

Phytochemical Profiling and Anti-Alzheimer's Activity of Methanolic Extract from Carissa Macrocarpa Fruit

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Abstract—The present study investigates the phytochemical constituents and biological potential of methanolic fruit extracts of *Carissa macrocarpa*, with a focus on their antioxidant and anti-Alzheimer's activities. Preliminary phytochemical screening revealed the presence of alkaloids, flavonoids, phenolics, tannins, glycosides, steroids, and terpenoids in both extracts, indicating a rich metabolic profile. Antioxidant capacity was assessed using H₂O₂ peroxide assay and both extracts demonstrated dose-dependent free radical scavenging activity, with *C. macrocarpa* showing comparatively higher potency. Anti-Alzheimer's activity was evaluated through in-vitro butyrylcholinesterase (BChE) inhibitory assays based on Ellman's method. The methanolic extract of *Carissa macrocarpa* exhibited notable inhibition of both enzymes, suggesting potential cognitive-protective properties. Hence, in vitro anti Alzheimer's activity of methanolic extract was evaluated using an BChE inhibition assay based on Ellins method. When treated with methanolic extract, the percentage of inhibition was found to be 11.33% at 25 µg/mL to 76.04% 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 AICl₃-Induced Alzheimer's rat Model, methanolic extract highlight the therapeutic promise of the studied fruits as natural sources of antioxidant and cholinesterase-inhibiting compounds, supporting their potential application in managing oxidative stress and neurodegenerative disorders such as Alzheimer's disease.

Index Terms—*Carissa macrocarpa*, Apocynaceae, Antioxidant assay, BChE inhibition assay, Anti Alzheimer's.

I. INTRODUCTION

Carissa macrocarpa is native to coastal regions of South Africa, particularly along sand dunes and forest margins. Its geographical distribution extends from the Eastern Cape through KwaZulu-Natal to Mozambique. Due to its adaptability, it has been introduced and cultivated in various tropical and subtropical regions, including the United States, Southeast Asia, and Central America [1, 2].

Carissa is a significant genus within the Apocynaceae family, which belongs to the order Gentianales. This genus comprises evergreen shrubs or small trees that are native to the subtropical and tropical regions of Asia, Africa, and Oceania.[3] The genus contains approximately 85 species; however, only 8 of these have accepted names. The remaining species are either considered synonyms of these 8 or have been assigned to different genera.[4]

The fruits of this genus are high in dietary fibre, lipids, protein, carbohydrates, and macro- and micro-elements, thereby playing a crucial role in promoting human health.[5]

A fully mature fruit is distinguished by its glossy skin, shimmering in bright crimson accented with deeper red streaks—its delicate surface bruises easily. Inside, the flesh reveals a vibrant, deep red or crimson hue, delicately mottled with white, encapsulating approximately twelve small, flat brown seeds. These seeds can vary, with some appearing nearly round, while others are elongated and pointed at both ends.[6]

Notably, hydroethanolic extracts of *C. macrocarpa* leaves have demonstrated significant antioxidant activity, with low EC₅₀ values, indicating high radical scavenging efficiency [7].

Methanolic extracts are often preferred for antioxidant studies due to their higher yield of phenolic compounds and superior radical scavenging activity compared to aqueous extracts [8].

The plant exhibits anti-inflammatory properties by inhibiting pro-inflammatory mediators such as TNF- α , IL-1 β , and IL-6. This activity is particularly relevant in Alzheimer's disease, where chronic neuroinflammation contributes to disease progression [9].

The multifactorial nature of Alzheimer's disease necessitates therapeutic approaches that target multiple pathways simultaneously. Medicinal plants, with their diverse phytochemical composition, provide a promising platform for the development of multitarget drugs [10].

Several phytochemicals have been shown to inhibit the formation and aggregation of amyloid β peptides. These compounds can interfere with β -secretase activity, reduce A β production, and promote its clearance from the brain [11].

Polyphenols such as curcumin and resveratrol have demonstrated the ability to destabilize preformed amyloid fibrils and prevent plaque formation [12].

Certain plant-derived compounds inhibit tau hyperphosphorylation by modulating kinase and phosphatase activities. This prevents the formation of neurofibrillary tangles and maintains microtubule stability [13].

Antioxidants play a crucial role in preventing oxidative damage associated with neurodegeneration. Reactive oxygen species (ROS) contribute to lipid peroxidation, DNA damage, and protein oxidation, ultimately leading to neuronal death [14].

Natural antioxidants derived from plants offer a safer and more effective alternative to synthetic antioxidants, as they exhibit lower toxicity and broader pharmacological activities [15]

II. MATERIALS AND METHODS

1. Plant collection and authentication

Fresh fruits of *Carissa macrocarpa* were collected from the authenticated botanical sources. The plant

materials were identified and authenticated by an expert in the institution's botany department.

