Ellman et al., 1961)). Reaction mixtures contained 0.1 M phosphate buffer (pH 8.0), DTNB (0.5 mM), acetylthiocholine iodide (ATCI, 0.71 mM), and electric eel AChE (0.2 U/mL). Samples were pre-incubated with the enzyme for 15 minutes at 25°C before substrate addition. Absorbance was monitored at 412 nm, and percentage inhibition was calculated relative to the enzyme-only control. Donepezil was the positive control.

Butyrylcholinesterase (BChE) Inhibition

BChE inhibitory activity was determined using the same Ellman-based protocol (Section 2.5.1), substituting horse serum BChE (0.2 U/mL) as the enzyme source and butyrylthiocholine iodide (BTCl, 0.71 mM) as the substrate. Galantamine was the positive control.

Statistical Analysis

All experiments were performed in triplicate and results are expressed as mean ± standard deviation (SD, n = 3). The half-maximal inhibitory concentration (IC₅₀) for each inhibitory assay was determined by log-linear regression of the dose-response data ($y = m \cdot \log[x] + b$; $IC_{50} = 10^{((50 - b)/m)}$). One-way analysis of variance (ANOVA) was used to assess the statistical significance of concentration-dependent differences in activity for each assay. All assays yielded F-statistics exceeding 1,100 ($p < 0.0001$), confirming that activity varied significantly across the tested concentration range. Statistical calculations were performed using Python (SciPy v1.x), and differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

DPPH Radical Scavenging Activity

AlZnONPs exhibited concentration-dependent DPPH radical scavenging activity across the tested range of 6.25–100 µg/mL (Table 1; Figure 1). Percentage inhibition increased progressively from $7.27 \pm 0.53\%$ at 6.25 µg/mL to $37.86 \pm 0.29\%$ at 100 µg/mL, yielding an IC₅₀ of 290.96 µg/mL ($R^2 = 0.9968$). Ascorbic acid demonstrated substantially greater scavenging capacity, with inhibition values from $29.85 \pm 0.12\%$ to $83.25 \pm 0.16\%$ and an IC₅₀ of 17.08 µg/mL. The dose-dependent profile and high regression linearity confirm a genuine scavenging effect attributable to the nanoparticles ($F = 1938.81$, $p < 0.0001$).

ABTS Radical Scavenging Activity

The ABTS assay revealed a similar concentration-dependent pattern, with AlZnONPs recording inhibition values from $4.07 \pm 1.18\%$ at 6.25 µg/mL to $42.38 \pm 0.65\%$ at 100 µg/mL, corresponding to an IC₅₀ of 210.21 µg/mL ($R^2 = 0.9763$; Table 1; Figure 1). The relatively stronger ABTS activity compared to DPPH at equivalent concentrations suggests preferential

reactivity toward the hydrophilic ABTS radical cation. Ascorbic acid achieved an IC₅₀ of 16.12 µg/mL. One-way ANOVA confirmed significant concentration-dependence ($F = 1413.32$, $p < 0.0001$).

Ferric Reducing Antioxidant Power (FRAP)

AlZnONPs demonstrated measurable ferric reducing capacity, with absorbance values at 593 nm increasing from 0.34 ± 0.002 at 6.25 µg/mL to 0.61 ± 0.005 at 100 µg/mL (Table 2; Figure 2). The reducing power was markedly lower than that of ascorbic acid (0.48–3.30 across the same range), but the concentration-dependent increase in absorbance ($F = 2106.80$, $p < 0.0001$) confirms genuine electron-donating activity attributable to the surface-bound phytochemical capping agents.

Nitric Oxide Scavenging Activity

Nitric oxide scavenging by AlZnONPs increased significantly with concentration, from $1.78 \pm 0.31\%$ at 6.25 µg/mL to $28.53 \pm 0.29\%$ at 100 µg/mL, with an IC₅₀ of 734.04 µg/mL ($R^2 = 0.9815$; Table 1; Figure 1). Ascorbic acid recorded an IC₅₀ of 29.57 µg/mL. The high IC₅₀ indicates moderate activity against reactive nitrogen species, though the dose-dependence was statistically robust ($F = 3596.02$, $p < 0.0001$).

