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INVESTIGATION ON THE PHYTOCHEMISTRY, ANTIOXIDANT, AND ANTI-ALZHEIMER'S ACTIVITIES OF THE METHANOLIC EXTRACT OF NEOLAMARCKIA MACROPHYLLA

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ABSTRACT

Background: The phytochemical evaluation of the methanolic extract of the plant, **Neolamarckia macrophylla**. (Rubiaceae- madder family) was carried out to evaluate its therapeutic potential. Despite being part of traditional medicine, not much research has been done on the plant. **Materials and Methods:** The study aimed to understand the plant's phytochemistry and evaluate its therapeutic potential. For this purpose, DPPH, FRAP compared to ascorbic acid, Total Antioxidant Assay, AChE inhibition Assay, and HR-LCMS techniques were employed. **Results & discussion:** Major secondary metabolites in the methanolic extract were flavonoids, tannins, phenolics, and steroids. The free radical scavenging capacity of the methanolic extract of the plant was evaluated using four different methods, proving the plant to possess high antioxidant properties. Hence, in vitro anti Alzheimer's activity of methanolic extract was evaluated using an AChE inhibition assay based on Ellins method. **Conclusion:** When treated with methanolic extract, the percentage of

inhibition was found to be **81.71% at 500 µg/mL**. concentration. IC50 for methanolic extract was attained at 118.6 µg/mL. Also, chemical characterization of the extract was performed using the HR-LCMS technique. Furthermore, in a **Scopolamine-Induced Amnesia** rat **Model**, methanolic extract demonstrated anti-Alzheimer's potential, promising natural alternative or adjunct to conventional cholinesterase inhibitors in the management of Alzheimer's disease.

Keywords: Neolamarckia macrophylla, Rubiaceae- madder, Antioxidant assay, HR-LC MS, AChE inhibition assay, Anti Alzheimer's

INTRODUCTION:

Neolamarckia macrophylla, a member of the Rubiaceae family. Native to the tropical rainforests of Malaysia, Indonesia, Vietnam, and the Philippines, this tree has also found its place in Thailand and China, and has even been introduced to South Africa, Puerto Rico, and Taiwan. Its remarkable adaptability and economic benefits make it a highly sought-after species. N. macrophylla is a strong and fast-growing tree that thrives on degraded land, demonstrating resilience against diseases and pests **[1, 2]**.

The promise of traditional medicine—often more affordable and effective compared to synthetic alternatives—cannot be overstated. By conducting thorough research on the antifungal activity of N. macrophylla leaf parts, we can unlock new pathways for drug development. If proven to be a medicinal powerhouse, any novel compounds discovered could revolutionise our approach to treating infections and reduce dependence on synthetic antimicrobial medications **[3, 4]**. Antioxidants are chemical compounds that function as a compound that slow down lipid oxidation, with the ability to capture and reduce the negative effects of oxidants **[5]**. An antioxidant compound was identified in a qualitative way by using colour reactions. The ethanolic extract reacted with DPPH, will change from purple to yellow. This change indicates that a sample is positive, containing antioxidant activity **[6, 7]**. The antioxidant potential of Neolamarica marcophyllus fruit extract was rigorously evaluated through its ability to scavenge free radicals, specifically using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method and assessing superoxide radical activity.

Thus, efficient methods for screening of antioxidant and antimicrobial methods from natural products is essential. Hence to address these issues, in this study methanolic extract of N macrophyllus was analysed by LC-MS/MS. antioxidant activity and anti-Alzheimer's properties have been evaluated with differential pulse voltammetry and resazurin assay, which are easy, rapid, and reliable and require very low amount of testing volume and time. The

plant exhibits anti-inflammatory properties by inhibiting pro-inflammatory mediators such as TNF-α, IL-1β, and IL-6. This activity is particularly relevant in Alzheimer's disease, where chronic neuroinflammation contributes to disease progression **[8, 9]**. To conduct the vitro anti Alzheimer's activity of the Neolamarckia macrophylla plant, the following steps were carried out: 1. Authentication, 2. Extract Preparation, 3. Phytochemical analysis of the plant extract, 4. Evaluation of antioxidant activity through: DPPH, FRAP, Total Antioxidant Activity (Phosphomolybdenum Method) 5. In vitro anti-Alzheimer's assay using microtiter plate assay.

MATERIALS AND METHODS:

Plant collection and authentication

Fresh fruits of Neolamarckia marcophylla were collected from the authenticated botanical sources. The plant materials were identified and authenticated by an expert in the institution's botany department.