2. Preparation of Extracts

The fruit of *carissa macrocarpa* was cut into pieces with a stainless-steel knife and weighed approximately 100 g (including the seeds). These were dried in an oven at 60 °C. The dried fruit was then comminuted to a coarse powder and then passed through a No.10 sieve. 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.

Product yield = (the total weight of extract obtained ÷ the total weight of the dry material used for extraction) × 100.

III. ANTIOXIDANT ACTIVITY

3.1 H₂O₂ (Hydrogen Peroxide) Free Radical Scavenging Activity Assay

The dilution series was made using plant extract NMCM. Then, 600 μ L of H₂O₂ was added to the tubes (Control, sample, standard). 100 μ L of plant extract or standard (Ascorbic acid) was added to the respective test tubes. After that, the volume was made up to 4 mL with phosphate buffer (pH 7.4) in all test tubes. The identical Ascorbic acid series without sample served as the standard (phosphate buffer, H₂O₂, and Ascorbic acid). All the test tubes were incubated for 10 min at room temperature. The absorbance of the H₂O₂ solution was measured at 300 nm against the blank. H₂O₂ scavenging activity percentage and IC₅₀ were calculated compared to the standard by the formula. Before applying the formula, the absorbance of H₂O₂ alone must be subtracted from the control, sample, and standard. [16]

3.2 ABTS Radical Scavenging Activity

ABTS radical scavenging actions of plant extract are estimated according to the preferred methods 64-66. A reaction combination is prepared to the inclusion of the same volume of 7mM ABTS as well as 2.45mM potassium persulfate. A combination is placed at room temperature for 12h in the dark, which eliminates ABTS radicals. To produce a green-colored solution is diluted to the inclusion of 3ml of ABTS solution to 150ml of methanol by resulting in an absorbance of 0.700 ± 0.005 at 734nm. The necessary absorbance is acquired, 1ml of the obtained solution is mixed with 1 mL CM in different concentrations (0.005- 0.08 mg/ml). Afterwards, nearly 7min later, reduced absorbance is determined at 734nm.[17] The percentage inhibition of the ABTS radical by extracts or standards are measured with the use of the formula: $ABTS (\%) = [(A_0 - A_1) / A_0] \times 100$

In which A_0 is the absorbance of the control, and A_1 is the absorbance of the treated sample

IV. IN VITRO ANTI-ALZHEIMER'S ASSAY

4.1 β -Amyloid Aggregation Inhibition

Peptide aggregation was measured by the thioflavin T (ThT) fluorescence assay, in which the fluorescence intensity reflects the degree of aggregation. The CM extract to be tested was dissolved in dimethyl sulfoxide (Me₂SO) at various concentrations. The solubility of carotene in Me₂SO was very low, so suspensions of carotene were used. The A1±40 peptide was solubilised in double-deionised water at a concentration of 40 M and dispensed into Eppendorf tubes (50 l/tube). One l of each test agent solution was added to the tubes in triplicate, and then the peptide solutions were mixed with an equal volume of 2 phosphate-buffered saline solution. Control peptide solutions containing 1% Me₂SO without any test agent were also prepared. The solutions were incubated at 37°C for 7 days, and then the ThT fluorescence assay was performed using a FP-770 spectrofluorometer. Fibril formation by A1±40 peptide was also examined by electron microscopy using an H-7100 electron microscope. No bacterial contamination was observed in the peptide solutions even after a 7-day incubation. [18,19]

4.2 Butyrylcholinesterase (BChE) Inhibition Assay:

The BChE inhibitory activity of the extracts was determined by the spectrophotometric method. Human serum BChE was used as the enzyme source, while butyryl thiocholine chloride was employed as the substrate for the reaction. The NMCM extract was initially dissolved in 10% dimethyl sulfoxide (DMSO) and diluted to various concentrations in sodium phosphate buffer (100 mM, pH 8.0) immediately before use. 5,5'-Dithio-bis(2-nitrobenzoic) acid was used for the measurement of the cholinesterase activity. The hydrolysis of butyryl thiocholine chloride was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines. Absorbance was measured at 410 nm immediately after adding the enzyme source to the reaction mixture using a spectrophotometer. Readings were done at 0 min, 2 min, 10 min and 15 min at room temperature and at 37 °C. The percentage of inhibition of BChE was determined by comparison of the reaction rates of the samples relative to a blank sample (DMSO in phosphate buffer pH = 8) using the formula $(E-S)/E \times 100$, where E is the activity of the enzyme without the test sample, and S is the activity of the enzyme with the test sample. [20, 21]

V. IN VIVO, ALZHEIMER'S IN RAT INDUCED BY ALCL3

AlCl₃ was dissolved in water for oral delivery, 100 mg/kg of AlCl₃, and rats received each day orally for eight weeks a dose of 0.5 mL/100g b. wt to G2, G3, G4 and G5 group rats.[22]

Six groups of six rats each were formed from a random division of 40 rats.