Inhibition of Lipid Peroxidation

LPO inhibition was the weakest antioxidant activity recorded for AlZnONPs, with inhibition values from $0.68 \pm 0.20\%$ to $26.64 \pm 0.28\%$ at the extreme concentrations and an IC₅₀ of 1437.10 µg/mL ($R^2 = 0.8828$; Table 1; Figure 1). Ascorbic acid achieved $79.94 \pm 0.34\%$ inhibition at 100 µg/mL with an IC₅₀ of 24.96 µg/mL. Despite this disparity, the dose-response relationship remained statistically significant ($F = 4836.04$, $p < 0.0001$).

Metal Chelation Activity

Iron (Fe²⁺) Chelation

AlZnONPs exhibited moderate iron chelation activity, with inhibition of ferrozine-Fe²⁺ complex formation increasing from $2.05 \pm 0.29\%$ to $28.75 \pm 0.43\%$ across the concentration range (IC₅₀ = 666.22 µg/mL, $R^2 = 0.9671$; Table 1; Figure 2). EDTA demonstrated considerably stronger chelation (IC₅₀ = 25.03 µg/mL). Activity was concentration-dependent ($F = 3004.34$, $p < 0.0001$).

Copper (Cu²⁺) Chelation

Copper chelation was the strongest metal-related activity of AlZnONPs, with inhibition from $4.83 \pm 0.37\%$ to $40.36 \pm 0.24\%$ across the range and an IC₅₀ of 168.76 µg/mL ($R^2 = 0.9534$; Table 1; Figure 2). This represented the lowest IC₅₀ across all antioxidant assays, indicating relative selectivity for cupric ions. The excellent reproducibility of the data was reflected in the high F-statistic ($F = 6617.64$, $p < 0.0001$). EDTA recorded an IC₅₀ of 16.90 µg/mL.

Cholinesterase Inhibitory Activity

Acetylcholinesterase (AChE) Inhibition

AlZnONPs demonstrated notable AChE inhibitory activity with percentage inhibition increasing from $4.79 \pm 0.61\%$ to $38.11 \pm 0.23\%$ across the concentration range, corresponding to an IC₅₀ of 245.70 µg/mL ($R^2 = 0.9776$; Table 1; Figure 3). This represents the second lowest IC₅₀ overall, placing AChE inhibition among the stronger bioactivities of AlZnONPs. Donepezil, the reference standard, recorded an IC₅₀ of 22.08 µg/mL. One-way ANOVA confirmed significant concentration-dependence ($F = 1347.06$, $p < 0.0001$).

Butyrylcholinesterase (BChE) Inhibition

In contrast, AIZnONPs showed substantially weaker BChE inhibitory activity, from $0.15 \pm 0.04\%$ to $25.76 \pm 1.14\%$ across the concentration range, with an IC_{50} of $1160.85 \mu\text{g/mL}$ ($R^2 = 0.8867$; Table 1; Figure 3). Galantamine recorded an IC_{50} of $31.61 \mu\text{g/mL}$. The markedly higher IC_{50} for BChE compared to AChE (1160.85 vs. $245.70 \mu\text{g/mL}$) indicates selective inhibitory preference for AChE ($F = 1153.63$, $p < 0.0001$).

