Original Research Report

Centella asiatica NANOPARTICLES AS POTENTIAL ACETYLCHOLINESTERASE INHIBITOR FOR COGNITIVE DECLINE THERAPY USING ELLMAN'S METHOD: AN IN VITRO STUDY

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ABSTRACT

Cognitive impairment, caused by neurocognitive changes and neuroinflammation, affects 65.6 million elderly people worldwide and can interfere with their quality of life. *Centella asiatica* is recognized for its neuroprotective potential due to its active compounds. This study aimed to investigate the acetylcholinesterase inhibitory properties of *Centella asiatica* as potential therapeutic agents for cognitive decline. Polyethylene glycol 400 (PEG-400) was used to achieve an effective drug delivery system of *Centella asiatica* extract, facilitating the inhibition of the apoptosis signaling pathway and allowing neuroprotective agents to cross the blood-brain barrier (BBB). This research involved several testing stages, including gas chromatography-mass spectrometry (GC-MS) to identify active compounds (e.g., tryptamine, γ-sitosterol, and β-sitosterol) that contribute to cognitive function improvement. Particle size analysis (PSA) tests were conducted on three formulations of the extract and PEG-400, with ratios of 1:100, 100:1, and 1:1, to determine the optimal formulation for subsequent testing. Scanning electron microscopy (SEM) was utilized to observe the morphology and surface structure of the samples, while Ellman's method was employed to test the ability of acetylcholine (ACh) in improving cognitive abilities. The results subsequently underwent descriptive analysis, particle distribution analysis, analysis of variance (ANOVA), nonparametric tests, image analysis, regression tests, multivariate analysis, and correlation tests. The synthesis demonstrated that the 1:100 formulation produced ideal-sized nanoparticles (5–7 nm), optimal for penetrating the BBB. The PSA and SEM analyses supported this finding by demonstrating homogeneous particle morphology and consistent chemical composition. The in vitro Ellman's assay revealed a high inhibitory rate of 97.63% for the 100:1 formulation. The 1:1 and 1:100 formulations demonstrated a very high effectiveness as acetylcholinesterase inhibitors. The combination of PEG-400 and *Centella asiatica* extract has great potential as an innovative pharmacological therapy for cognitive decline. However, further research is required to ensure the right dosage and development of the research findings.

Keywords: Cognitive function; gotu kola (*Centella asiatica* (L.) Urban); nanoparticles; geriatrics; herbal medicine

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Highlights:

1. This research contributes novel data to current studies by combining the potential of *Centella asiatica* extract with PEG-400 to develop a therapeutic agent aimed at improving cognitive function through an effective drug delivery system capable of penetrating the blood-brain barrier.
2. The findings of this study revealed that the combination of *Centella asiatica* extract and PEG-400 at an adequate ratio exhibits great potential as an acetylcholinesterase inhibitor.

INTRODUCTION

Cognitive decline is a significant problem among the aging population, with millions of older adults worldwide experiencing it. The World Health Organization (2021) reported that approximately 65.6 million older adults around the world experience cognitive impairment, which is primarily caused by neurocognitive and neuroinflammatory changes. These changes not only reduce the older adults' quality of life, but also present challenges to healthcare systems. Consequently, there is an urgent need for effective therapeutic interventions that can mitigate these impacts.

Recent studies, including those conducted by Wright et al. (2022) and Liu et al. (2022), have highlighted the potential of *Centella asiatica* extract in improving cognitive function, mainly due to its active ingredients. *Centella asiatica* is commonly known as *gotu kola* or *vallarai* and is locally referred to as *pegagan* in Indonesia. The plant is a perennial herb from the Apiaceae family and is widely found in tropical countries, such as Indonesia. *Centella asiatica* contains chemical contents, such as tryptamine, which have been proven effective in accelerating brain regeneration by reducing neuroinflammation levels Chen et al. (2014). The mechanism involves the inhibition of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and brain-derived neurotrophic factor (BDNF). In addition, this plant can prevent apoptotic death in the central nervous system and improve cognitive function, memory capacity, and antidepressant effects (Puttarak et al. 2017). However, the delivery of active compounds derived from *Centella asiatica* to the brain remains a challenge. Polyethylene glycol 400 (PEG-400) has emerged as a promising carrier for drug delivery due to its ability to increase the penetration of active compounds across the blood-brain barrier (BBB). The combination of *Centella asiatica* extract with PEG-400 may have the potential to increase the efficacy of the extract by facilitating better delivery to the brain.