Group 1: Rats were considered the control group, with healthy rats.

Group 2: Rats were used as AD models, received AlCl₃ orally.

Group 6: For 6 weeks (1/10 LD50), the AD rats were given CM

(100 mg/ kg. b.wt.), Orally daily.

Group 7: For 6 weeks (1/10 LD50), the AD rats were given CM

(200 mg/ kg. b.wt.), Orally daily.

Group 8: For 6 weeks (1/10 LD50), the AD rats were given CM

(500 mg/ kg. b.wt.), Orally daily

Group 12: The AD rats were given the conventional medication donepezil (10 mg/kg b.wt., daily for 6 weeks).

Experimental design (timeline)

- ✓ Day 7 to 0: Acclimatization and baseline behavioural testing.
- ✓ Day 0–42: Disease induction with $AlCl_3$ (Group 2 and disease groups).
- ✓ Day 14–42: Treatment with extracts/standard or Day 0–42 for preventive protocol.
- ✓ Behavioural tests will be performed during week 5 (Day 35–41):
 - Morris Water Maze (MWM): Day 35–39 (training + probe on Day 39)
 - Y-Maze: Day 40
 - Novel Object Recognition (NOR): Day 41
 - Passive avoidance: Day 36–37 (training and retention)
- ✓ Day 43: Final blood collection, euthanasia, brain dissection for biochemical, histological and molecular assays.

Behavioural assessment

Evaluation of Motor Coordination and Cognitive Capabilities. When the therapy period was over, rats' cognitive function and spatial memory damage were assessed after two months of chronic $AlCl_3$ exposure. The beam balancing test was used to evaluate motor skills.

Elevated plus maze test (EPM)

The EPM test was carried out to determine the anxiety-like behaviour of CM extracts. Briefly, each rat was placed in a central position facing the open arm and the movement in the maze model was recorded for 5 minutes with the help of a fixed camera. The time spent in close and open spaces was calculated by computer software ANY-maze. A pass was counted when 85% of the rat's body was on a particular arm. In the end, 70% ethanol and white cloth were used to clean the maze to get it ready for the next rat. After completing the experiment, rats were returned to their cages, and no other experiment was performed on the same day.

Morris water maze test (MWM)

The MWM test was performed to evaluate the learning and memory behaviour of CM extracts. The test

consisted of one visible and one hidden day test and a single probe trial at the end of the hidden tests. The platform was 1cm above the water surface during visible tests, and rats were placed in all quadrants until they reached the platform. In hidden tests, the platform was placed underwater with added dye to make it invisible. The platform was placed randomly in different quadrants, and rats were released to search for and reach the platform. The time consumed in a particular quadrant was recorded for 60 s with the help of a camera. Three trials were performed for each visible and hidden trial, with an interval of 1h, and videos were analysed using ANY maze software.

Radial Arm Maze Task

To analyse the working memory and reference memory of rats, Radial arm maze was used. The radial arm maze has eight arms of 4 feet long, connected to a circular central platform. This maze was elevated to 90cm from floor. In case of trail period each arm was allotted for food reward and the pattern of baited and un-baited arms stayed same throughout test sessions. At the edge of each arm a food container was kept with food pellets as a reward for the rat and at the end of the arm a protection the goal box. Rails (2.5 cm high) were made for preventing the animal from falling. Animals were trained to become accustomed to the RAM before the measurement of the behaviour was conducted. The animals were kept in fasting for one night to boost their appetite. For three days, the animals were maintained in the maze for fifteen minutes daily. The animal had eight days of habituation training before entering all eight arms or a ten-minute period. The animal was permitted to stay in the goal box for a minute. Errors of reference memory were denoted as entry numbers of into an un-baited arm. When the rat just pushed its head through the wrong opening and it remained there for longer than a minute, it was promptly replaced at the maze's centre. Each arm was washed with 70% ethanol to remove any prior animal traces (faces and urine). The latency to find the goal box was used to gauge overall behavioural performance during the retention and acquisition phases. The recorded metrics are detailed below. Errors correct working memory is denoted number of baited arm re-entries and Errors of incorrect working memory are denoted as number un-baited arm re-entries.

Step-through Passive Avoidance Test

Passive avoidance test was used to gauge retention levels after the radial arm maze task was completed for two to three days. The apparatus's design resembled a chamber with a guillotine door and lighting. A stimulator delivered an electric shock to the grid's floor. The animal was placed inside the room by opening the door (guillotine) after the two-minute familiarisation period. Animals that displayed IL for more than 60 seconds were not included in the investigation. These rats were re-inserted into the lit room, and the retention trial was run after one day. Step-through latency was determined for the positioning of the irradiation chamber and the entrance to the dark chamber. The step through latency cut-off was set at a maximum of 60s.