Comparative IC_{50} Profile

Comparison of IC_{50} values across all inhibitory assays (Figure 4) highlights that copper chelation ($168.76 \mu\text{g/mL}$), ABTS scavenging ($210.21 \mu\text{g/mL}$), AChE inhibition ($245.70 \mu\text{g/mL}$), and DPPH scavenging ($290.96 \mu\text{g/mL}$) represent the strongest activities of AIZnONPs, while LPO inhibition

($1437.10 \mu\text{g/mL}$) and BChE inhibition ($1160.85 \mu\text{g/mL}$) were weakest. Across all assays, AIZnONPs recorded higher IC_{50} values than the respective reference standards, consistent with a moderate but multi-faceted bioactivity profile. It should be noted that none of the AIZnONP treatments reached 50% inhibition within the tested range (maximum 42.38% for ABTS at $100 \mu\text{g/mL}$). All IC_{50} values were therefore estimated by extrapolating the log-linear regression beyond $100 \mu\text{g/mL}$ and should be regarded as comparative indices rather than exact potencies. The uncertainty is largest where regression fit was weakest, namely LPO ($R^2 = 0.8828$) and BChE ($R^2 = 0.8867$), whose extrapolated values exceed the tested range by more than tenfold. The percentage inhibition values at $100 \mu\text{g/mL}$ (Table 1) provide a directly measured basis for comparison.

Table 1: Percentage Inhibition (mean $\pm$ SD, $n = 3$) and IC_{50} Values of AIZnONPs and Reference Standards across all Inhibitory Assays (Concentration range: 6.25 – $100 \mu\text{g/mL}$)

Assay	Sample	100 $\mu\text{g/mL}$	50 $\mu\text{g/mL}$	25 $\mu\text{g/mL}$	12.5 $\mu\text{g/mL}$	6.25 $\mu\text{g/mL}$	IC_{50} ($\mu\text{g/mL}$)	R^2
DPPH	AIZnONPs	37.86 ± 0.29	31.30 ± 0.79	21.82 ± 0.20	15.55 ± 0.34	7.27 ± 0.53	290.96	0.9968
	Ascorbic acid	83.25 ± 0.16	77.54 ± 0.65	57.41 ± 0.56	41.28 ± 0.25	29.85 ± 0.12	17.08	—
ABTS	AIZnONPs	42.38 ± 0.65	29.33 ± 0.38	19.92 ± 0.19	15.71 ± 0.47	4.07 ± 1.18	210.21	0.9763
	Ascorbic acid	87.39 ± 0.26	80.37 ± 0.21	63.20 ± 0.14	43.64 ± 0.38	25.92 ± 0.05	16.12	—
Nitric Oxide	AIZnONPs	28.53 ± 0.29	24.56 ± 0.32	14.27 ± 0.47	6.58 ± 0.21	1.78 ± 0.31	734.04	0.9815
	Ascorbic acid	71.16 ± 0.43	65.77 ± 0.18	45.62 ± 0.58	31.20 ± 0.29	19.57 ± 0.60	29.57	—
LPO Inhibition	AIZnONPs	26.64 ± 0.28	17.87 ± 0.25	4.42 ± 0.37	1.84 ± 0.31	0.68 ± 0.20	1437.10	0.8828
	Ascorbic acid	79.94 ± 0.34	70.18 ± 0.74	48.26 ± 0.17	30.40 ± 0.28	21.38 ± 0.27	24.96	—
Iron Chelation	AIZnONPs	28.75 ± 0.43	25.80 ± 0.39	14.20 ± 0.23	6.10 ± 0.46	2.05 ± 0.29	666.22	0.9671
	EDTA	77.16 ± 0.36	68.15 ± 0.35	48.16 ± 0.28	34.49 ± 0.49	21.90 ± 0.49	25.03	—
Copper Chelation	AIZnONPs	40.36 ± 0.24	36.22 ± 0.08	28.31 ± 0.33	19.81 ± 0.39	4.83 ± 0.37	168.76	0.9534
	EDTA	81.26 ± 0.49	72.85 ± 0.31	55.38 ± 0.19	43.32 ± 2.59	32.85 ± 0.43	16.90	—
AChE Inhibition	AIZnONPs	38.11 ± 0.23	33.03 ± 0.51	20.16 ± 0.35	16.04 ± 1.09	4.79 ± 0.61	245.70	0.9776
	Donepezil	71.21 ± 0.32	64.56 ± 0.32	54.30 ± 0.39	45.75 ± 0.53	24.29 ± 0.45	22.08	—
BChE Inhibition	AIZnONPs	25.76 ± 1.14	20.71 ± 0.50	4.94 ± 0.57	0.41 ± 0.11	0.15 ± 0.04	1160.85	0.8867
	Galantamine	78.67 ± 0.75	60.71 ± 0.34	42.90 ± 0.82	29.60 ± 0.79	9.44 ± 0.71	31.61	—

