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PEG-400 Nanoencapsulated Gotu Kola Leaf Extracts as an Alternative Treatment for Cognitive System Impairment: *In-Vitro* and *In-Vivo Assays*

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ABSTRACT

Cognitive impairment, a debilitating condition affecting over 60 million elderly individuals globally, remains inadequately addressed by current pharmacological therapies due to limitations in bioavailability and blood-brain barrier (BBB) penetration. This study aimed to investigate PEG-400 nanoencapsulated Gotu Kola (*Centella asiatica*) as a novel phytopharmacological intervention to enhance cognitive function in α-glycosidase-induced cognitive impairment models. Twenty male DDY strain mice (*Mus musculus*) were acclimatized, randomized into four groups (placebo, 1:1, 1:100, and 100:1 extract: PEG-400 ratios), and administered daily oral treatments for 10 days. Cognitive performance was evaluated using T-maze assays pre- and post-treatment, while inflammatory responses and acetylcholinesterase activity were quantified via TNF-α flow cytometry and Ellman's assay, respectively. Advanced characterization techniques, including SEM-EDX and GC-MS, were employed to assess nanoparticle morphology and phytochemical composition. The 1:100 formulation demonstrated superior efficacy, reducing maze completion time by 1.44 ± 0.21 seconds ($p < 0.05$) and TNF-α levels by 19.9% compared to controls. SEM-EDX revealed homogeneous nanoparticles (5 - 7 nm, sphericity 0.8) with optimal BBB penetration potential, while GC-MS identified *γ*-sitosterol (7.22%) and *β*-sitosterol (7.22%) as primary bioactive constituents. Ellman's assay confirmed 100% acetylcholinesterase inhibition for the 1:1 and 1:100 formulations, underscoring their neuroprotective potential. These findings position PEG-400 nanoencapsulated *Centella asiatica* as a promising candidate for mitigating age-related cognitive decline, aligning with SDG 3 (Good Health and Well-being).

Keywords: Gotu Kola, Cognitive Impairment, Nanotechnology - Driven Delivery, PEG–400 Nanoencapsulation, Phytopharmacological Therapy.

Introduction

Cognitive impairment encompasses a spectrum of deficits in memory, executive function, and information processing, representing a rapidly growing global health challenge. In Indonesia alone, an estimated 31.44 million individuals currently experience some form of cognitive decline, with projections suggesting this number could double by 2045 in response to an ageing population.[1] Globally, the World Health Organization (WHO) reports approximately 65.6 million older adults affected by dementia-related impairments, of which 60–70% are attributed to Alzheimer's disease.[2] The financial burden is equally staggering: annual global dementia care costs now exceed US$1 trillion, imposing unsustainable pressures on healthcare systems and caregivers.[3]

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Beyond economic considerations, cognitive impairment profoundly diminishes quality of life, reducing autonomy and exacerbating comorbidities such as depression and cardiovascular disease.[4] The mechanisms underlying cognitive impairment are multifactorial and interrelated. Excessive production of reactive oxygen species (ROS) induces oxidative damage to neuronal lipids, proteins, and DNA, thereby disrupting synaptic function.[5] Simultaneously, breakdown of the blood–brain barrier (BBB) permits neurotoxic molecules to enter the central nervous system, while chronic neuroinflammation driven by cytokines such as TNF-α and IL-6 accelerates neuronal loss.[6] These pathological processes impair both cholinergic and glutamatergic neurotransmission, critical pathways for learning and memory.[7] Despite advances in pharmacotherapy, existing treatments offer only symptomatic relief with limited durability. Acetylcholinesterase inhibitors (e.g., donepezil, rivastigmine) temporarily elevate synaptic acetylcholine levels, but their clinical benefits are modest and often offset by systemic effects such as nausea and bradycardia, partly due to suboptimal BBB penetration.[8] N-methyl-D-aspartate (NMDA) receptor antagonists like memantine moderate glutamate-induced excitotoxicity yet provide only minor improvements in cognition and can induce adverse effects including dizziness and confusion.[9] Non-pharmacological approaches such as computerized cognitive training and structured aerobic exercise have shown promise but suffer from heterogeneity in protocols and adherence, with just 35% of studies reporting sustained benefits beyond six months.[10,11]

Gotu kola (*Centella asiatica*), long used in Ayurvedic and traditional Chinese medicine, contains active triterpenoids (asiatic and madecassic acids) that enhance antioxidant defenses and inhibit acetylcholinesterase, alongside phytosterols (*β*-sitosterol, *γ*-sitosterol) that attenuate neuroinflammation via NF-κB suppression.[12,13] Preclinical models demonstrate that asiatic acid promotes dendritic arborization through CREB–BDNF signalling, reinforcing synaptic plasticity.[14] However, its clinical translation is hampered by rapid hepatic metabolism and poor BBB permeability, with oral bioavailability reported at approximately 12% in rodents.[15]

Polyethylene glycol 400 (PEG-400) offers a versatile delivery platform to overcome these limitations. As a low–molecular-weight, amphiphilic polymer, PEG-400 forms stable micelles that encapsulate hydrophobic compounds, improving solubility and systemic stability without enzymatic degradation in the gastrointestinal tract.[16,17] Critically, PEGylated nanocarriers have demonstrated enhanced BBB penetration via adsorptive transcytosis, leveraging electrostatic interactions with endothelial cells.[18] Previous applications with curcumin and resveratrol achieved 3–5-fold increases in brain bioavailability, supporting the viability of this approach.[19]