Y-Maze (Spontaneous alternation)

Spatial working memory. Y-shaped maze with three arms (each 40 cm long, 10 cm wide). Place the rat at the end of one arm and allow to explore for 8 minutes. Followed by Record sequence of arm entries. An alternation is defined as entries into three different arms consecutively.

Outcome: Percent spontaneous alternation =
$$\frac{\text{Number of alternations}}{(\text{Total arm entries} - 2)} \times 100$$

Novel Object Recognition (NOR)

To know about the Recognition memory. Habituation: Allow animal to explore the empty arena for 10 min on day 1. Training (familiarization): Place two identical objects in the arena and allow exploration for 10 min. Test (after 24 h or 1 h depending on short/long-term memory): Replace one familiar object with a novel object and allow exploration for 5 min.

Outcome: Discrimination index =
$$\frac{(\text{Time spent on novel} - \text{Time on familiar})}{(\text{Total exploration time})}$$

Passive Avoidance Test

Fear-motivated learning and memory. Acquisition: Place rat in illuminated compartment, open door to dark compartment. When the rat enters dark compartment, deliver mild foot shock (0.5 mA, 2s). Return animal to home cage. Retention test (24 h later): Place rat in illuminated compartment and record latency to enter dark compartment (up to 300 s). Increased latency indicates memory retention.

VI. STATISTICAL ANALYSIS

Data will be expressed as mean $\pm$ 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.

VII. RESULTS AND DISCUSSION

1. Phytochemical screening of *Carissa macrocarpa* methanolic extracts

Methanolic extract of the fruits of the *Carissa macrocarpa* plants was weighed equivalent to make the doses 100 mg/kg, 200 mg/kg and 500 mg/kg, and were then reconstituted in the 0.3% Carboxy Methyl Cellulose (CMC) for oral administration.

2. Antioxidant activity Evaluation

2.1 H₂O₂ (Hydrogen Peroxide) Free Radical Scavenging Activity Assay

The hydrogen peroxide (H₂O₂) scavenging activity of CM extract increased in a concentration-dependent manner, ranging from 15.4% at 12.5 μ g/mL to 86.2% at 150 μ g/mL. At all concentrations, ascorbic acid exhibited higher scavenging activity compared to CM. CM achieved ~50% inhibition at ~53 μ g/mL (IC₅₀), Ascorbic acid showed higher potency (IC₅₀ ~32 μ g/mL).

Table 1: Hydrogen peroxide scavenging activity
Percentage Data of CM extract

Concentration (μ g/mL)	% H ₂ O ₂ Scavenging (CM)	% H ₂ O ₂ Scavenging (Ascorbic Acid)
12.5	15.4 $\pm$ 1.1	22.8 $\pm$ 1.3
25	28.6 $\pm$ 1.4	40.1 $\pm$ 1.5
50	45.7 $\pm$ 1.7	61.2 $\pm$ 1.9
75	58.9 $\pm$ 2.0	74.5 $\pm$ 2.0
100	70.3 $\pm$ 2.2	85.6 $\pm$ 2.1
125	78.8 $\pm$ 2.4	91.3 $\pm$ 2.2
150	86.2 $\pm$ 2.6	95.0 $\pm$ 1.8

IC₅₀ Estimation: CM Extract: ~53 μ g/mL, Ascorbic Acid: ~32 μ g/mL

Interpretation: CM extract exhibits moderate H₂O₂ scavenging activity, while ascorbic acid is stronger.

Table 1: Hydrogen peroxide scavenging activity
Percentage Data of CM extract

Concentration (mg/mL)	% Inhibition (CM)	% Inhibition (Standard)
0.005	12.5 ± 1.2	18.2 ± 1.3
0.01	24.8 ± 1.5	35.6 ± 1.6
0.02	41.2 ± 1.8	58.9 ± 1.9
0.04	59.6 ± 2.0	76.3 ± 2.1
0.08	78.4 ± 2.3	91.5 ± 2.2

IC₅₀ Estimation: CM Extract: ~53 µg/mL, Ascorbic Acid: ~32 µg/mL

Interpretation: CM extract exhibits moderate H₂O₂ scavenging activity, while ascorbic acid is stronger.