Table 2: Ferric Reducing Antioxidant Power (FRAP) of AIZnONPs and Ascorbic Acid Expressed as Absorbance at 593 nm (mean $\pm$ SD, $n = 3$; $F = 2106.801$, $p < 0.0001$)

Sample	100 $\mu\text{g/mL}$	50 $\mu\text{g/mL}$	25 $\mu\text{g/mL}$	12.5 $\mu\text{g/mL}$	6.25 $\mu\text{g/mL}$
AIZnONPs	0.6099 ± 0.005	0.4858 ± 0.003	0.4272 ± 0.005	0.3915 ± 0.004	0.3439 ± 0.002
Ascorbic Acid	3.3037 ± 0.014	2.1517 ± 0.042	1.1861 ± 0.005	0.6062 ± 0.007	0.4832 ± 0.001

Table 3: One-way ANOVA Results for Concentration-dependent Bioactivity of AIZnONPs Across all Assays ($n = 3$ per group; $df = 4, 10$). * $p < 0.0001$**

Assay	df (between)	df (within)	F-statistic	p-value	Significance
DPPH radical scavenging	4	10	1938.812	< 0.0001	***
ABTS radical scavenging	4	10	1413.321	< 0.0001	***
Nitric oxide scavenging	4	10	3596.024	< 0.0001	***
Lipid peroxidation inhibition	4	10	4836.041	< 0.0001	***
Iron chelation	4	10	3004.339	< 0.0001	***
Copper chelation	4	10	6617.644	< 0.0001	***
FRAP (reducing power)	4	10	2106.801	< 0.0001	***

Assay	df (between)	df (within)	F-statistic	p-value	Significance
AChE inhibition	4	10	1347.055	< 0.0001	***
BChE inhibition	4	10	1153.633	< 0.0001	***

*** $p < 0.0001$.

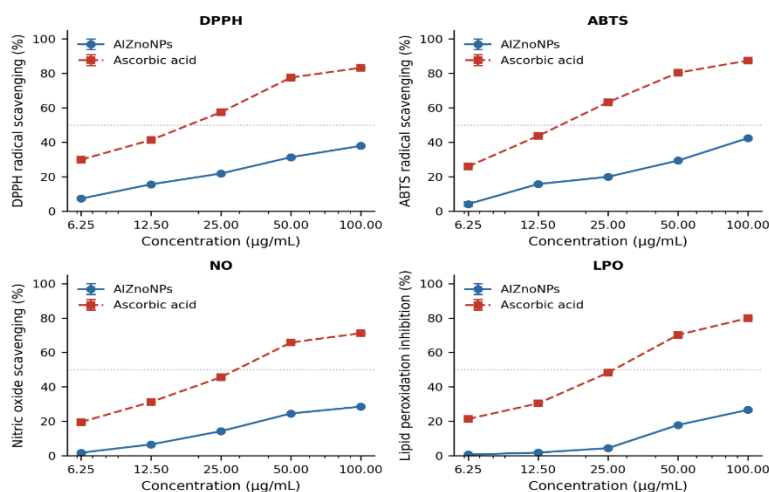


Figure 1: Concentration-dependent Antioxidant Activity of AlZnONPs and Reference Standards: (a) dpph Radical Scavenging, (b) abts Radical Scavenging, (c) nitric Oxide Scavenging, and (d) Lipid Peroxidation Inhibition. Values are Mean $\pm$ sd (n = 3). Dotted line Indicates 50% Inhibition (ic₅₀ Threshold)