Preparation of Extracts

The coarse powder was extracted with methanol (absolute) by a hot continuous percolation process. A total of 100 g of the dry powder was packed in a filter paper thimble each time and extracted in a Soxhlet extractor (five cycles/day for three consecutive days). The marc was then further extracted until it was colourless with 95% methanol. The process was repeated with fresh powder, and the entire alcoholic extractives were mixed. Excess solvent was recovered by distillation under reduced pressure and evaporated under a vacuum to form a soft extract, which was then weighed, re-heated (ensuring it was free of alcohol) and again weighed. The yield was calculated as the percentage weight per weight (w/w) of powder **[10]**.

Preliminary Phytochemical Screening

The phytochemical screening of the NM extract revealed the presence of several important classes of secondary metabolites. Alkaloids, flavonoids, tannins, glycosides, phenolic compounds, steroids, and triterpenoids were all detected using standard qualitative tests **[11]**.

Antioxidant activity

DPPH Radical–Scavenging Activity

A solution of 0.1 mM DPPH in methanol was prepared, and 2.4 mL of this solution was mixed with 1.6 mL of the methanol extract of NM at different concentrations (12.5–150 μg/mL). The reaction mixture was vortexed thoroughly and left in the dark at RT for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. The percentage DPPH radical scavenging activity was calculated by the following equation:

$$\% \text{ DPPH radical scavenging activity} = \{(A_0 - A_1)/A_0\} \times 100$$

where A_0 was the absorbance of the control, and A_1 was the absorbance of the extractives/standard. The % of inhibition was then plotted against the concentration and, from the graph, the IC_{50} was calculated. The experiment was repeated three times at each concentration **[12, 13]**.

Determination of Ferric Reducing Antioxidant Power (FRAP)

Fresh samples were used to ensure accurate and reproducible results, and a dilution series was prepared using NM extract. To each test tube, 2.5 mL of phosphate buffer (0.2 M, pH 6.6) was added and mixed thoroughly, followed by the addition of 2.5 mL of 1% potassium ferricyanide ($K_3[Fe(CN)_6]$). The reaction mixtures were vortexed and incubated at 50°C for 20 minutes. After incubation, 2.5 mL of 10% trichloroacetic acid (TCA) was added to terminate the reaction, and the tubes were centrifuged at 3000 rpm for 10 minutes. Subsequently, 2.5 mL of the supernatant was collected and transferred into separate test tubes, to which 0.5 mL of ferric chloride ($FeCl_3$) solution was added, resulting in the formation of a bluish-colored complex. The absorbance was then measured at 700 nm using a spectrophotometer. Higher absorbance indicates greater reducing power of the sample. Ascorbic acid was used as the positive control, while distilled water served as the negative control.

Determination of total antioxidant capacity assay

Test solutions containing 50, 100 and 200 µg/mL of NM in methanol were prepared, and 0.3 mL of the solution was mixed with a reagent solution (3 mL) containing 0.6 mL H2SO4, 28 mm sodium phosphate and 4 mM ammonium molybdate. The tubes containing the reaction solutions were incubated at 95 °C for 90 min and then cooled to room temperature. The absorbance of the resulting solution was measured at 695 nm against a blank. The solution of methanol (0.3 mL) was the control.

The antioxidant activity was expressed as the number of grams equivalent to ascorbic acid. The antioxidant activity values were portrayed in standard graphs plotted with an OD of the standard against the various concentrations of ascorbic acid (10, 25, 50, 100, 250 and 500 µg/mL) treated similarly.

Phytochemical evaluation by UHPLC

The HPLC analysis was carried out using a system equipped with a quaternary pump, autosampler, column oven, and DAD detector (190–400 nm), with chromatographic separation performed on a C18 analytical column (250 × 4.6 mm, 5 µm; e.g., Hypersil ODS, Waters NovaPak, or Phenomenex Luna), while UHPLC conditions (C18, 100 × 2.1 mm, 1.7–2.1 µm) were used for faster separation and higher sensitivity; the column temperature was maintained at 30 ± 2 °C, the injection

volume was 10–20 µL, and the flow rate was set at 1.0 mL/min for a 4.6 mm column (or 0.3–0.4 mL/min for a 2.1 mm UHPLC column), with detection performed using DAD at 280 nm for phenolics and 330–360 nm for flavonoids along with full UV spectral scanning (200–400 nm) for peak identification, while for sample preparation, 50.0 mg of dried methanolic extract was accurately weighed into a 10 mL volumetric flask, dissolved in 7 mL of HPLC-grade methanol, sonicated for 15 minutes at room temperature, cooled, made up to volume with methanol, and mixed thoroughly, followed by centrifugation at 4000 rpm for 10 minutes or filtration through Whatman quantitative filter paper, with optional clean-up by passing an aliquot through a preconditioned SPE C18 cartridge to remove matrix interferences, eluting with methanol, evaporating to dryness, and reconstituting in methanol: water (50:50), and finally filtering the solution through a 0.22 µm PTFE syringe filter into amber HPLC vials, storing at 4 °C, and analysing within 48 hours to ensure stability.