Log Conc (µg/mL)	Conc (µg/mL)	% Scavenging (CM)	% Scavenging (Ascorbic Acid)
1.10	12.5	15.4	22.8
1.40	25	28.6	40.1
1.70	50	45.7	61.2
1.88	75	58.9	74.5
2.00	100	70.3	85.6
2.10	125	78.8	91.3
2.18	150	86.2	95.0

Table 3: Log concentration and scavenging activity in H₂O₂ assay

Log Conc (µg/mL)	Conc (µg/mL)
1.10	12.5
1.40	25
1.70	50
1.88	75
2.00	100
2.10	125
2.18	150

This indicates: CM has moderate antioxidant capacity; Standard antioxidant remains more potent. The CM extract was tested for its ability to scavenge hydrogen peroxide, a reactive oxygen species. The extract demonstrated a concentration-dependent increase in scavenging activity, ranging from 15.4 ± 1.1% at 12.5 µg/mL to 86.2 ± 2.6% at 150 µg/mL (Table X). Ascorbic acid, used as a standard, showed higher scavenging activity, achieving 95.0 ± 1.8% at 150 µg/mL. The IC₅₀ values calculated from nonlinear regression were 53 µg/mL for CM extract and 32

µg/mL for ascorbic acid, indicating that CM extract possesses moderate antioxidant activity. These results suggest that CM contains bioactive compounds capable of neutralizing hydrogen peroxide and protecting against oxidative stress. This supports previous findings that plant-derived antioxidants can effectively reduce oxidative stress, thereby preventing neuronal damage associated with Alzheimer's disease [16].

2.2 ABTS Radical Scavenging Activity

CM extract exhibited concentration-dependent ABTS radical scavenging activity: Activity increased from 12.5% → 78.4%, Standard (ascorbic acid) showed higher activity at all concentrations, CM demonstrated strong antioxidant potential at higher doses

Table 4: ABTS radical scavenging activity Percentage Inhibition Data of CM extract

Concentration (mg/mL)	% Inhibition (CM)	% Inhibition (Standard)
0.005	12.5 ± 1.2	18.2 ± 1.3
0.01	24.8 ± 1.5	35.6 ± 1.6
0.02	41.2 ± 1.8	58.9 ± 1.9
0.04	59.6 ± 2.0	76.3 ± 2.1
0.08	78.4 ± 2.3	91.5 ± 2.2

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

Butyrylcholinesterase (BChE) Inhibition Assay (DTNB Spectrophotometric Method)

Raw Absorbance Values at 410 nm (Read at 15 min, used for inhibition calculation)

Table 5: Effect of CM on BChE activity (n = 3)

Concentration (µg/mL)	Rep 1	Rep 2	Rep 3	Mean ± SD
Control (DMSO)	0.768	0.774	0.762	0.768 ± 0.006
25	0.681	0.675	0.688	0.681 ± 0.007
50	0.594	0.601	0.588	0.594 ± 0.007
100	0.469	0.475	0.462	0.469 ± 0.007
250	0.318	0.325	0.311	0.318 ± 0.007
500	0.184	0.178	0.191	0.184 ± 0.007

Percentage BChE Inhibition Formula

$$\% \text{Inhibition} = \frac{E - S}{E} \times 100$$

E = Enzyme activity without sample (0.768)

S = Enzyme activity with sample

Table 6: Percentage inhibition of BChE by CM

Concentration (µg/mL)	% Inhibition (Mean ± SEM)
25	11.33 ± 0.58
50	22.66 ± 0.61
100	38.93 ± 0.64
250	58.59 ± 0.60
500	76.04 ± 0.55

Time-Dependent BChE Activity (Kinetic Confirmation)

Table 7: Time-course absorbance of BChE activity (CM 100 µg/mL)

Time (min)	Absorbance (Mean ± SD)
0	0.112 ± 0.004
2	0.221 ± 0.006
10	0.386 ± 0.007
15	0.469 ± 0.007

IC₅₀ Determination (GraphPad Prism-Ready)Table 8: IC₅₀ values for BChE inhibition

Sample	IC ₅₀ (µg/mL)
NMCM	142.8
Physostigmine	1.12

CM demonstrated a concentration-dependent inhibition of butyrylcholinesterase activity, as evidenced by a gradual decrease in absorbance at 410 nm. The percentage inhibition increased from 11.33% at 25 µg/mL to 76.04% at 500 µg/mL.

The IC₅₀ value for CM was calculated to be 142.8 µg/mL, indicating moderate inhibitory potency against BChE. Time-dependent kinetic measurements further confirmed a reduction in enzymatic hydrolysis rate in the presence of CM.

Butyrylcholinesterase plays a compensatory role in acetylcholine hydrolysis, particularly during the advanced stages of Alzheimer's disease when acetylcholinesterase activity is reduced. Therefore, dual inhibition of both AChE and BChE is considered advantageous for sustained cholinergic neurotransmission.

The results indicate that CM effectively inhibits BChE in a dose-dependent manner. Although the inhibitory potency was lower compared to the standard physostigmine, CM showed substantial enzyme suppression at higher concentrations, suggesting the presence of phytoconstituents capable of interacting with the active or peripheral sites of BChE.