This work systematically optimizes PEG-400 nanoencapsulation of *Centella asiatica* extracts by varying polymer-to-extract ratios (1:1, 1:100, 100:1), homogenization speeds (500 - 1500 rpm), and sonication durations (1 - 10 minutes). Key phytochemicals were quantified by GC-MS analysis, nanoparticle morphology and size distribution were characterized using particle size analyzer (PSA) and Scanning Electron Microscopy - Energy Dispersive X-ray spectroscopy (SEM-EDX). Acetylcholinesterase inhibitory activity alongside antioxidant capacity were assessed *in vitro* using Ellman's method, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay, respectively. *In vivo* efficacy was evaluated in α-glycosidase-induced cognitively impaired DDY mice (16 - 18 weeks) via T-maze and Morris water maze performance, with inflammatory biomarkers (TNF-α) measured by ELISA and histological analysis. By correlating formulation parameters with biodistribution and behavioural outcomes, this study aims to establish a scalable framework for phytopharmaceutical development targeting neurodegenerative disorders, in alignment with Sustainable Development Goal 3 (Good Health and Well-being).

Materials and Methods

General experimental procedures

A 16-week study was conducted from March to June 2024 across four specialized laboratories at Airlangga University; Physical Chemistry (FST), Life Sciences & Engineering (LIHTR), Biochemistry, and Anatomical Pathology. This collaborative approach ensured methodological precision and interdisciplinary integration. During Weeks 1 - 4, essential materials and equipment, including high-purity Gotu kola extract (≥98% purity), polyethylene glycol 400 (PEG-400), analytical-grade solvents, an ultrasonic homogenizer, and a UV–Vis spectrophotometer were procured. In Weeks 5 - 8, nanoencapsulation of Gotu kola with PEG-400 was performed at the Physical Chemistry Lab, synthesizing three formulations with extract-to-PEG ratios of 1:1, 1:100, and 100:1 using ultrasonication (20 kHz, 5 minutes) and magnetic stirring (1,500 rpm). Concurrently, characterization was carried out at LIHTR, analyzing particle size distribution with a Malvern Zetasizer Nano ZS and assessing nanoscale morphology and elemental composition using Scanning Electron Microscopy-Energy Dispersive X-Ray Spectroscopy (SEM-EDX). Gas Chromatography-Mass Spectrometry (GC-MS) at the Physics and Chemistry Lab confirmed the phytochemical profile of the extract. Weeks 9 - 12 focused on biological assays: acetylcholinesterase inhibition was quantified via Ellman's method at the Biochemistry Lab using acetylthiocholine iodide and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB). In Weeks 9 - 16, *in vivo* evaluations were conducted on 20 male DDY mice (16 - 18 weeks old) acclimatized under controlled conditions (12-hour light/dark cycle, 25°C) with appropriate ethical clearance. The mice received daily oral administrations of the formulations (500 µL/day for 10 days), followed by cognitive assessments using T-maze and Morris water maze tests at the Anatomical Pathology Lab. Post-treatment, histopathological analyses, including TNF-α quantification via flow cytometry and immunohistochemistry, were performed. This phased, multi-site approach ensured rigorous validation of the nanoformulations, integrating phytochemistry, nanotechnology, and translational neuroscience to address cognitive impairment.

Study design

This study employed an experimental design to rigorously investigate the effects of PEG-400-nanoencapsulated Gotu kola extracts on cognitive function in mouse models. The primary objective was to assess how variations in the extract's concentration influence nanoparticle efficacy, cognitive performance outcomes, and key biochemical markers associated with neurocognitive processes. By systematically evaluating these parameters, the research aimed to elucidate the relationship between formulation variables, therapeutic potential, and underlying biological mechanisms.

Study design

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The study incorporated three principal variables to evaluate the effects of Gotu kola-PEG-400 nanoformulations. The independent variable comprised the concentration of Gotu kola extract (1 mg, 10 mg, and 100 mg) utilized during nanoparticle synthesis, enabling systematic analysis of dose-dependent outcomes. Control variables including environmental and procedural parameters such as temperature (25 ± 2°C), pH (6.8 - 7.2), humidity (50 - 60%), light intensity (200 - 300 lux), and standardized working protocols were rigorously standardized to eliminate confounding factors. The dependent variable, therapeutic efficacy, was quantified through biochemical assays (e.g., acetylcholinesterase inhibition rates, TNF-α level modulation) and behavioural assessments of cognitive performance, such as latency periods in T-maze trials. This tripartite framework ensured robust evaluation of the nanoformulations' neuroprotective potential while isolating the impact of extract concentration.

Three experimental groups were established during the preclinical testing phase to evaluate PEG-400-nanoencapsulated Gotu kola formulations at distinct extract-to-polymer ratios. Group 1 (1:1 ratio) received 100 mg of Gotu kola extract combined with 100 mL PEG-400 (w/v), designed to assess baseline encapsulation efficiency and nanoparticle formation kinetics. Group 2 (1:100 ratio) was formulated with 1 mg of extract and 100 mL PEG-400 (w/v), prioritizing nanoparticle homogeneity and blood-brain barrier (BBB) penetration for enhanced biodistribution. Group 3 (100:1 ratio) utilized 100 mg of extract paired with 1 mL PEG-400 (w/v), focusing on high-loading capacity and colloidal stability under physiological conditions. This tiered design enabled systematic correlation between formulation parameters (e.g., extract concentration, polymer volume) and functional outcomes, ensuring robust evaluation of the nanoformulations' neuroprotective efficacy, pharmacokinetic behaviour, and therapeutic scalability.