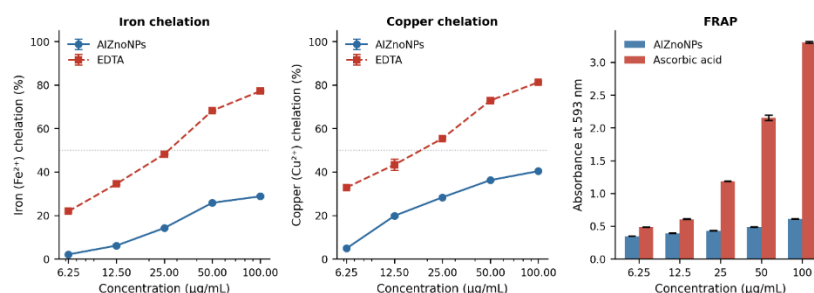


Figure 2: Metal Chelation and Ferric Reducing Antioxidant Power of AlZnONPs: (A) Iron Chelation, (B) Copper Chelation, and (C) FRAP Reducing Power Expressed as Absorbance at 593 nm. Values are Mean $\pm$ SD (n = 3)

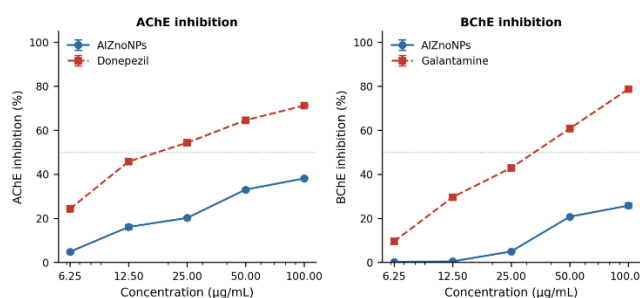


Figure 3: Cholinesterase Inhibitory Activity of AlZnONPs: (A) Acetylcholinesterase (AChE) Inhibition vs. Donepezil, and (B) butyrylcholinesterase (BChE) Inhibition vs. Galantamine. Values are Mean $\pm$ SD (n = 3). Dotted Line Indicates IC₅₀ Threshold

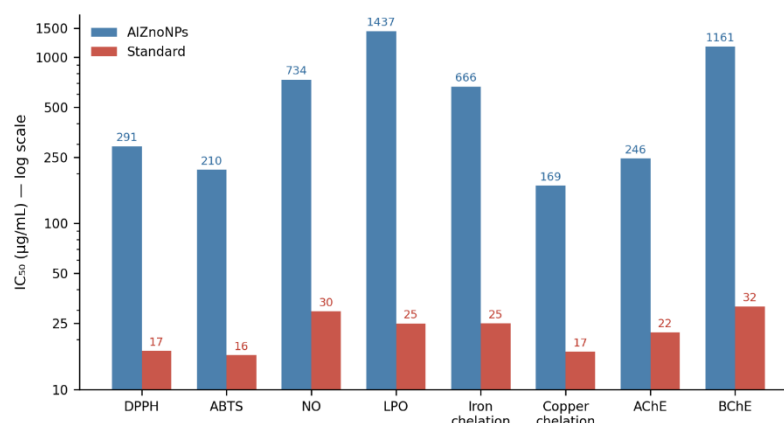


Figure 4: Comparative IC₅₀ Values (µg/mL, Log Scale) of AlZnONPs and Reference Standards Across all Eight Inhibitory Assays. Lower IC₅₀ Values Indicate Stronger Bioactivity

The present study evaluated the *in vitro* antioxidant and cholinesterase inhibitory activities of AlZnONPs across nine biochemical assays. The results collectively demonstrate a moderate but reproducible, concentration-dependent bioactivity profile, with the strongest activities recorded for copper chelation, ABTS and DPPH radical scavenging, and AChE inhibition.