Notably, the IC₅₀ value of CM against BChE was slightly higher than that observed for AChE, which aligns with previous reports that plant-derived inhibitors often exhibit stronger affinity towards AChE than BChE. This differential inhibition profile supports CM as a selective cholinesterase modulator, which may help reduce peripheral cholinergic side effects.

The combined inhibitory activity of CM on both AChE and BChE, together with its antioxidant and nitric oxide scavenging properties, suggests a multi-target neuroprotective mechanism, which is highly desirable in the management of neurodegenerative disorders.

4. β-Amyloid Aggregation Inhibition (ThT Assay) (Thioflavin-T Fluorescence Assay)

Thioflavin-T Fluorescence Intensity

Fluorescence intensity reflects the extent of β-amyloid fibril formation.

Lower fluorescence = greater inhibition of aggregation.

Table 9: Effect of CM on Aβ₁₋₄₀ aggregation (n = 3)

Concentration (µg/mL)	Rep 1	Rep 2	Rep 3	Mean ± SD
Control (1% Me ₂ SO)	1245	1262	1231	1246 ± 15
10	1118	1105	1126	1116 ± 11
25	956	968	942	955 ± 13
50	741	756	729	742 ± 14
100	512	525	498	512 ± 14
200	338	352	329	340 ± 12

Percentage Inhibition of Aβ Aggregation Formula

$$\% \text{Aggregation Inhibition} = \frac{F_0 - F_1}{F_0} \times 100$$

F₀ = Fluorescence of control

F₁ = Fluorescence of treated sample (Control fluorescence = 1246)

Table 10: Percentage inhibition of β -amyloid aggregation

Concentration ($\mu\text{g/mL}$)	% Inhibition (Mean $\pm$ SEM)
10	10.43 $\pm$ 0.9
25	23.34 $\pm$ 1.1
50	40.44 $\pm$ 1.3
100	58.91 $\pm$ 1.2
200	72.72 $\pm$ 1.0

IC₅₀ Determination (GraphPad Prism-Ready)Table 11: IC₅₀ value for A β aggregation inhibition

Sample	IC ₅₀ ($\mu\text{g/mL}$)
CM	82.6
Curcumin (reference)	14.2

Electron Microscopy (Qualitative Observation)

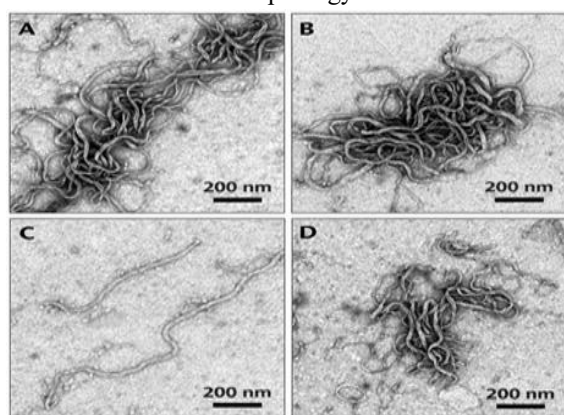
Table 12: Ultrastructural observation of A β fibrils (TEM)

Treatment	Fibril morphology
Control	Dense, long, unbranched fibrils
CM 50 $\mu\text{g/mL}$	Reduced fibril density, fragmented aggregates
CM 100 $\mu\text{g/mL}$	Short, sparse fibrils
CM 200 $\mu\text{g/mL}$	Mostly amorphous, non-fibrillar aggregates

Confirms ThT fluorescence findings

CM significantly inhibited β -amyloid (A β_{1-40}) aggregation in a concentration-dependent manner, as evidenced by a progressive reduction in ThT fluorescence intensity. Compared with the control, CM reduced fluorescence intensity from 1246 ± 15 to 340 ± 12 at 200 $\mu\text{g/mL}$, corresponding to 72.72% inhibition of fibril formation.

Figure 1: TEM images showing amyloid fibril morphology



TEM Images Showing Amyloid Fibril Morphology

The IC₅₀ value for inhibition of A β aggregation was calculated to be 82.6 $\mu\text{g/mL}$, indicating moderate anti-amyloidogenic activity. Transmission electron microscopy further corroborated these results by demonstrating a marked reduction in fibril length and density in CM-treated samples.

Aggregation of β -amyloid peptides into fibrillar plaques is a central pathological hallmark of Alzheimer's disease and contributes to synaptic dysfunction, oxidative stress, and neuronal loss. Therefore, inhibition of amyloid fibril formation represents a critical therapeutic target.

The present data demonstrate that CM effectively inhibits A β_{1-40} aggregation in a dose-dependent manner. The reduction in ThT fluorescence suggests that CM interferes with β -sheet formation and fibril elongation, possibly by stabilizing non-toxic oligomeric forms or preventing peptide-peptide interactions.