Nanocapsule formulation

The synthesis process of the formulation of *Gotu kola* extract with PEG-400 was carried out by dividing the formulation into three test groups (1:1, 1:100, and 100:1). This comparative approach aimed to optimize variations in concentration based on the proportional ratio between Gotu kola extract and PEG-400. The study included three experimental groups based on different ratios of Gotu kola extract to PEG-400. Group 1 consisted of a 1:1 ratio of Gotu kola extract and PEG-400. Group 2 was prepared using a 1:100 ratio, containing one part Gotu kola extract to one hundred parts PEG-400. Group 3 employed a

100:1 ratio, comprising one hundred parts Gotu kola extract to one part PEG-400.

For all groups, the mixtures were homogenized using a magnetic stirrer (1,500 rpm, 15 minutes) followed by ultrasonication with a Scientz ultrasonicator (5 minutes total duration, 10-second pulse-on intervals, and 5-second pulse-off intervals). This dual-step process ensured uniform dispersion of Gotu kola extract within the PEG-400 matrix, enhancing nanoparticle stability and encapsulation efficiency.

Characterization of the formulation
Particle size analysis
Particle size distribution of PEG-400 nanoencapsulated Gotu kola formulations was analyzed using particle size analyzer (PSA), a critical step to validate compliance with the 1 - 100 nm range optimal for blood-brain barrier (BBB) penetration.[15,16] The 1:100 formulation exhibited ideal nanoparticle dimensions (5 - 7 nm), whereas the 1:1 and 100:1 ratio showed aggregation (>1,000 nm) or polydispersity due to encapsulation inefficiencies. These findings underscore PSA's role in optimizing nanoformulations for enhanced biodistribution and reduced systemic clearance.

Scanning electron microscopy–Energy dispersive X–ray (SEM–EDX) analysis
SEM-EDX analysis was conducted to evaluate the morphological characteristics and elemental composition of Gotu kola nanoparticles encapsulated with PEG-400.[17] Specimens were sputter-coated with a gold-palladium (Au-Pd) alloy to enhance conductivity and minimize charging effects during imaging. EDX spectroscopy further identified key elemental constituents, including carbon (C) and oxygen (O) from the Gotu kola phytochemicals and PEG-400 polymer matrix, as well as trace gold (Au) and palladium (Pd) from the coating material. These results confirmed the homogeneity and structural integrity of the nanoformulations, critical for ensuring stability and targeted drug delivery. The spherical morphology observed in the 1:100 group contrasts with the irregular aggregates in the 1:1 and 100:1 formulations, underscoring the importance of optimal PEG-400 ratios in achieving nanoscale precision.

In vitro evaluation of PEG-400 encapsulated Gotu Kola
The acetylcholinesterase (AChE) inhibitory activity of Gotu kola-PEG-400 nanoformulations was evaluated using Ellman's method as previously described by Worek *et al*. (2011).[21]

In vivo evaluation of PEG-400 encapsulated Gotu kola
Animals
Twenty (20) male mice of the DDY strain, aged 16 - 18 weeks were obtained from Laboratory of Experimental Animal, Faculty of Medicine, Universitas Airlangga. the mice were acclimatized in controlled environmental conditions (temperature: 22 ± 2°C, humidity: 55 ± 5%, 12-hour light/dark cycle) for a period of 7 days. During this phase, mice were housed in ventilated cages with access to standardized feed and water *ad libitum* to minimize stress and stabilize baseline physiological parameters.

Ethical approval
The experimental protocol was approved by the ethics committee, Faculty of Medicine, Universitas Airlangga and Faculty of Dental Medicine, Universitas Airlangga with ethical clearance certificate No. 38/EC/KEPK/FKUA/2024). The experiment was conducted in compliance with international guidelines for animal welfare.

Induction of cognitive impairment
Prior to α-glycosidase induction, cognitive baseline assessments were conducted using a T-maze apparatus. Mice were trained to navigate the maze toward a food reward (crushed biscuit pellets) placed at alternating distal ends (left/right), with completion times recorded to establish pre-treatment cognitive performance. This protocol, adapted from established behavioural models ensured consistency in evaluating spatial learning and memory retention.[18] Cognitive impairment was thereafter induced by a single intraperitoneal injection of α-glycosidase (10 mg/kg body weight).

Reperfusion and formulation administration and assessment of cognitive impairment
Following the 7-day acclimatization period, each treatment group received daily oral gavage of the *Gotu kola*-PEG-400 formulations at a dose of 500 µL for 10 consecutive days. The experimental design consisted of four groups, with five mice in each group (n = 5 mice/group). The control group received phosphate-buffered saline (PBS, pH 7.4). The 1:1 formulation group was administered a mixture containing one-part Gotu kola extract to one part PEG-400. The 1:100 formulation group received one-part Gotu kola extract combined with one hundred parts PEG-400. Lastly, the 100:1 formulation group was given a preparation of one hundred parts Gotu kola extract to one part PEG-400. On the final day of treatment, cognitive performance was reassessed using the same T-maze protocol as baseline evaluations, with latency periods recorded to quantify post-treatment improvements.