The high increase in cases of cognitive function disintegration all over the world underlies the need for research into the mechanism of age-related changes that reduce cognitive function (memory) in older adults. There is also a necessity to determine the risk factors of these changes to facilitate the development of preventive and therapeutic interventions as new innovations. Research on *Centella asiatica* nanoparticles (NanoCentella) must be carried out to assess their potential in improving cognitive function and quality of life in individuals over 60 years of age. The use of PEG-400 is important in drug delivery systems because of its excellent delivery capabilities. The active constituents of *Centella asiatica*, such as asiatic acid, act as neuroprotective agents through the inhibition of apoptotic signaling pathways (Fan et al. 2018). The formulation of *Centella asiatica* extract and PEG-400 may contribute to the realization of Sustainable Development Goals, specifically good health and well-being. This new formulation has the potential as a therapeutic approach for cognitive decline. Further research is necessary to optimize the dosage and fully understand the long-term effects of this combination, paving the way for the development of innovative pharmacological therapies for neurodegenerative diseases. The aim of this research was to evaluate the acetylcholinesterase inhibitory properties of *Centella asiatica* active constituents as potential therapeutic agents for cognitive decline.

MATERIALS AND METHODS

Conducted at Universitas Airlangga, Surabaya, Indonesia, this study focused on the synthesis and characterization of nanoparticle formulations that combined *Centella asiatica* extract with PEG-400. The research commenced with the preparation of *Centella asiatica* extract formulations. Given that the extraction process could alter the structure and composition of the chemical compounds within *Centella asiatica*, gas chromatography-mass spectrometry (GC-MS) was selected for the identification of active compounds. This technique was chosen due to its capacity to analyze complex mixtures without the need for reference standards, relying on an extensive compound library instead (Wei et al. 2014, Špadina et al. 2019).

As outlined by Olivia et al. (2021), *Centella asiatica* extract is typically dissolved in methanol or ethanol prior to the GC-MS analysis. Before analysis, the extracted sample underwent filtration to remove particulate matter that could obstruct the gas chromatography (GC) column. The sample was then introduced into the GC system via an autosampler, which automatically performed the injection. The sample was heated in the injector until it vaporized, at which point it was carried into the GC column by a carrier gas, usually helium or hydrogen. The GC column facilitated the separation of the compounds according to their volatility differences and interaction with the stationary phase of the column. Compounds having greater volatility, or those that exhibit minimal interaction with the stationary phase, would elute from the column more rapidly. The column temperature was generally increased in a stepwise manner to enhance the separation of compounds with varying boiling points. After exiting the GC column, the separated compounds were introduced into the mass spectrometer (MS) for ionization. The resulting ions were subsequently

sorted according to their mass-to-charge (m/z) ratio by an analyzer, such as a quadrupole or ion trap. These ions were detected, and a mass spectrum was generated for each one. The mass spectra were analyzed and compared to a library of known spectra, enabling the identification of the compounds through their distinctive fragmentation patterns.

After the completion of the GC-MS analysis and the collection of appropriate results, the process was continued with the synthesis of *Centella asiatica* formulations. The process of synthesizing *Centella asiatica* extract formulations with PEG-400 was carried out by creating three test groups with different ratios of 1:1, 1:100, and 100:1. The comparison was conducted with the aim of optimizing the concentration variations depending on the proportion between *Centella asiatica* extract and PEG-400. The three test groups consisted of group 1 (one part *Centella asiatica* extract to one part PEG-400), group 2 (one part *Centella asiatica* extract to 100 parts PEG-400), and group 3 (100 parts *Centella asiatica* extract to one part PEG-400). These three formulation groups were mixed using a magnetic stirrer for 15 minutes at a speed of 1,500 rpm and sonicated using a Scientz ultrasonicator for 5 minutes, with pulse modes of 10 seconds on and 5 seconds off to achieve particle homogenization. After sonication, the formulations were left for an hour, after which the solution was separated from the sediment and stored in a closed glass container at room temperature (Jookjantra & Wongwuttanasatian 2019).