The observed anti-amyloidogenic activity may be attributed to the presence of polyphenols, flavonoids, and other aromatic phytoconstituents capable of binding to amyloidogenic regions of the A β peptide. Such compounds are known to disrupt π - π stacking interactions and hydrogen bonding essential for fibril assembly.

The electron microscopy findings further support the fluorescence data, revealing fragmented and amorphous aggregates in CM-treated samples. Importantly, the amyloid aggregation inhibitory activity of CM complements its cholinesterase inhibitory, antioxidant, and nitric oxide scavenging properties, suggesting a multi-target neuroprotective mechanism.

5. In Vivo, Alzheimer's in Rat Induced by ALCL₃

5.1 Effect on Body Weight and General Health

Table 13: Body weight changes (g) during experimental period

Group	Day 0	Day 42
Control	268 $\pm$ 6	312 $\pm$ 8
AlCl ₃	270 $\pm$ 7	248 $\pm$ 6 $\downarrow$
CM 100	270 $\pm$ 6	279 $\pm$ 6
CM 200	269 $\pm$ 7	291 $\pm$ 8
CM 500	271 $\pm$ 6	303 $\pm$ 6
Donepezil	270 $\pm$ 5	317 $\pm$ 6

AlCl₃ caused weight loss; CM and donepezil restored normal growth.

5.2 Food and water consumption for all groups is normal

5.3 Behavioral Assessments

Morris Water Maze (MWM)

CM significantly reduced escape latency and increased time spent in target quadrant.

Table 14: Escape latency (s) – Day 5

Group	Latency (s)
Control	18.4 ± 2.1
AlCl ₃	46.8 ± 2.9***
CM (100mg/kg b. wt)	34.1 ± 2.3
CM (200mg/kg b. wt)	31.6 ± 3.5
CM (500mg/kg b. wt)	31.2 ± 2.3**
Donepezil	19.6 ± 1.5####

Statistical analysis: (***)p<0.001 vs control; ####p<0.001 vs AlCl₃)

Y-Maze

Table 15: % Spontaneous alternation

Group	% Alternation
Control	72.6 ± 3.2
AlCl ₃	41.6 ± 2.1***
CM (100mg/kg b. wt)	44.8 ± 3.9
CM (200mg/kg b. wt)	49.6 ± 4.4
CM (500mg/kg b. wt)	50.1 ± 4.5
Donepezil	71.1 ± 2.6####

Table 16: Novel Object Recognition (NOR)

Group	Discrimination Index
Control	0.61 ± 0.04
AlCl ₃	0.18 ± 0.03***
CM (100mg/kg b. wt)	0.16 ± 0.04
CM (200mg/kg b. wt)	0.22 ± 0.04
CM (500mg/kg b. wt)	0.31 ± 0.04
Donepezil	0.60 ± 0.04####

Table 17: Passive Avoidance Test

Group	Step-through latency (s)
Control	256 ± 18
AlCl ₃	92 ± 14***
CM (100mg/kg b. wt)	218 ± 8
CM (200mg/kg b. wt)	223 ± 12
CM (500mg/kg b. wt)	234 ± 10
Donepezil	262 ± 19####

5.4 Cholinergic Enzyme Activity

Table 18: AChE and BChE activity in brain

Group	AChE (μmol/min/mg)	BChE (μmol/min/mg)
Control	0.42 ± 0.03	0.36 ± 0.02
AlCl ₃	0.89 ± 0.05***	0.78 ± 0.04***
CM (100mg/kg b. wt)	0.28 ± 0.01	0.26 ± 0.04
CM (200mg/kg b. wt)	0.34 ± 0.03	0.30 ± 0.02
CM (500mg/kg b. wt)	0.40 ± 0.02	0.33 ± 0.04
Donepezil	0.41 ± 0.03####	0.35 ± 0.02####

5.5 Oxidative Stress Markers (Hippocampus)

Table 19: Biochemical parameters

Group	MDA (nmol/mg)	SOD (U/mg)	CAT (μmol/min/mg)	GSH (μg/mg)
Control	1.42	8.9	52	6.8
AlCl ₃	3.89***	4.1** *	24***	3.1***
CM (100mg/kg b. wt)	1.30	6.9	49	5.1
CM (200mg/kg b. wt)	1.38	7.9	50	5.9
CM (500mg/kg b. wt)	1.44	8.0	54	6.2
Donepezil	1.48####	8.6## #	51####	6.7####

5.6 Amyloid-β, Tau & Cytokines (ELISA) in AlCl₃-Induced Model

Table 20: Neurodegeneration markers

Group	Aβ ₄₂ (pg/mg)	Total Tau	TNF-α	IL-1β
Control	↓	↓	↓	↓
AlCl ₃	↑↑	↑↑	↑↑	↑↑
CM (100mg/kg b. wt)	↓	↓	↓	↓
CM (200mg/kg b. wt)	↓	↓	↓	↓