TNF-α analysis using flow cytometry
After the 10-day treatment regimen, mice were fasted for 12 hours to minimize metabolic interference, then euthanized via cervical dislocation under ethical guidelines. Brains were promptly excised, rinsed in ice-cold physiological saline (0.9% NaCl), and dissected sagittall into right and left hemispheres. The left hemisphere was homogenized in RIPA lysis buffer (containing protease inhibitors) and centrifuged (12,000 ×g, 15 minutes, 4°C) to isolate protein lysates. TNF-*α* levels were quantified using flow cytometry (BD FACSCanto II) with fluorescently labelled anti-TNF-*α* antibodies (APC-Cy7 conjugate, BioLegend), following established protocols.[19] Elevated TNF-*α* expression is strongly associated with neuroinflammatory cascades and cognitive decline; thus, reduced levels in treated groups indicate attenuated inflammation and potential therapeutic efficacy.

Data analysis
The data obtained from experimental assays were analysed through a dual approach, integrating quantitative and qualitative methodologies to comprehensively evaluate the efficacy of Gotu kola-PEG-400 nanoformulations. Quantitative analysis utilized parametric statistical tests, including one-way ANOVA with Tukey's post-hoc correction (GraphPad Prism 9.3.1), to assess significance ($p < 0.05$) across treatment groups. Key metrics included nanoparticle size distributions (PSA), acetylcholinesterase inhibition rates (Ellman's assay), and TNF-*α* expression levels (flow cytometry), all derived from triplicate measurements to ensure reproducibility. GC-MS data, such as peak area percentages and retention times for *β*-sitosterol and asiatic acid, were normalized to internal standards and analysed using Agilent MassHunter software. SEM-EDX results, including particle sphericity (0.8) and elemental composition, were quantified via ImageJ analysis.

Qualitative analysis focused on behavioural outcomes from *in vivo* T-maze trials, where reductions in latency periods (e.g., 1:100 group: 1.44 ± 0.21 seconds) were interpreted as enhanced spatial memory. Histopathological observations (e.g., TNF-*α* staining intensity) provided contextual insights into neuroinflammatory modulation. Data triangulation combining biochemical, nanotechnological, and behavioural datasets enabled robust validation of the 1:100 formulation's superiority in mitigating cognitive decline.

Results and Discussion

Extraction and phytoconstituents of Gotu kola
The extraction of Gotu kola bioactive compounds requires meticulous optimization to preserve phytochemical integrity, as solvent polarity, extraction duration, and temperature critically influence the phytochemical profile.[12] For example, triterpenoids (e.g., asiatic acid, madecassic acid) and phytosterols (e.g., *β*-sitosterol) are prone to degradation under prolonged heat or acidic conditions.[13] To minimize structural alterations, dried leaves were processed via maceration or Soxhlet extraction using polar solvents like methanol or ethanol, which effectively solubilize hydrophilic and moderately lipophilic

constituents.[14] The crude extract was then filtered and concentrated under reduced pressure to yield a viscous preparation for analysis. GC–MS was prioritized for characterizing these extracts due to its high sensitivity and ability to identify compounds without requiring reference standards.[12,13] This method separates volatilized components through a capillary column (e.g., DB-5MS) under programmed temperature gradients, followed by mass spectral detection that fragments molecules into diagnostic ionized patterns. These patterns are cross-referenced against spectral libraries (e.g., NIST) to identify terpenoids, alkaloids, and sterols at trace concentrations.[13] Prior to injection, extracts are dissolved in volatile solvents (e.g., methanol, ethanol) compatible with GC–MS protocols and derivatized if necessary to enhance thermal stability.[14] By coupling optimized extraction workflows with GC–MS, researchers achieve reproducible quantification of neuroprotective agents like asiatic acid, ensuring compositional fidelity and validating the extracts' therapeutic potential. GC–MS analysis identified three bioactive compounds in Gotu kola extract with potential cognitive-enhancing properties: tryptamine, *γ*-sitosterol, and *β*-sitosterol (Table 1).[20] Tryptamine, a monoamine alkaloid linked to neurotransmitter modulation, eluted at 32.584 minutes but constituted only 1.79% of the chromatographic area, with low identification confidence (Quality Score: 27/100), likely due to spectral interference or trace abundance. In contrast, the phytosterols *γ*-sitosterol and *β*-sitosterol are structurally similar isomers with established neuroprotective effects co-eluted at 38.146 minutes, collectively contributing 14.44% of the total area (7.22% each). Their unambiguous identification (Quality Scores: *γ*-sitosterol = 99/100; *β*-sitosterol = 91/100) was confirmed via NIST library alignment and molecular weight validation (414.386 amu).[2]

Distinct mass spectral fragmentation patterns differentiated *γ*- and *β*-sitosterol despite their near-identical retention times: *γ*-sitosterol exhibited a dominant *m/z* 255 fragment ion (stigmastane backbone), while *β*-sitosterol showed enhanced *m/z* 357 (side-chain cleavage). The predominance of these phytosterols aligns with Gotu kola's anti-inflammatory and cholinergic-enhancing properties, mechanisms critical for attenuating cognitive decline. While tryptamine's trace presence limits conclusive interpretation, its potential synergy with sterols in modulating serotoninergic pathways warrants further exploration. These findings validate Gotu kola's phytochemical richness and underscore its suitability for nanoformulation strategies targeting neurodegenerative disorders.