The subsequent stage of this research was the characterization of the formulation results. Particle size analysis (PSA) was selected as the technique for determining the particle size distribution in the samples (Zinatloo-Ajabshir 2022). In the context of *Centella asiatica* extract mixed with PEG-400, PSA facilitated the characterization of particle size, which was important for product formulation and application. PSA tests were carried out on the three formulations to determine the most optimal formulation for subsequent testing. Before the measurement, the PSA equipment was calibrated to ensure accurate results. The sample suspension was injected into the measuring cell of the PSA equipment. Laser diffraction was the technique employed to measure the particle size distribution in the suspension (Kulkarni & Shaw 2016). Measurement data were collected in three replicates to ensure result consistency. The data obtained from the PSA were evaluated to determine the average and distribution of particle size. A graph depicting the particle size distribution was generated to visualize the results.

Scanning electron microscopy (SEM) served as the analytical technique for observing the morphology and surface structure of the sample at a microscopic level (Gutierrez et al. 2017). In the characterization of *Centella asiatica* extract mixed with PEG-400, this technique provided high-resolution images of particle morphology, which aided in understanding the surface structure and shape of the particles. In addition to qualitative visualization, SEM also facilitated the quantitative measurement of particle size and distribution analysis (Inkson 2016). The formulation evaluated through SEM was 1:100, as it produced ideal-sized nanoparticles according to the PSA test results. The mixture of *Centella asiatica* extract and PEG-400 was placed in a bottle and frozen at ultra-low temperatures (-40°C to -80°C). The frozen sample was then dried in a freeze dryer to remove water and solvents, resulting in a dry powder. The freeze-dried powder of the mixture was taken in small amounts and placed on the SEM stub (sample support) using carbon tape or conductive adhesive. The sample was coated with a thin layer of platinum metal using a sputter coater. This coating improved the conductivity of the sample and the quality of the SEM images. The coating process required several minutes to achieve a metal layer with a thickness of 5–10 nm. The SEM equipment was calibrated in accordance with its specifications to ensure accurate measurements. Subsequently, the sample was loaded into the SEM vacuum chamber. The sample surface was observed at various magnifications to identify relevant morphological features. Additionally, images were captured at different magnifications to analyze the morphology and particle distribution in detail.

The ability of acetylcholine (ACh) to improve cognitive abilities was evaluated through Ellman's method. This test served as an initial assessment prior to clinical trials, considering the significant role of acetylcholine as a neurotransmitter in the nervous system (Decker & Duncan 2020). The role of acetylcholine in increasing the conductance of neuron cells could indicate its impact on the acceleration of information transmission, specifically in increasing the ability and function of the brain comprehensively as well as delaying the aging process of neuronal cells (Huang et al., 2022). Dinitrobenzoic acid (DTNB), also known as Ellman's reagent, was used as an indicator in this test. The test began by dissolving the three formulations of *Centella asiatica* extract and PEG-400 in a pH 8 solution. Acetylcholinesterase (AChE) enzyme solution was added to each test tube, followed by incubation at room temperature for a few minutes to facilitate the interaction between the enzyme and the inhibitor. Thereafter, Ellman's reagent was added to the mixtures. DTNB would react with thiocholine produced from the breakdown of acetylthiocholine iodide (AChI) by

acetylcholinesterase, resulting in a yellow color. The second incubation took around 10–30 minutes. Absorbance measurements were performed at a wavelength of 412 nm using a spectrophotometer to assess enzyme activity. A reduced absorbance indicated stronger acetylcholinesterase inhibition by the formulation.

The data from the tests were subjected to statistical analysis to conclude the results. The statistical analysis for the PSA included descriptive analysis, particle distribution analysis, analysis of variance (ANOVA), nonparametric tests, regression tests, and correlation tests to evaluate particle size and distribution. The analysis for the SEM comprised descriptive statistical analysis, ANOVA, nonparametric tests, image analysis, and multivariate analysis that were carried out to analyze surface morphology and particle shape (van der Pol et al. 2014).