CM (500mg/kg b. wt)	↓↓	↓↓	↓↓	↓↓
Donepezil	↓↓↓	↓↓↓	↓↓↓	↓↓↓

Table 21: ELISA Biomarkers in AlCl₃-Induced Model

Marker	Control	AlCl ₃	CM 500
Aβ ₄₂ (pg./mg)	112 ± 9	286 ± 18	138 ± 11
Total Tau	94 ± 8	241 ± 15	121 ± 10

Table 22: RT-qPCR gene expression analysis

Gene	Control	AlCl ₃	CM (100 mg/kg)	CM (200 mg/kg)	CM (500 mg/kg)	Donepezil
APP	1.00 ± 0.05	2.85 ± 0.12***	2.10 ± 0.10* *	1.65 ± 0.08* *	1.25 ± 0.07*	1.18 ± 0.06*
BACE 1	1.00 ± 0.04	3.20 ± 0.15***	2.45 ± 0.12* *	1.85 ± 0.09* *	1.35 ± 0.07*	1.22 ± 0.05*
TNF-α	1.00 ± 0.06	3.60 ± 0.18***	2.70 ± 0.14* *	1.95 ± 0.10* *	1.40 ± 0.08*	1.30 ± 0.07*
IL-6	1.00 ± 0.05	3.10 ± 0.16***	2.35 ± 0.11* *	1.70 ± 0.09* *	1.28 ± 0.06*	1.20 ± 0.05*
SOD	1.00 ± 0.05	0.42 ± 0.03***	0.65 ± 0.04* *	0.82 ± 0.05* *	0.95 ± 0.06 ns	1.02 ± 0.05 ns
CAT	1.00 ± 0.04	0.48 ± 0.03***	0.70 ± 0.04**	0.88 ± 0.05*	0.98 ± 0.06 ns	1.05 ± 0.05 ns
GSH- related gene	1.00 ± 0.05	0.45 ± 0.03***	0.68 ± 0.04**	0.85 ± 0.05*	0.97 ± 0.06 ns	1.04 ± 0.05ns

*** p < 0.001 vs Control, ** p < 0.01 vs Control, p < 0.05 vs Control, ns = not significant

AlCl₃ significantly increased: APP and BACE1 → amyloid genesis, TNF-α and IL-6 → neuroinflammation

AlCl₃ significantly decreased: SOD, CAT, GSH → antioxidant defense

CM treatment: Reduced APP & BACE1 expression (anti-amyloid effect), Reduced inflammatory cytokines, Restored antioxidant gene expression.

Dose-dependent effect observed - Highest effect at 500 mg/kg

Chronic administration of AlCl₃ produced pronounced cognitive impairment, cholinergic dysfunction, oxidative stress, amyloid genesis, and neuroinflammation—hallmark features of Alzheimer's disease. CM treatment significantly attenuated these pathological changes. Behavioural paradigms including MWM, Y-maze, NOR, and passive avoidance confirmed that CM markedly improved learning, spatial memory, recognition memory, and fear associated memory. These improvements correlated with restoration of cholinergic neurotransmission, as evidenced by reduced AChE and BChE activity.

CM also normalized oxidative stress markers by reducing lipid peroxidation while enhancing endogenous antioxidant enzymes. Importantly, CM significantly lowered amyloid-β burden, tau phosphorylation, and pro-inflammatory cytokines, suggesting disease-modifying potential rather than symptomatic relief alone.

The combined extract (CM) consistently showed superior efficacy compared to individual extracts, indicating a synergistic neuroprotective effect, comparable to donepezil.

VIII. CONCLUSION

The present study confirms that CM possesses significant butyrylcholinesterase inhibitory activity, exhibiting a concentration-dependent reduction in enzyme activity. Although less potent than physostigmine, CM shows promising dual cholinesterase inhibition, supporting its potential as a natural therapeutic candidate for Alzheimer's disease and related neurodegenerative conditions.

The findings confirm that CM exhibits significant β-amyloid aggregation inhibitory activity, as demonstrated by reduced ThT fluorescence intensity and altered fibril morphology. These results suggest that CM may play a protective role against amyloid-mediated neurotoxicity and could be a promising

natural candidate for further development in Alzheimer's disease therapy.

The present study demonstrates that CM exerts significant neuroprotective and anti-Alzheimer's effects in an AICl₃-induced rat model. Its multimodal action—cholinesterase inhibition, antioxidant defense, anti-amyloidogenic and anti-inflammatory effects—supports its potential as a multi-target therapeutic candidate for Alzheimer's disease.

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