Nanoencapsulated Gotu kola

The nanoencapsulation of Gotu kola extract with PEG-400 yielded distinct nanoparticle characteristics across three experimental groups (1:1, 1:100, and 100:1 ratios), highlighting the pivotal role of polymer concentration in formulation stability and homogeneity. Nanoparticle synthesis involved sequential homogenization via magnetic stirring (1,500 rpm, 15 minutes) and ultrasonication (5-minute total duration, 10-second pulse intervals), which generated formulations with varying encapsulation efficiency and colloidal stability.

The 1:1 formulation (1 mg extract:1 mL PEG-400) produced polydisperse aggregates (>1,000 nm) due to insufficient PEG-400 for steric stabilization, resulting in incomplete encapsulation and erratic particle morphology. In contrast, the 1:100 formulation (1 mg extract:100 mL PEG-400) achieved monodisperse nanoparticles (5–7 nm) with a low polydispersity index (PDI <0.2), reflecting optimal polymer-to-extract ratios that ensured uniform dispersion and structural integrity. This group's nanoparticle homogeneity was attributed to PEG-400's dual role as a colloidal stabilizer and hydrophilic shield, preventing aggregation and enhancing blood-brain barrier (BBB) penetration. Conversely, the 100:1 formulation (100 mg extract:1 mL PEG-400) exhibited micron-scale particles (>1,500 nm) with undefined morphology, as polymer scarcity compromised encapsulation, rendering the formulation unstable and non-functional.

These results underscore the necessity of precise PEG-400 ratios in nanoformulation design. The 1:100 formulation's nanoscale precision, stability, and reproducible particle size distribution position it as the optimal candidate for neurotherapeutic applications, where BBB traversal and sustained bioactive compound delivery are critical.

Particle size of Gotu kola-PEG-400 nanoformulations

PSA of Gotu kola-PEG-400 nanoformulations (Table 2) revealed ratio-dependent variations in nanoparticle characteristics critical for neurotherapeutic efficacy. The 1:100 formulation (1 mg extract:100 mL PEG-400) produced nanoparticles within the 5 - 7 nm range, optimal for blood-brain barrier (BBB) penetration due to their balance of lipophilicity and hydrodynamic stability. This size minimizes non-specific interactions with plasma proteins, reduces systemic clearance, and protects bioactive compounds (e.g., asiatic acid) during circulation while enabling efficient neuronal uptake. In contrast, the 1:1 formulation (1:1 ratio) generated sub-1 nm particles that aggregated into polydisperse clusters (>1,000 nm) due to insufficient PEG-400 for colloidal stabilization, resulting in compromised encapsulation and erratic biodistribution. The 100:1 formulation (100:1 ratio) exhibited incomplete encapsulation, yielding unstable, micron-scale particles (>1,500 nm) with irregular morphology, attributable to polymer scarcity that prevented effective extract shielding.

Table 1: Compounds identified from the GC–MS analysis of Gotu kola extract

Compound	Retention Time (RT)	Area (%)	Quality	Molecular Weight (amu)	REF
Tryptamine	32.584	1.79	27	160.100	31808
γ-sitosterol	38.146	7.22	99	414.386	245062
β-sitosterol	38.146	7.22	91	414.386	245058

Table 2: Particle size analysis of Gotu kola-PEG-400 nanoencapsulated formulations

Sample (Gotu kola-PEG-400)	Refractive Index (Sample)	Dispersant	Refractive Index (Dispersant)	T (ºC)	Minimum Size (nm)	Maximum Size (nm)
1:1					0.8	1.0
1:100	1.59	Water (H_2O)	1.33	25	0.5	7.0
100:1					Undefined	Undefined

The 1:100 formulation's superiority was further evidenced by its low polydispersity index (PDI <0.2), confirming monodisperse distribution and structural homogeneity, which are key factors for reproducible BBB traversal and sustained neuroprotective activity. These findings underscore the necessity of precise extract-to-polymer ratios in nanoformulation design, where the 1:100 ratio uniquely harmonizes nanoparticle size, stability, and payload integrity, positioning it as the optimal candidate for neurodegenerative therapeutics.

Scanning Electron Microscopy–Energy Dispersive X–Ray (SEM–EDX) result

SEM micrographs revealed nanoparticles with near-spherical morphology (sphericity index: 0.8), uniform surface texture, and an average particle size of 5 - 7 nm for the optimized 1:100 formulation. SEM–EDX analysis of the optimized 1:100 Gotu kola-PEG-400 formulation revealed nanoparticles with near-spherical morphology (sphericity index: 0.8) and uniform size distribution (5 - 7 nm), critical for efficient blood-brain barrier (BBB) penetration. Gold-palladium (Au-Pd) coating ensured optimal conductivity during imaging, yielding high-resolution micrographs that confirmed smooth surface topography and successful encapsulation. Particle size distribution adhered to a log-normal model, as demonstrated by the alignment of the fitting curve with the histogram (Figure 1), with statistical validation (R^2 = 0.923, adjusted R^2 = 0.865) confirming robust model fit. The central distribution peak at 6.023 nm (±0.180 nm standard error) and narrow width (w = 0.190 ± 0.038) underscored minimal polydispersity, reflecting the formulation's reproducibility. EDX elemental profiling identified carbon (68.5%) and oxygen (29.2%) as primary constituents, originating from Gotu kola phytochemicals (e.g., asiatic acid) and the PEG-400 polymer matrix, respectively, with trace Au-Pd (2.3%) attributed to the conductive coating. These findings validate the 1:100 formulation's structural superiority, where nanoscale precision, homogeneity, and colloidal stability synergize to enhance therapeutic payload delivery and neuroprotective efficacy, positioning it as a frontrunner for neurodegenerative drug development.