Antioxidant Activity

DPPH and ABTS Radical Scavenging

The IC₅₀ values of 290.96 µg/mL (DPPH) and 210.21 µg/mL (ABTS) are consistent with moderate radical scavenging activity reported for other plant-mediated ZnO nanoparticles, including those synthesised from *Dennettia tripetala* (IC₅₀ = 201.21–227.18 µg/mL; (Oluwaniyi & Oyewo, 2025) and *Aronia melanocarpa* (IC₅₀ = 154.2 µg/mL; (Donmez, 2025). The lower IC₅₀ values of ascorbic acid (17.08 and 16.12 µg/mL, respectively) reflect the direct hydrogen atom transfer mechanism available to pure antioxidant molecules a kinetic pathway less accessible to surface-mediated nanoparticle systems.

The relatively stronger ABTS activity compared to DPPH is consistent with the hydrophilic character of the neem phytochemical capping layer, which interfaces more favourably with the water-soluble ABTS radical cation. This interpretation is supported by the antioxidant chemistry of *A. indica* secondary metabolites, particularly quercetin and rutin, which are potent hydrogen atom donors in aqueous environments (Brahim Mahamat et al., 2025). The radical scavenging activity of AlZnONPs is attributable to two complementary mechanisms: the intrinsic redox activity of surface oxygen vacancies on the ZnO lattice, and the bioactive phytochemicals retained on the nanoparticle surface as capping agents (Murali et al., 2021). The nanoscale dimensions further amplify this activity through the large surface-area-to-volume ratio (Samrot et al., 2022).

Ferric Reducing Antioxidant Power (FRAP)

The concentration-dependent increase in FRAP absorbance ($F = 2106.80$, $p < 0.0001$) confirms genuine electron-donating capacity, consistent with related studies reporting moderate reducing power for polyphenol-capped ZnO nanoparticles (Hussain et al., 2024). The modest FRAP values relative to ascorbic acid may partly reflect competition between the ZnO surface and the Fe³⁺/Fe²⁺ detection chemistry, which is a recognised confound in metal oxide nanoparticle FRAP assays.

Nitric Oxide and Lipid Peroxidation Inhibition

The high IC₅₀ values for NO scavenging (734.04 µg/mL) and LPO inhibition (1437.10 µg/mL) indicate that AlZnONPs are least effective against reactive nitrogen species and lipid radical chain reactions. This selectivity pattern has been observed in other green-synthesised metal oxide nanoparticle studies (Rananaware et al., 2025). The weak LPO inhibition is mechanistically explained by the hydrophilic character of the nanoparticle surface, which limits penetration into the lipid phase of the egg yolk substrate where peroxidation chain reactions propagate (Repetto et al., 2012). Future studies employing liposome-based LPO models may better capture the membrane-protective potential of these nanoparticles.

Metal Chelation Activity

The selective copper chelation activity of AlZnONPs (IC₅₀ = 168.76 µg/mL) over iron chelation (IC₅₀ = 666.22 µg/mL) is consistent with the hard-soft acid-base (HSAB) theory, wherein Cu²⁺, a borderline Lewis acid forms more stable complexes with the oxygen and nitrogen-containing functional groups of surface polyphenols than does Fe²⁺ under ferrozine competition conditions (Masternak et al., 2021). Importantly, copper dyshomeostasis has been implicated in the amyloid aggregation processes central to Alzheimer's disease pathology (Cong et al., 2025), providing a direct mechanistic link between the copper chelation activity of AlZnONPs and the neuroprotective angle of this study. The moderate iron chelation may also reflect competitive interference from zinc ions released from the nanoparticle lattice, which can occupy ferrozine binding sites under assay conditions (Lakey-Beitia et al., 2021).