RESULTS

Gas chromatography-mass spectrometry (GC-MS) analysis of compounds derived from *Centella asiatica*

The GC-MS analysis identified several compounds from *Centella asiatica*, including tryptamine, gamma-sitosterol (γ-sitosterol), and beta-sitosterol (β-sitosterol). The detailed results of this analysis are shown in Table 1. The data showed that the sample contained three distinct compounds with different relative quantities and varying levels of identification reliability.

The relative quantity of tryptamine was minimal, at 1.79%, indicating its lesser presence compared to the other two compounds. On the other hand, γ-sitosterol and β-sitosterol exhibited identical area percentages of 7.22%, signifying that these two compounds were present in larger and relatively equal amounts in the sample. Tryptamine had a low identification quality score of 27, suggesting that the identification result might not be very reliable. The identification quality score for γ-sitosterol was very high at 99, indicating a very reliable identification. Similarly, β-sitosterol also had a high identification quality score of 91, indicating a reliable identification.

Particle size analysis (PSA) of *Centella asiatica* extract and PEG-400 formulations

The formulation of *Centella asiatica* extract with a 1:100 ratio to PEG-400 was chosen due to its ability to produce nanoparticles of approximately 5–7 nm in size. This size was optimal for penetrating the lipophilic blood-brain barrier without becoming too small, preventing it from interacting easily with the external environment or disintegrating before reaching the target cells, namely neurons in the brain. The consistency of the nanoparticle size rendered the 1:100 formulation the most optimal choice for neuroprotective drugs.

Table 1. Results of gas chromatography-mass spectrometry (GC-MS) analysis.

Compounds	RT	Area (%)	Quality	MW (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

Notes: RT=retention time; MW=molecular weight; REF=reference number

The 1:1 formulation produced nanoparticles that were too small due to the difficulty of achieving a homogenous dispersion of *Centella asiatica* extract with PEG-400. The data showed that the particle size in this formulation was undefined, indicating the failure of PEG-400 to encapsulate the extract perfectly. As a result, the 1:1 formulation was not homogeneous and ineffective as a drug due to the uneven distribution of active substances. The 100:1 formulation was not deemed completely homogeneous because of the unbalanced amount of PEG-400 compared to the *Centella asiatica* extract, leading to its classification as "undefined" in Table 2. This resulted in a formulation that was not encapsulated perfectly, rendering it ineffective as well. Thus, the 1:100 formulation was selected as the most favorable option because it produced ideal-sized nanoparticles and exhibited homogeneous distribution of active substances, establishing it as a potential formulation for neuroprotective drug development.

Table 2. Characteristic interpretation of the particle size analysis (PSA) results.

Samples	Sample refraction index	Dispersant	Dispersant refractive index	Temperature (°C)	Minimum particle size (nm)	Maximum particle size (nm)
1:1	1.59	Water	1.33	25	0.8	1
1:100	1.59	Water	1.33	25	0.5	7
100:1	1.59	Water	1.33	25	Undefined	Undefined

Scanning electron microscopy (SEM) of *Centella asiatica* extract nanoparticles

The SEM analysis revealed that the specimen coated with Au-Pd had a particle morphology with a sphericity of 0.8 (approaching 1). Furthermore, an

average size of 5-7 nm was detected. These results indicated a homogeneous and uniform size distribution.

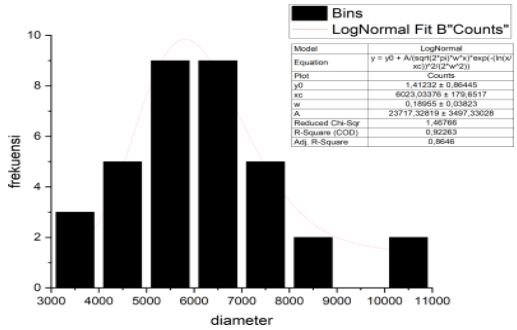

Figure 1. Analysis of the scanning electron microscopy (SEM) results.