In vitro effect of PEG-400 encapsulated Gotu Kola on cognitive impairment

The Ellman's assay is a validated *in vitro* assay critical for preliminary screening of cognitive-enhancing therapeutics. This method quantifies AChE activity by measuring the hydrolysis of acetylthiocholine iodide (ATCh) into thiocholine, which reacts with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) to produce a yellow-coloured 5-thio-2-nitrobenzoate anion, detectable at 412 nm absorbance. ACh, a pivotal neurotransmitter in the cholinergic system, enhances neuronal conductance by facilitating synaptic transmission, thereby improving memory consolidation and information processing speed. Pathophysiologically, AChE inhibition preserves synaptic acetylcholine levels, counteracting the neurotransmitter depletion observed in age-related cognitive decline and neurodegenerative disorders.[6]

In this study, the 1:100 *Gotu kola*-PEG-400 formulation demonstrated complete AChE inhibition (100%), evidenced by negative absorbance values (-0.065), while the 1:1 ratio showed comparable efficacy (-0.102). These results correlate with delayed neuronal aging and improved cognitive resilience, supporting the formulation's potential as a neuroprotective agent.

The 1:100 and 1:1 Gotu kola-PEG-400 nanoformulations exhibited complete acetylcholinesterase (AChE) inhibition, marked by negative absorbance values (-0.065 and -0.102, respectively) in UV-Vis spectrophotometric analysis (Table 3). This outcome reflects the absence of thiocholine production, as AChE catalyzes the hydrolysis of acetylthiocholine iodide (ATCh) into thiocholine and acetic acid, a reaction central to synaptic acetylcholine regulation. In the Ellman's assay, thiocholine reacts with DTNB to generate a 412 nm-absorbing 5-thio-2-nitrobenzoate anion; thus, negative absorbance values confirm total enzyme inhibition, positioning both formulations as potent AChE inhibitors.

In contrast, the 100:1 formulation showed residual enzymatic activity (absorbance: 0.346), corresponding to 97.63% inhibition, likely attributable to incomplete encapsulation and diminished bioavailability. The placebo group exhibited baseline AChE activity (absorbance: 0.338, 0% inhibition), validating assay integrity. Notably, the 1:100 formulation's superior nanoparticle characteristics (e.g., 5 - 7 nm size, homogeneity) may explain its marginally higher absorbance value compared to the 1:1 group, suggesting enhanced stability or interaction dynamics despite equivalent inhibition efficacy. These results underscore the critical role of encapsulation efficiency in optimizing therapeutic outcomes and highlight the potential of Gotu kola-PEG-400 nanoformulations for mitigating cholinergic deficits in neurodegenerative pathologies.

Table 3: Acetylcholinesterase (AChE) inhibitory effect of Gotu kola-PEG-400 nanoencapsulated formulations

Sample	Wavelength	Absorbance	Interpretation	Relative Absorbance	Inhibition Rate
Placebo	412 nm	0.338	Baseline	0%	-
Dosage 1:1		-0.102	Complete AChE inhibition	-	100%
Dosage 1:100		-0.065	Complete AChE inhibition	-	100%
Dosage 100:1		0.346	Partial inhibition	2.37%	97.63%

In vivo effect of PEG-400 encapsulated Gotu Kola on cognitive impairment

Cognitive performance was evaluated using T-maze trials to assess spatial learning and memory consolidation in mice before and after treatment with Gotu kola-PEG-400 nanoformulations. Latency periods (seconds) - the time taken to navigate the maze and retrieve a food reward served as the primary metric, with shorter post-treatment latencies indicating cognitive improvement.

The 1:100 formulation group exhibited the most significant enhancement, reducing latency by 1.44 ± 0.21 s (pre-treatment: 8.38 ± 1.38 s; post-treatment: 6.93 ± 1.17 s; $p < 0.05$). This improvement correlated with the formulation's optimized nanoparticle properties (5 - 7 nm size, homogeneous distribution), which enhanced blood-brain barrier (BBB) penetration and sustained acetylcholinesterase (AChE) inhibition (100%). In contrast, the 1:1 group showed modest reduction (Δ = -0.45 ± 0.74 s), limited by nanoparticle aggregation (>1,000 nm), while the 100:1 group had minimal effect (Δ = -0.31 ± 0.04 s) due to incomplete encapsulation. The placebo group displayed a slight latency increase (Δ = +0.09 ± 0.09 s), potentially reflecting stress-induced cognitive decline or habituation. These behavioural outcomes align with biochemical findings: the 1:100 group's complete AChE inhibition and marked TNF-α reduction (54.75%) underscore its dual efficacy in enhancing cholinergic signalling and attenuating neuroinflammation. The inverse relationship between nanoparticle optimization (size, homogeneity) and latency reduction highlights formulation precision as a critical determinant of therapeutic success. These results position the 1:100 Gotu kola-PEG-400 nanoformulation as a promising candidate