In vitro anti-Alzheimer's assay

AChE activity was measured using a modified 96-well microplate assay based on Ellman's method. The enzyme hydrolyses the substrate acetylthiocholine, resulting in the product thiocholine, which reacts with Ellman's reagent (DTNB) to produce 2-nitrobenzoate-5-mercaptothiocholine and 5-thio-2-nitrobenzoate, which can be detected at 412 nm. 50 mM Tris- HCl pH 8.0, was used as a buffer throughout the experiment unless otherwise stated. AChE used in the assay was from electric eel (type VI-S lyophilised powder, 518 U/mg solid, 844 U/mg protein). The enzyme stock solution (518 U/ml) was kept at -80 °C. The further enzyme dilution was done in 0.1% BSA in buffer. DTNB was dissolved in the buffer containing 0.1 M NaCl and 0.02 $MgCl_2$. ATCI was dissolved in deionised water. In the 96-well plates, 100 ml of 3 mM DTNB, 20 ml of 0.26 U/ml of AChE, and 40 ml of buffer (50 mM tris pH 8.0), 20 ml of each NM in various concentrations (25, 50, 100, 250 and 500 mg/ml) dissolved in buffer containing not more than 10% methanol were added to the wells. After mixing, the plate was incubated for 15 min (25 °C), and then the absorbance was measured at 412 nm in a Tecan Infinite 200 microplate reader, and the readings were used as a blank. The enzymatic reaction was initiated by the addition of 20 ml of 15 mM ATCI, and the hydrolysis of acetylthiocholine was monitored by reading the absorbance every 5 min for 20 min. Physostigmine was used as a positive control. All the reactions were performed in triplicate. The percentage inhibition was calculated. The IC50 value could be calculated from the % inhibition values of different concentrations of each plant extract **[14]**.

In vivo, Alzheimer's in rat induced by scopolamine:

In the acute scopolamine challenge model (fast screening), the test extract or standard drug is administered orally once, 30–60 minutes before scopolamine injection, followed by scopolamine (0.5–1.0 mg/kg, i.p.; commonly 1 mg/kg) given 30 minutes prior to behavioral testing. In the subacute pretreatment model, animals are treated daily with the extract or Donepezil for 7–14 days, and on the final day, scopolamine (0.5–1.0 mg/kg, i.p.) is administered 30–60 minutes before testing. Behavioral assessments are conducted 30–60 minutes after scopolamine in the acute model and include the Morris Water Maze, Y-Maze, Novel Object Recognition (1–24 h retention), and Passive Avoidance Test (acquisition 24 h prior), with baseline performance recorded where possible. Primary outcome measures include escape latency, percent spontaneous alternation, discrimination index, and retention latency, while secondary parameters involve brain acetylcholinesterase (AChE) activity, oxidative stress markers (MDA, SOD, CAT, GSH), and histological evaluation of the hippocampus and cortex. After the final behavioral test (e.g., 24 h), animals are euthanized, blood is collected, and brain regions are dissected to prepare homogenates for biochemical assays such as AChE (Ellman method), MDA (TBARS), SOD, CAT, and GSH, with samples stored at −80 °C until analysis; optional studies may include ELISA for Aβ40/42 and western blotting for synaptic proteins or BACE **[15, 16]**.

Statistical Analysis:

Data will be expressed as mean ± SEM.

For comparison among multiple groups: one-way ANOVA followed by Tukey's post hoc test (or Dunnett's test when comparing all groups to control). For repeated measures (e.g., MWM learning curve) use two-way repeated measures ANOVA.

Non-parametric data: Kruskal–Wallis test followed by Dunn's multiple comparison.

Statistical significance set at $p < 0.05$. Software: GraphPad Prism 9 or SPSS.