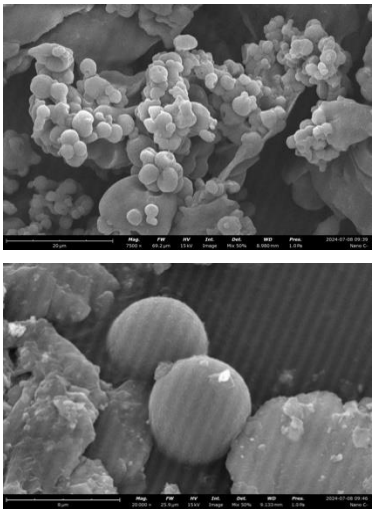

Figure 2. The scanning electron microscopy (SEM) imaging of *Centella asiatica* nanoparticles.

As shown by the data in Figure 1, the xc value was 6,023.03376 with a standard error of 179.6517, indicating that around 6,023 particle units were frequently distributed log-normally within the sample. The w value was 0.18955 with a standard error of 0.03823, suggesting that the distribution was rather narrow, while the particle size in the sample was quite uniform and did not vary drastically. The histogram depicted the particle size distribution, with most particles being around 5,000–7,000 units. The red log-normal fitting curve closely aligned with the histogram, indicating that the particle size distribution in this sample conformed to a log-normal distribution. The fit statistics revealed an R-squared value of 0.92263 and an adjusted R-squared of 0.8646, signifying that the log-normal model accounted for approximately 92.26% of the variance in the data. Overall, the results of the analysis demonstrated that the particle size distribution was accurately described by the log-normal model, with a distribution center at around 6,023 units and a significant variance in particle size. These results indicated that the 1:100 formulation produced nanoparticles with optimal size and distribution. Images of *Centella asiatica* nanoparticles obtained using SEM are shown in Figure 2.

In vitro test of *Centella asiatica* extract and PEG-400 formulations using Ellman's assay

The formulations of *Centella asiatica* extract and PEG-400 in ratios of 1:100 and 1:1 exhibited effectiveness in inhibiting the acetylcholinesterase reaction that converts the acetylthiocholine (ATCh) substrate from acetylthiocholine iodide into intermediate compounds of thiocholine and acetic acid. This was confirmed by the negative absorbance values obtained from the ultraviolet-visible (UV-Vis) spectrophotometer for both formulations, as shown in Table 3.

Table 3. Interpretation of experimental data from the Ellman's assay.

Samples	Wavelength	Absorbance	Interpretation	Relative absorbance	Inhibition Ability
Blank	412 nm	0.338	-	0%	-
1:1	412 nm	-0.102	No change: enzymatic reaction (-)	-	100%
1:100	412 nm	-0.065	No change: enzymatic reaction (-)	-	100%
100:1	412 nm	0.346	No change: enzymatic reaction (+)	2.37%	97.63%

Ellman's reagent (DTNB) added to the test tubes containing the 1:1 and 1:100 formulations failed to yield yellow luminescence due to the absence of thiocholine resulting from the formulations' role as an acetylcholinesterase inhibitor. This eliminated the precursor for converting DTNB into 2,5-mercapto thiocholine nitrobenzoate, which could be responsible for the yellow luminescence. The difference in absorbance resulted from the varying concentrations of *Centella asiatica* extract used. The 100:1 formulation demonstrated high inhibition with an effectiveness of 97.63%, highlighting the potential of *Centella asiatica* extract as an acetylcholinesterase inhibitor. However, the formulation with a higher ratio showed a decrease in effectiveness, possibly because of its nanoparticles. The 1:1 and 1:100 formulations exhibited a more ideal and realistic structure for competing with the substrate to occupy the enzyme site as an inhibitor. Further validation through characterization tests might clarify the specifications of an improved formulation.

DISCUSSION

Compounds analysis through gas chromatography-mass spectrometry (GC-MS)

The GC-MS analysis identified three essential compounds: tryptamine, gamma-sitosterol (γ-sitosterol), and beta-sitosterol (β-sitosterol), each contributing differently to the sample's composition and potentially boosting cognitive performance. Tryptamine was detected at a retention time (RT) of 32.584 minutes, covering only 1.79% of the overall area, thus rendering it the least abundant compound among the three analyzed. Its identification quality score was 27, indicating reduced confidence in the outcome, potentially attributable to lowered peak intensity, probable interference, or limits in library matching. Despite its low abundance and low reliability rating, tryptamine is a well-known bioactive molecule. It serves as a precursor for neurotransmitters such as serotonin and melatonin, and even in small quantities, it may have a significant role in modulating cognitive function (Nguyen et al. 2021).