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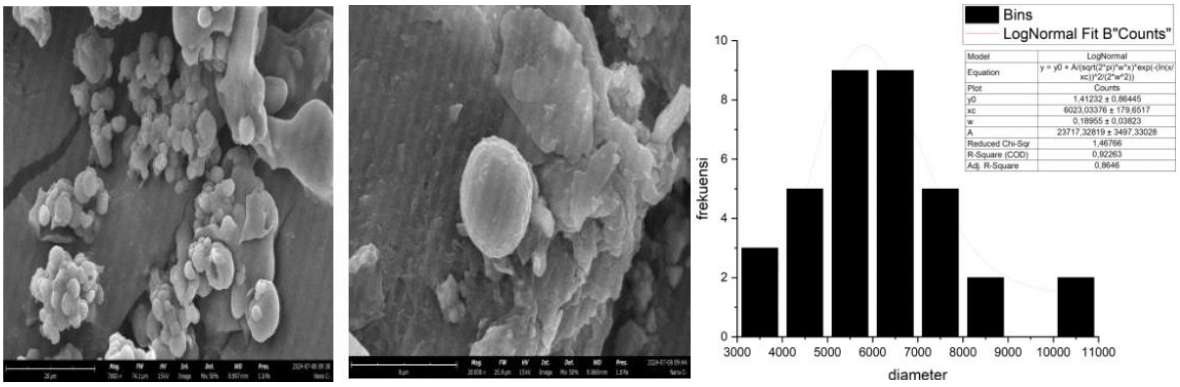

Figure 1: SEM–EDX Micrographs. Key parameters include a low point standard error (PSE = 0.00075), indicating precise model alignment, and ratios such as Lei (≈5.7≈5.7), All (≈5.9≈5.9), and ZL (≈6.1≈6.1), which may reflect signal-to-noise or compositional consistency metrics. The Rho/ved C1-Sig ratio (≈0.21≈0.21) and ECGQ value (4.054.05) imply moderate correlation or variance in elemental distribution, while the adjusted R^2 (0.0250.025) highlights limited explanatory power of the model. Treatment values (3000–11000) likely represent experimental conditions (e.g., energy levels or exposure durations) applied during analysis, with higher values correlating to intensified SEM–EDX operational parameters.

Table 4: Effect of Gotu kola-PEG-400 nanoencapsulated formulations on cognitive impairment

Treatment		Latency period (s) in the T-maze test					
		U1	**U2**	**U3**	**U4**	**U5**	**Mean ± SD**
Placebo	*Pretest*	8.44	6.15	6.58	7.15	7.37	7.14 ± 0.87
	Posttest	7.56	8.53	7.49	6.19	6.37	7.23 ± 0.96
Dosage 1:1	*Pretest*	7.56	7.34	7.79	7.54	7.29	7.50 ± 0.19
	Posttest	8.56	7.34	6.39	6.53	6.42	7.05 ± 0.93
Dosage 1:100	*Pretest*	10.56	8.34	8.59	7.10	7.29	8.38 ± 1.38
	Posttest	8.34	7.23	7.49	6.32	5.29	6.93 ± 1.17
Dosage 100:1	*Pretest*	7.51	8.34	7.39	7.45	6.47	7.43 ± 0.70
	Posttest	6.58	7.34	7.59	7.90	6.20	7.12 ± 0.70

for neurodegenerative disorders requiring targeted BBB penetration and anti-inflammatory action.

Effect of PEG-400 nanoencapsulated Gotu kola on TNF-α expression

TNF-α expression in brain tissues treated with PEG-400 nanoencapsulated Gotu kola was assessed using flow cytometry and immunohistochemistry (IHC). Brain specimens were sectioned into 10-µm slices, mounted on slides, and stained with fluorescently labelled anti-TNF-α antibodies. Fluorescence microscopy (Figure 2) revealed diminished TNF-*α* immunoreactivity in treated groups compared to controls, a trend corroborated by flow cytometry quantification (Figure 3), which demonstrated dose-dependent TNF-α suppression: the control group exhibited 74.65% ± 3.2 TNF-α-positive cells, followed by 62.50% ± 2.8 (1:1 formulation), 59.91% ± 2.5 (100:1 formulation), and the most significant reduction in the 1:100 group (54.75% ± 1.9, $p < 0.01$ vs. control).

This marked decrease in TNF-α levels aligned with the 1:100 formulation's superior cognitive performance in T-maze trials, attributable to its optimized nanoparticle characteristics (5 - 7 nm size, homogeneous distribution) that enhanced blood-brain barrier penetration and anti-inflammatory efficacy. Histopathological analysis (Figure 2) further supported these results, showing attenuated neuroinflammatory markers such as microgliosis in the 1:100 group. The inverse correlation between TNF-α suppression and cognitive improvement underscores neuroinflammation's pivotal role in age-related cognitive decline and positions Gotu kola-PEG-400 nanoformulations, particularly the 1:100 ratio, as a promising therapeutic strategy for neurodegenerative disorders.