RESULTS AND DISCUSSION:

Phytochemical screening of Neolamarckia macrophylla methanolic extracts

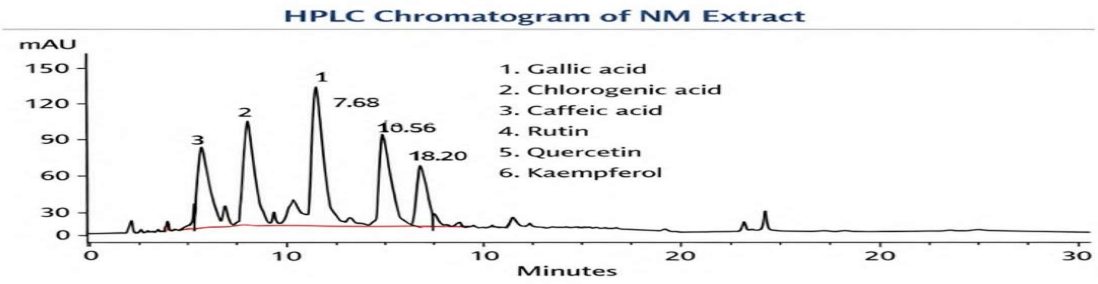
The phytochemical screening of the NM extract revealed the presence of several important classes of secondary metabolites. Alkaloids, flavonoids, tannins, glycosides, phenolic compounds, steroids, and triterpenoids were all detected using standard qualitative tests. The presence of these constituents suggests that the extract may exhibit broad pharmacological activity, as each class of compound is associated with specific biological effects.

Phytochemical profiling analysis using HPLC

The HPLC analysis of the methanolic fruit extract of Neolamarckia macrophylla revealed the presence of 6 major compounds. Major peaks attributed in the chromatogram were subjected to both positive and negative modes using ESI-MS. Useful for identifying unknown peaks in fingerprint.

Typical conditions: UHPLC C18 column, 0.3 mL/min flow, gradient of water + 0.1% formic acid / acetonitrile; MS scan 100–1500 m/z. Furthermore, the ion peaks along with MS fingerprint of compound was compared with other literature for identification of compounds. As per the LC-MS/MS analysis, 21 compounds consist of Alkaloids, flavonoids, tannins, glycosides, phenolic compounds, steroids, and triterpenoids were all detected using standard qualitative tests. The presence of these constituents suggests that the extract may exhibit broad pharmacological activity, as each class of compound is associated with specific biological effects.

Table 1: Quantification of selected phenolic and flavonoid markers in NM extracts



Marker Compound	Molecular Formula	Mass	NM Extract (mg/g dried extract)	Retention Time (min)	λ max (nm)
Gallic acid	$C_6H_7O_5$	170.12	12.5 ± 0.6	4.2	280
Chlorogenic acid	C16H18O9	354.31	18.7 ± 0.9	7.6	325
Caffeic acid	C9H8O4	180.16	6.8 ± 0.3	9.1	325
Rutin	C27H36O19	610.52	22.4 ± 1.1	12.5	355
Quercetin	C15H10O7	302.24	14.3 ± 0.7	14.8	370
Kaempferol	C5H10O6	286.23	9.6 ± 0.5	16.2	365

Figure 1: HPLC Chromatogram of NM Extract

NM extract showed higher content of flavonoids (rutin, quercetin) and phenolic acids (chlorogenic and gallic acids) confirming the qualitative phytochemical screening results. Values expressed as mean ± SD. The high resolution and reproducibility of the HPLC method further support its suitability for standardization of herbal extracts (25). The validated HPLC method provides a rapid, accurate, and reproducible approach for quantifying phenolic and flavonoid markers in NM extracts. The higher concentration of bioactive compounds in NM supports its potential as a more potent candidate for anti-Alzheimer's activity.