Compared to tryptamine, γ-sitosterol was present in a higher proportion, with an area percentage of 7.22%, reflecting a more significant contribution to the sample. It had the same retention time (RT) as β-sitosterol (38.146 minutes), indicating structural similarities. This molecule received a quality score of 99, signifying a very reliable identification, most likely due to well-matched mass spectral data (Ambavade et al. 2014). As γ-sitosterol is a phytosterol with neuroprotective, anti-inflammatory, and antioxidant properties, it potentially contributes to cognitive advantages. Lastly, we also highlighted that β-sitosterol accounted for 7.22% of the total area and exhibited the same molecular weight (414.386 amu) and retention duration as γ-sitosterol. It achieved a somewhat lower quality score of 91, which nevertheless signified a high level of confidence in its identification. This phytosterol offers numerous benefits, including reducing inflammation, improving brain health, and enhancing cognitive performance (Sharma et al. 2021).

The presence of γ-sitosterol and β-sitosterol in equal amounts suggests potential synergistic actions or equal contributions to the sample's overall effects on cognitive performance. Tryptamine, despite its low abundance, still has the potential to exert major neurochemical effects due to its direct role in neurotransmitter synthesis. The analysis through GC-MS identified several compounds from *Centella asiatica*, including tryptamine, γ-sitosterol, and β-sitosterol. These compounds play a role in improving cognitive function (Ambavade et al. 2014).

Particle size analysis (PSA) across several formulations

The PSA results provided important information about the formulation and activity of *Centella asiatica* extract nanoparticles when combined with PEG-400 as a dispersant. The 1:100 formulation exhibited an obvious advantage, creating nanoparticles in the appropriate size range of 5–7 nm. This range is crucial for breaching the blood-brain barrier, which is a major obstacle in drug administration for neurodegenerative illnesses. Nanoparticles of this size range have demonstrated improved penetration across the lipophilic blood-brain barrier while remaining stable enough to avoid premature breakdown in systemic circulation (Teleanu et al. 2018). Therefore, the 1:100 formulation is ideal for administering neuroprotective medications to neurons. The 1:1 formulation failed to generate relevant findings because the particle size was too small, at less than 1 nm. This indicated that PEG-400 could not evenly encapsulate the *Centella asiatica* extract at such a high ratio of dispersant. The efficiency of encapsulating active substances directly impacts the uniformity of their distribution within the formulation. Poor encapsulation in the 1:1 formulation led to an inefficient drug delivery system, since the nanoparticles might undergo rapid degradation or removal before reaching the target site. The 100:1 formulation encountered issues on the opposite end of the spectrum. The undefined particle size statistics showed that this ratio did not provide sufficient dispersion (PEG-400) to achieve nanoparticle stability and uniformity. This might result in bigger, irregularly shaped aggregates or an uneven distribution of the active component, compromising the formulation's efficacy.

As outlined by Obadimu et al. (2024), the findings of this study also highlighted the need of maintaining an appropriate balance between the extract and the dispersion to produce stable, consistently sized nanoparticles. Furthermore, the refractive index data and dispersion properties underscored the crucial role of homogeneity in nanoparticle formulation. Finally, the PSA results emphasized the importance of formulation ratios in nanoparticle development. While the 1:1 and 100:1 formulations failed to meet the criteria for stable and effective drug administration, the 1:100 formulation achieved the optimum balance of size, stability, and homogeneity, rendering it a promising choice for neuroprotective drug development. Nevertheless, future research should investigate additional factors, including encapsulation efficiency, drug release kinetics, and in vivo performance, to confirm the therapeutic potential of *Centella asiatica* extract and PEG-400 formulations.