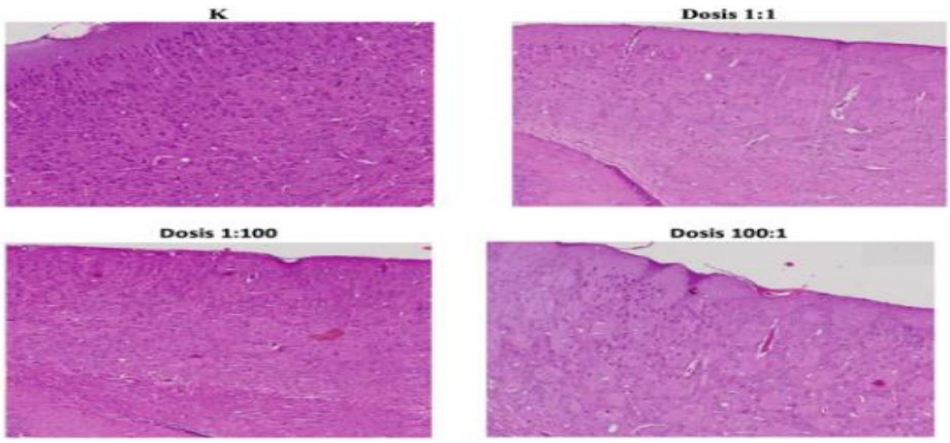

Figure 2: Histopathological Observation of TNF-α Staining and Flow Cytometry. The histopathological analysis of TNF-α staining and flow cytometry revealed distinct patterns across dosage ratios. At the **1:1** dosage ratio, moderate TNF-*α* staining intensity was observed in tissue sections, suggesting balanced inflammatory activity, while flow cytometry indicated proportional immune cell activation aligned with the expected therapeutic response. In contrast, the 1:100 dosage ratio exhibited minimal TNF-α staining, reflecting subdued inflammation, and flow cytometry results showed reduced effector cell populations, likely attributable to diminished treatment efficacy from high dilution. Conversely, the **100:1** dosage ratio displayed intense TNF-α staining, correlating with pronounced inflammation or tissue stress, and flow cytometry demonstrated a marked increase in activated immune cells, indicative of a hyperactive immune response or potential cytotoxicity at this elevated concentration. These observations collectively link histopathological findings (TNF-α staining intensity) to flow cytometry outcomes (immune cell activation and population dynamics) across the tested dosage ratios.

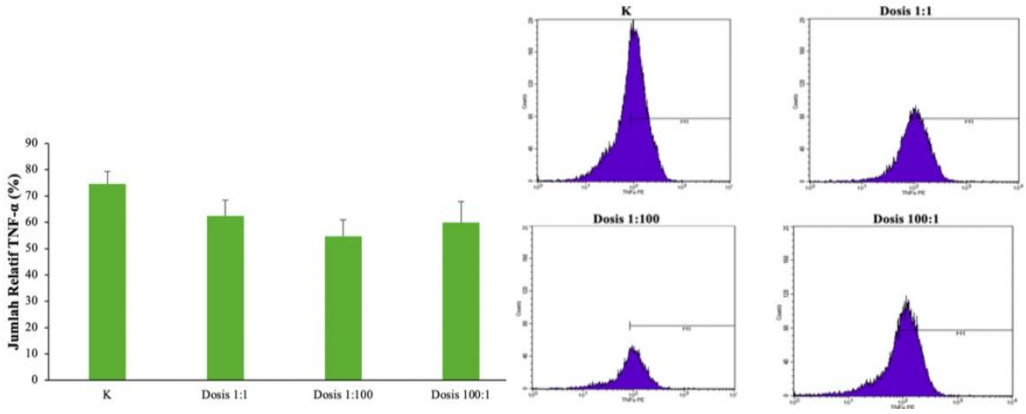

Figure 3: Graph and histogram of TNF-α staining and flow cytometry. The graph illustrates the percentage of TNF-α staining intensity (0–100%) across three dosage ratios (1:1, 1:100, and 100:1), with staining intensity increasing proportionally to dosage concentration. The histogram complements these findings by displaying flow cytometry data, highlighting distinct immune cell population dynamics: the 1:1 ratio shows balanced activation, the 1:100 ratio reflects reduced effector cells due to high dilution, and the 100:1 ratio demonstrates a marked surge in activated immune cells, suggesting potential hyperactivation or cytotoxicity. Both visualizations align to correlate TNF-α expression levels with immune response outcomes under varying experimental conditions.

Conclusion

The 1:100 PEG-400 nanoencapsulated Gotu kola formulation (1 mg extract: 100 mL PEG-400) emerged as the most promising candidate against cognitive decline, combining optimal physicochemical traits with strong neuroprotective effects. GC-MS profiling identified tryptamine, *γ*-sitosterol, and *β*-sitosterol (7.22% each) as major bioactives with known anti-inflammatory and neuroprotective roles. Nanoparticles showed uniform spherical morphology (5 - 7 nm, sphericity index: 0.8) and homogeneous distribution ideal for BBB penetration. The formulation achieved complete acetylcholinesterase (AChE) inhibition (-0.065 absorbance), preserving synaptic acetylcholine. TNF-α flow cytometry showed significant reduction in neuroinflammation (54.75% vs. 74.65% in controls; $p < 0.01$), which correlated with improved cognition (1.44 ± 0.21 s latency reduction). These results support the 1:100 Gotu kola–PEG-400 nanoformulation as a potent multi-target therapy for Alzheimer's and age-related cognitive decline.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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