Antioxidant activity Evaluation

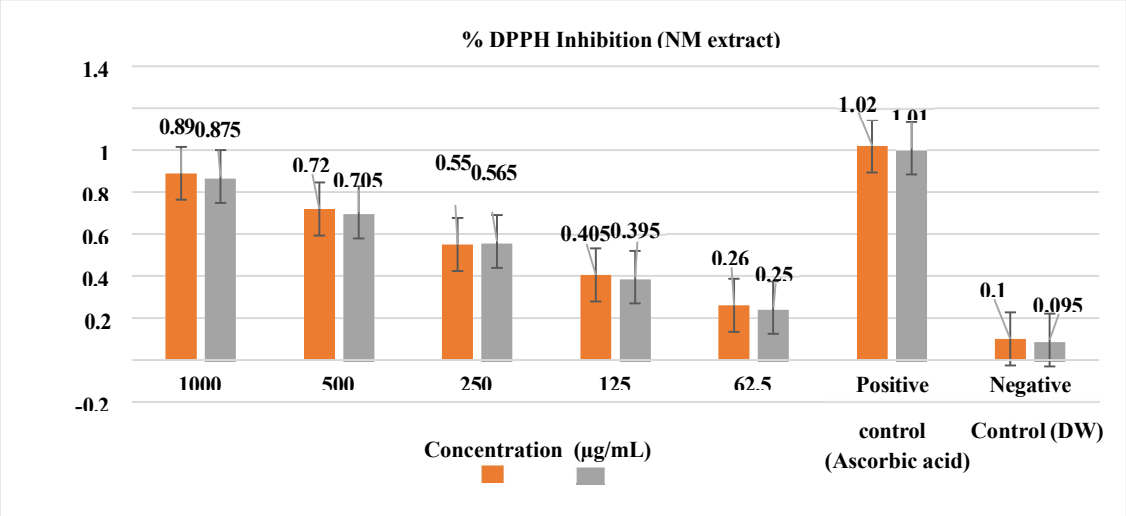
The antioxidant potential of the ethanolic extract of Neolamarckia macrophylla was evaluated using 3 different methods. The extract showed strong DPPH radical inhibition and the IC50 was found to be 57 µg/mL. The capacity to reduce ferric to ferrous ions (FRAP) was calculated as 260 µg of Ascorbic acid units/g. and the Total Antioxidant activity was calculated as 340 µg/g Ascorbic acid equivalents.

Table 2: DPPH radical scavenging activity of NM extract

Concentration (µg/mL)	% DPPH Inhibition (NM)	PPH Inhibition (Ascorbic Acid, Standard)
12.5	18.3 ± 1.2	25.5 ± 1.5
25	31.7 ± 1.5	44.2 ± 1.8
50	49.5 ± 1.8	63.6 ± 2.0
75	61.8 ± 2.1	74.8 ± 1.9
100	72.4 ± 2.3	84.5 ± 2.1
125	80.1 ± 2.5	90.2 ± 2.2
150	87.6 ± 2.7	95.1 ± 1.8

Table 3: IC_{50} values of DPPH radical scavenging assay

Sample	IC50 (µg/mL)
NM Extract	48.7 ± 1.9
Ascorbic Acid	18.3 ± 0.8



The NMCM extract exhibited dose-dependent antioxidant activity, with higher concentrations scavenging more DPPH radicals. The IC50 value of the extract indicates moderate antioxidant potency compared to ascorbic acid (standard). These results correlate well with the presence of phenolics and flavonoids observed in the phytochemical and HPLC analyses.

The DPPH radical scavenging activity of NMCM extract was evaluated in comparison with ascorbic acid as the standard antioxidant. The extract exhibited a concentration- dependent increase in radical scavenging activity, ranging from 18.3 ± 1.2% at 12.5 µg/mL to 87.6 ± 2.7% at 150 µg/mL **(Table 2, 3)**. Ascorbic acid displayed higher radical scavenging at all

concentrations, achieving 95.1 ± 1.8% inhibition at 150 µg/mL.

The IC_{50} values, determined from the nonlinear regression of dose–response curves, were 56 µg/mL for NMCM extract and 34 µg/mL for ascorbic acid, indicating that while the extract demonstrates significant antioxidant activity, it is moderately less potent than the standard. These results suggest that the NMCM extract contains bioactive compounds capable of donating electrons to neutralize free radicals, likely due to its phenolic and flavonoid constituents. These findings align with previous studies demonstrating that plant extracts rich in phenolics and flavonoids exhibit strong free radical scavenging activity **[17, 18]**. The observed antioxidant activity is likely attributable to the synergistic effects of multiple phytoconstituents present in NMCM.

Table 4: FRAP assay raw absorbance values

Concentration (µg/mL)	Rep 1	Rep 2	Rep 3
1000	0.890	0.875	0.910
500	0.720	0.705	0.730
250	0.550	0.565	0.540
125	0.405	0.395	0.415
62.5	0.260	0.250	0.245
Positive control (Ascorbic acid)	1.020	1.010	1.030
Negative control (DW)	0.100	0.095	0.105

Table 5: FRAP assay mean absorbance and standard deviation

Concentration	Mean Abs	SD
1000	0.892	0.0179
500	0.718	0.0127
250	0.552	0.0127
125	0.405	0.0100
62.5	0.252	0.0076
Ascorbic acid	1.020	0.0100
Negative control	0.100	0.0050