Nanoparticle analysis using scanning electron microscopy (SEM)

The SEM analysis provided essential information on the morphological properties and particle size distribution of the nanoparticles generated in the 1:100 formulation. The observed particle morphology, with a sphericity value of 0.8, suggests that the particles were almost spherical, which is a desirable feature for drug delivery systems. Spherical particles often have lower aggregation tendencies and better flow properties, hence improving their stability and bioavailability upon delivery. These dimensions are ideal for applications that require passage through the blood-brain barrier, as particles within this size range are more likely to penetrate the barrier effectively while maintaining stability (Teleanu et al. 2018, Obadimu et al. 2024).

The histogram analysis confirmed the uniformity of size, with most particles falling between 5,000 and 7,000 nanometers and following a log-normal distribution. The statistical metrics obtained from the SEM study validated the homogeneity of the particle distribution. The particle size distribution was narrow, as evidenced by the central size value of 6,023 nm and a tiny standard error (179.6517 nm). This suggests low variation across the sample, implying that the formulation process effectively created nanoparticles of constant size and morphology. Such homogeneity is critical for establishing predictable pharmacokinetics and regulated drug release, which are important qualities for neuroprotective medication formulations (Obadimu et al. 2024).

The log-normal fitting curve in the analysis significantly aligned with the histogram data, exhibiting an adjusted R-squared of 0.8646. The log-normal model accurately characterized the particle size distribution, as evidenced by its high R-squared value of 92.26%. Log-normal distributions are frequently seen in nanoparticle systems because of the multiplicative factors that influence particle size throughout the synthesis process, such as nucleation and growth dynamics (Wu et al. 2022). The w value of 0.18955 with a standard error of 0.03823 reinforced the conclusion that the distribution was limited and stable. Nanoparticles with a narrow w value have low size fluctuation and are more likely to be distributed uniformly in vivo. The combination of spherical form, optimized size, and thin size distribution established the 1:100 formulation as a strong candidate for drug delivery applications, particularly in neuroprotective therapies targeting the brain.

Analysis of acetylcholinesterase inhibition using Ellman's method

Ellman's method involves the use of a chromogenic substrate, typically acetylthiocholine iodide, which is hydrolyzed by acetylcholinesterase to produce thiocholine. Thiocholine subsequently reacts with 5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB), generating a yellow-colored product, 5-thio-2-nitrobenzoic acid, which can be quantitatively measured through spectrophotometry at 412 nm (Patel 2023). The intensity of the yellow color is directly proportional to the enzymatic activity of acetylcholinesterase. The results of this study showed acetylcholinesterase inhibition rates ranging from 97.63% to 100%, indicating the ability of *Centella asiatica* constituents in maintaining higher levels of acetylcholine in the brain, thereby improving cognitive functions such as memory, attention, and learning (Jusril et al. 2020). This mechanism aligns with the traditional use of *Centella asiatica* in enhancing cognitive health and provides a scientific basis for its potential therapeutic use in neurodegenerative diseases, such as Alzheimer's disease.

The mechanisms for inactivating acetylcholinesterase may be reversible or irreversible, with compounds likely exerting their inhibitory effect by binding to the active site of acetylcholinesterase. The active site of acetylcholinesterase contains a catalytic triad composed of serine, histidine, and glutamate residues, which are crucial for its enzymatic activity (Walczak-Nowicka & Herbet 2021). The substrate, acetylcholine, normally binds to this site, where it is hydrolyzed into choline and acetate. Molecular docking suggests that asiatic acid can form hydrogen bonds with the catalytic triad and surrounding amino acids, thereby occupying the binding site of acetylcholine. Additionally, the hydrophobic tail of asiatic acid may interact with the hydrophobic pockets of acetylcholinesterase, enhancing its binding affinity and inhibitory potency.

Efficacy of different concentrations analyzed in this study

The results of this study provided important insights into the development of nanoparticles using *Centella asiatica* extract combined with PEG-400, particularly in addressing the challenges of drug delivery through the blood-brain barrier for neuroprotection purposes. The findings suggested that the 1:100 formulation is optimal in producing nanoparticles with an ideal size range of 5–7 nm, which is critical for blood-brain barrier penetration and effective neuroprotection. This formulation exhibited a homogeneous distribution of active compounds, enhancing the potential efficacy of the