Cholinesterase Inhibitory Activity

The AChE inhibitory activity of AlZnONPs (IC₅₀ = 245.70 µg/mL) falls within the range reported for related green-synthesised nanoparticles, including ZnO nanoparticles from *Vitex negundo* (Aseen et al., 2025). The proposed mechanism involves non-competitive or mixed-mode inhibition, wherein surface-bound flavonoids interact with both the catalytic active site (CAS) and the peripheral anionic site (PAS) of AChE through hydrophobic contacts and hydrogen bonding. Quercetin and rutin, prominent constituents of *A. indica*, have independently been documented as AChE inhibitors (Islas et al., 2020), providing phytochemical basis for the observed activity.(Cichon et al., 2024)

The substantially weaker BChE inhibition ($IC_{50} = 1160.85 \mu\text{g/mL}$) indicates selective preference for AChE, a profile mirroring that of first-generation cholinesterase inhibitors such as donepezil (Asiri & Mostafa, 2023). This selectivity may reflect structural differences in enzyme active site geometry: the narrower active site gorge of AChE accommodates the surface phytochemical ligands of AIZnONPs more favourably than the wider, more permissive active site of BChE (Islas et al., 2020). Although the absolute IC_{50} values are considerably higher than the clinical standards, the primary value of these nanoparticles may lie in sustained, surface-mediated release of bioactive phytochemicals, and their activity profile warrants further investigation through kinetic inhibition studies and molecular docking (Yu et al., 2026).

Implications and Limitations

The bioactivity profile reported here is best understood as a composite expression of the ZnO nanoparticle core and the *A. indica* phytochemical surface coat acting in concert. This synergistic relationship between inorganic nanoscale architecture and organic phytochemical capping is a defining feature of green-synthesised nanomaterials (Kamarajan et al., 2022). Several limitations should be acknowledged: all assays were conducted *in vitro*; IC_{50} extrapolation carries some uncertainty for LPO and BChE ($R^2 = 0.883$ and 0.887 , respectively); and the specific phytochemical species contributing to observed activities were not isolated, representing a productive direction for future mechanistic investigation.

CONCLUSION

The present study demonstrates that zinc oxide nanoparticles biosynthesised using *Azadirachta indica* leaf extract (AIZnONPs) possess a broad but moderate *in vitro* bioactivity profile encompassing antioxidant, metal chelation, and cholinesterase inhibitory activities. Across all nine assays evaluated, AIZnONPs exhibited consistent, statistically significant concentration-dependent responses ($p < 0.0001$), with the strongest activities recorded for copper chelation ($IC_{50} = 168.76 \mu\text{g/mL}$), ABTS radical scavenging ($IC_{50} = 210.21 \mu\text{g/mL}$), AChE inhibition ($IC_{50} = 245.70 \mu\text{g/mL}$), and DPPH radical scavenging ($IC_{50} = 290.96 \mu\text{g/mL}$). The selective inhibition of AChE over BChE, combined with the preferential chelation of copper ions — implicated in amyloid aggregation and oxidative neurodegeneration — collectively underscore the neuroprotective potential of these nanoparticles and provide a biochemical rationale for further investigation in the context of Alzheimer's disease. Although the bioactivity of AIZnONPs was consistently lower than that of the respective reference standards, this is consistent with the surface-mediated, multi-target mode of action characteristic of green-synthesised nanomaterials, wherein *A. indica*-derived phytochemicals — flavonoids, phenolic acids, and terpenoids — act cooperatively with the ZnO nanoparticle core. These findings, taken together with the physicochemical characterisation reported in our previous publication, provide a comprehensive foundation for understanding the functional properties of AIZnONPs. Further investigation through enzyme kinetics studies, molecular docking simulations, cell-based oxidative stress models, and *in vivo* evaluation is warranted to fully establish the therapeutic relevance of these green-derived nanomaterials.

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