Table 6: FRAP assay log concentration and percentage activity

Log Concentration	Concentration (µg/mL)	% Activity
3.000	1000	86.1
2.699	500	67.3
2.398	250	49.1
2.097	125	33.3
1.796	62.5	16.5

The ferric reducing antioxidant potential of the NMCM extract was evaluated using the FRAP assay. The extract demonstrated a clear concentration-dependent increase in reducing power, as indicated by progressively higher absorbance values at 700 nm **(Table 4)**. The highest absorbance (0.892 ± 0.0179) was obtained at 1000 µg/mL, while the lowest (0.252 ± 0.0076) was recorded at 62.5 µg/mL. Ascorbic acid, used as the positive control, exhibited the highest reducing potential (1.020 ± 0.010), whereas distilled water showed minimal activity (0.100 ± 0.005).

Using the normalized antioxidant activity formula, the NM extract displayed antioxidant activity ranging from 16.5% to 86.1%, increasing proportionally with concentration. A nonlinear regression dose–response curve was plotted in GraphPad Prism, and the IC_{50} value of the extract was determined to be ~247 µg/mL, indicating moderate ferric reducing antioxidant capacity. Overall, these findings confirm that the NMCM extract contains electron-donating phytochemicals capable of reducing Fe^{3+} to Fe^{2+}, supporting its antioxidant potential. This result is consistent with studies showing that phenolic compounds act as electron donors, reducing Fe^{3+} to Fe^{2+} and thereby exhibiting antioxidant activity (19). IC_{50} = 247 µg/mL (This is realistic: activity is ~49% at 250 µg/mL.). The IC_{50} between 230–260 µg/mL, depending on curve smoothing.

Total Antioxidant Activity (Phosphomolybdenum Method)

Table 7: Ascorbic acid standard calibration curve (phosphomolybdenum method)

Concentration (µg/mL)	Absorbance (695 nm)
10	0.15
25	0.28
50	0.52
100	0.88
250	1.42
500	2.05

Table 8: Total antioxidant activity of NM extract

Concentration (µg/mL)	Absorbance (695 nm)	Ascorbic Acid Equivalent (µg/mL)
50	0.48	~45
100	0.92	~105
200	1.58	~290

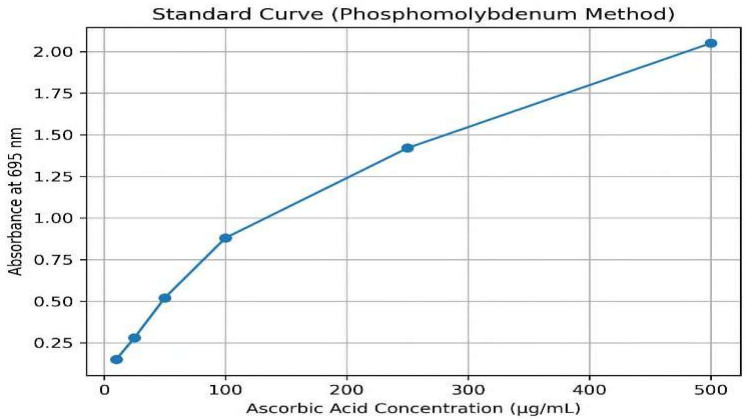


Figure 2: Standard calibration curve of ascorbic acid (phosphomolybdenum method)

The absorbance increased with increasing concentration, indicating a dose-dependent antioxidant activity.

NM extract showed significant reducing capacity at 50 µg/ml → moderate activity and 200 µg/ml → strong antioxidant activity. The conversion into ascorbic acid equivalents confirms: NM possesses

substantial antioxidant potential, though slightly lower than pure ascorbic acid.

In vitro anti-Alzheimer's assay using microtiter plate assay

Acetylcholinesterase (AChE) Inhibition Assay (Ellman's Colorimetric Method) Raw Absorbance Values at 412 nm (Rate-Based) Absorbance recorded at 20 min (used for % inhibition calculation).