drug in targeting neurons and reducing cognitive decline. The 1:100 formulation demonstrated an inhibitory efficacy of 97.63% as an acetylcholinesterase (AChE) inhibitor, underlining the therapeutic potential of *Centella asiatica* in neurodegenerative conditions such as Alzheimer's disease. These results are in line with previous research, such as a study conducted by Puttarak et al. (2017), which highlighted the neuroprotective properties of *Centella asiatica*, particularly in reducing neuroinflammation and supporting cognitive function. However, this study complemented previous studies by successfully encapsulating active compounds in a nanoparticle formulation with PEG-400, thereby improving their bioavailability and efficacy in crossing the blood-brain barrier, which was a significant challenge previously. In contrast to the 1:100 formulation, the 1:1 and 100:1 formulations did not produce nanoparticles with optimal characteristics. The 1:1 formulation produced particles that were too small and less homogeneous, rendering them ineffective for drug delivery. Similarly, the 100:1 formulation showed suboptimal encapsulation of the extract, leading to inconsistent distribution and reduced efficacy as a neuroprotective agent. These findings emphasized the importance of using the appropriate ratio in the formulation process to achieve nanoparticles with desired properties.

Strength and limitations

This study offers a novel approach by combining *Centella asiatica* extract with PEG-400, a combination that had not been widely explored previously. This enhances the effectiveness of drug delivery to the brain, especially for treating cognitive decline. With a focus on improving cognitive function in older adults, this study is highly relevant to current global health challenges, especially considering the high prevalence of cognitive impairment in this population. The use of various analytical techniques, including GC-MS, PSA, SEM, and in vitro assays using Ellman's method, provided in-depth and comprehensive data on the characteristics of the developed nanoparticles. This study successfully demonstrated the neuroprotective potential of a 1:100 nanoparticle formulation of *Centella asiatica* extract and PEG-400. The newly developed formulation may serve as an innovative pharmacological therapy for neurodegenerative diseases, supporting the third Sustainable Development Goal (SDG) of promoting good health and well-being.

Although this study offers a strong foundation for the development of new therapies for cognitive decline, further research is necessary to address limitations and ensure safe and effective clinical applications. While in vitro studies provide a favorable starting point, the results do not always translate directly to in vivo conditions. Further in vivo studies are necessary to confirm the efficacy and safety of the 1:100 nanoparticle formulation in humans, particularly concerning the supramolecular interactions of the drug with acetylcholine. This study did not include dose optimization of the *Centella asiatica* extract and PEG-400 combination, which is important for maximizing efficacy and minimizing side effects in clinical applications. The findings of this study may not be universally applicable without additional trials, necessitating consideration of individual response variations to the therapy, especially in vulnerable populations such as older adults with multiple medical conditions. Future research is required to investigate the long-term effects of using PEG-400 as a drug carrier for *Centella asiatica* extract to mitigate potential toxicity and other adverse effects associated with this therapy. Additional research is also recommended to explore the molecular mechanisms by which the formulation interacts with neuronal cells, aiming to expand understanding of its neuroprotective effects and facilitate its application in the treatment of various neurodegenerative diseases.

CONCLUSION

Centella asiatica extract in combination with PEG-400 exhibits outstanding potential for the treatment of cognitive decline. Nevertheless, the potential of adjuvant therapy using *Centella asiatica* extract combined with PEG-400 warrants further analysis. The standardization of herbal therapy for cognitive function is necessary due to its major impact on physiological processes and its potential to address global problems. *Centella asiatica* may substantially reduce neurodegeneration, especially in older adults, although additional clinical research is expected to validate this effect. This adjuvant therapy is anticipated to serve as an innovative pharmacological therapy with minimal adverse effects and improve the quality of life of older adults.

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Conflict of interest

None.

Ethical consideration

This research was conducted after receiving ethical clearance from the Health Research Ethics Committee of the Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia, under reference No. 119/EC/KEPK/FKUA/2024 dated 1/7/2024. This study adhered to the guidelines of the Committee on Publication Ethics (COPE), the World Association of Medical Editors (WAME), and the Declaration of Helsinki.

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Author contribution

nn contributed to the conception and design, analyzed and interpreted the data, and drafted the article. SCK, FK and RM analyzed and interpreted the data. RI contributed to the critical revision of the article for important intellectual content and final approval of the article.

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