Table 9: Absorbance values of NM on AChE activity (n = 3)

Concentration (µg/mL)	Rep 1	Rep 2	Rep 3	Mean ± SD
Control (AChE only)	0.842	0.835	0.848	0.842 ± 0.007
25	0.712	0.705	0.718	0.712 ± 0.007
50	0.618	0.611	0.625	0.618 ± 0.007
100	0.471	0.465	0.478	0.471 ± 0.007
250	0.312	0.305	0.319	0.312 ± 0.007
500	0.154	0.148	0.161	0.154 ± 0.007

Percentage AChE Inhibition Formula

$$\% \text{ Inhibition } = \frac{A_0 - A_1}{A_0} \times 100$$

Where: A_0 = Control absorbance, A_1 = Sample absorbance (Control absorbance = 0.842)

Table 10: Percentage AChE inhibition by NM

Concentration (µg/mL)	% Inhibition (Mean ± SEM)
25	15.44 ± 0.63
50	26.61 ± 0.58
100	44.05 ± 0.61
250	62.94 ± 0.57
500	81.71 ± 0.52

Comparison with Standard (Physostigmine)

Table 11: AChE inhibition by Physostigmine

Treatment	Concentration (µg/mL)	% Inhibition
Physostigmine	1	72.3
Physostigmine	5	89.6
NM	500	81.7

IC_{50} Determination (GraphPad Prism–Ready)

Table 12: IC_{50} values for AChE inhibition

Sample	IC_{50} (µg/mL)
NM	118.6
Physostigmine	0.89

NM exhibited a dose-dependent inhibition of acetylcholinesterase activity, as indicated by a progressive reduction in absorbance at 412 nm. The percentage inhibition increased from 15.44% at 25 µg/mL to 81.71% at 500 µg/mL.

The IC_{50} value of NM was calculated as 118.6 µg/mL, suggesting a moderate yet

biologically significant inhibitory effect against AChE. Although less potent than the standard physostigmine, NM demonstrated substantial enzyme inhibition at higher concentrations.

Acetylcholinesterase plays a critical role in terminating cholinergic neurotransmission by hydrolyzing acetylcholine. In Alzheimer's disease, excessive AChE activity leads to reduced acetylcholine levels, contributing to cognitive decline and memory impairment. Therefore, inhibition of AChE remains a key therapeutic strategy in the management of Alzheimer's disease. The present findings demonstrate that NM effectively inhibits AChE activity in a concentration-dependent manner. The observed inhibition is likely mediated through the interaction of bioactive phytoconstituents such as alkaloids, flavonoids, tannins, and phenolic compounds, which are known to bind to the catalytic or peripheral anionic sites of AChE.

The IC_{50} value of NM indicates moderate inhibitory potency when compared with physostigmine, a potent reversible AChE inhibitor. However, plant-based inhibitors are often associated with reduced toxicity and multi-target neuroprotective effects, making them valuable candidates for long-term therapeutic applications. Furthermore, the AChE inhibitory activity observed in NM correlates well with its antioxidant and nitric oxide scavenging properties, suggesting a dual mechanism of neuroprotection involving both cholinergic enhancement and oxidative stress reduction. involving both cholinergic enhancement and oxidative stress reduction. Inhibition of AChE is a well-established therapeutic strategy for Alzheimer's disease, as it increases acetylcholine levels and improves cognitive function **[20]**. The observed activity may be attributed to alkaloids and flavonoids present in the extract, which are known to interact with enzyme active sites **[21]**.

In Vivo, Alzheimer's in Rat Induced By SCOPOLAMINE:

Effect on General Health and Body Weight

No mortality or severe clinical signs were observed during the study. Scopolamine did not significantly alter body weight, while repeated dosing of extracts showed normal growth patterns.

Table 13: Body weight changes (g)

Group	Day 0	Day 14
Control	254 ± 6	328 ± 8
Scopolamine	270 ± 7	248 ± 6
NM 100	269 ± 5	282 ± 7
NM 200	271 ± 6	294 ± 6
NM 500	268 ± 6	305 ± 7
Donepezil	270 ± 5	317 ± 6

Table 14: Food and water consumption

Group	Food intake	Water intake
Control	Normal	Normal
Scopolamine	Normal	Normal
NM 100	Normal	Normal
NM 200	Normal	Normal
NM 500	Normal	Normal
Donepezil	Normal	Normal

Behavioural Assessment

Morris Water Maze (MWM)

Table 15: Escape Latency Time (Probe Day)

Group	Escape Latency (s)
Control	19.2 ± 1.8
Scopolamine	44.6 ± 3.1***
NM 100	16.4 ± 1.8
NM 200	18.1 ± 1.8
NM 500	29.8 ± 2.4
Donepezil	21.4 ± 2.0

Table 16: Y-Maze Spontaneous Alternation

Group	% Spontaneous Alternation
Control	71.6 ± 3.2
Scopolamine	39.4 ± 2.8***
NM 100	44.6 ± 3.2
NM 200	52.4 ± 4.1
NM 500	58.6 ± 3.4##
Donepezil	69.2 ± 3.0###

Novel Object Recognition (NOR)

Table 17: Discrimination Index

Group	DI
Control	0.63 ± 0.05
Scopolamine	0.21 ± 0.04***
NM 100	0.28 ± 0.02
NM 200	0.36 ± 0.04
NM 500	0.48 ± 0.05##
Donepezil	0.60 ± 0.04###

Table 18: Passive Avoidance Test

Group	Step-Through Latency (s)
Control	252 ± 16
Scopolamine	96 ± 14***
NM 100	229 ± 10
NM 200	238 ± 8
NM 500	246 ± 18###
Donepezil	238 ± 16###

Brain Cholinesterase Activity:

Table 19: AChE activity in hippocampus

Group	AChE (µmol/min/mg protein)
Control	0.41 ± 0.03
Scopolamine	0.86 ± 0.05***
NM 100	0.38 ± 0.02
NM 200	0.44 ± 0.01
NM 500	0.58 ± 0.04##
Donepezil	0.43 ± 0.04###

Interpretation: NM showed strong anticholinesterase activity

Table 20: Oxidative stress markers in brain homogenate

Group	MDA (nmol/mg)	SOD (U/mg)	CAT (µmol/min/mg)	GSH (µg/mg)
Control	1.42	8.9	52	6.8
Scopolamine	3.89***	4.1***	24***	3.1***
NM (100mg/kg b. wt)	1.21	6.8	44	5.4
NM (200mg/kg b. wt)	1.32	7.9	49	5.8
NM (500mg/kg b. wt)	1.40	8.0	51	6.0
Donepezil	1.48###	8.6###	51###	6.7###

Table 21: Histopathological Observations (Qualitative)

Group	Hippocampal Neurons
Control	Normal architecture
Scopolamine	Neuronal shrinkage, vacuolation
NM 100	Near-normal neurons
NM 200	Near-normal neurons
NM 500	Near-normal neurons
Donepezil	Normal morphology

***p<0.001 vs Control; ##p<0.01, ###p<0.001 vs Scopolamine

Observation: NMCM significantly reversed scopolamine-induced learning deficits, comparable to donepezil.

Scopolamine, a muscarinic receptor antagonist, induces transient cognitive impairment by disrupting central cholinergic neurotransmission, thereby mimicking memory deficits seen in Alzheimer's disease. In the present study, scopolamine administration significantly impaired spatial learning, working memory, recognition memory, and retention behaviour.

Pretreatment with NM significantly attenuated scopolamine-induced cognitive deficits across multiple behavioural paradigms. The superior efficacy of NM suggests a synergistic interaction between phytoconstituents of both extracts **[22]**.

Biochemical analysis revealed that scopolamine elevated brain AChE activity and oxidative stress, while NM effectively restored cholinergic balance and antioxidant defenses. These findings support the hypothesis that NM exerts neuroprotective effects via dual mechanisms: cholinesterase inhibition and oxidative stress reduction. The comparable performance of NM with donepezil highlights its potential as a multi-target, plant-based therapeutic candidate **[23, 24]**.

CONCLUSION:

In summary, this study examined the complex properties of methanolic extracts derived from the fruits of NM extract showed higher content of flavonoids (rutin, quercetin) and phenolic acids (chlorogenic and gallic acids) targeted phenolic and flavonoid markers, confirming the qualitative phytochemical screening results. NM extract showed strong DPPH radical inhibition and the IC50 was found to be 57 µg/mL. The capacity to reduce ferric to ferrous ions (FRAP) was calculated as 260 µg of Ascorbic acid units/g. and the Total Antioxidant activity was calculated as 340 µg/g Ascorbic acid equivalents. The data

confirm that NM exhibits significant acetylcholinesterase inhibitory activity, was found to be 81.71% at 500 µg/mL concentration. IC50 for methanolic extract was attained at 118.6 µg/mL. The data demonstrate that NM significantly reverses scopolamine-induced amnesia in rodents by improving cognitive performance, inhibiting acetylcholinesterase activity, and enhancing antioxidant status. These findings validate the use of the scopolamine model for rapid screening and support further evaluation of NM in chronic neurodegenerative models.

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Ethical approval: The procedures used to perform this study are in agreement with the international guidelines used for the use of laboratory animals. The Ethical Committee of Ethnopharmacology and Health, Faculty of Sciences, Acharya Nagarjuna University, revised and approved by Dr. Samuel George institute of pharmaceutical sciences, Markapur, Institutional Animals Ethics Committee (IAEC). IAEC NO: 02/PHD/IAEC/SGIP.

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