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Research Article

Piper cubeba L. f. Nanoemulsion: Formulation and Effect on Antioxidant Activity in Rat's Hippocampus and Prefrontal Cortex

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Abstract

The previous study showed that *Piper cubeba* extract increased brain antioxidant capacity, alleviating cognitive impairment in rats. However, brain-targeted drug delivery is often limited by the blood-brain barrier. One of the approaches to overcome this obstacle is nanocarrier. This study aimed to formulate a nanoemulsion (NE) of *P. cubeba* extract and determine the antioxidant activity in the brain. The research begins with the determination of excipients and the formula of the nanoemulsion. The formula was then tested for globule size, polydispersity index, thermodynamic stability, zeta potential, pH, and viscosity. The globule's shape was determined using transmission electron microscopy (TEM). The antioxidant activity was tested by catalase and lipid peroxidase activity in the brain after giving NE orally for seven days. Formulations of NE resulted in nano-size globules and were stable thermodynamically. The NE formula significantly increased catalase and inhibited lipid peroxidase activities in the Rat's hippocampus and prefrontal cortex. This study resulted in a stable formula and low-cost production of *P. cubeba* extract NE, which is active as the brain's antioxidant and is feasible to develop as a neuroprotective agent for various neurodegenerative diseases.

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INTRODUCTION

Neurodegenerative diseases are often marked by a damaging combination of heightened oxidative stress and subsequent inflammation within the brain. This one-two punch can wreak havoc on brain cells, as excessive free radicals generated by oxidative stress lead to cellular damage. In response, the brain's immune system can trigger an inflammatory cascade, which, while intended to protect, often exacerbates the damage in chronic neurodegenerative conditions. This vicious cycle of oxidative stress fueling inflammation, and vice-versa, is a key driver in the progression of these debilitating diseases[1-3]. Antioxidants have been widely studied for their potential to protect the brain in neurodegenerative conditions like Parkinson's and Alzheimer's disease, as well as in acute events such as ischemic stroke. However, a significant challenge in treating these conditions is the blood-brain barrier (BBB), which severely restricts the passage of therapeutic compounds into the brain[4]. To overcome this hurdle, researchers have developed various strategies to enhance drug delivery to the brain. These approaches include direct intraparenchymal injections, which deliver drugs directly into brain tissue; transient disruption of the BBB, temporarily opening it to allow drug entry; drug modification to alter compounds for better BBB permeability; nanocarrier systems that encapsulate drugs for targeted delivery; and intranasal administration, a non-invasive method that bypasses the BBB by delivering drugs directly to the brain via nasal pathways[5].

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Despite a wealth of preclinical evidence highlighting numerous potential neuroprotector candidates, clinical trials have not consistently yielded promising outcomes[6]. This discrepancy is largely attributed to factors such as poor drug bioavailability in the brain and the delayed administration of neuroprotective agents in human patients. To overcome these challenges, the development of neuroprotectors in nano-dosage forms presents a viable solution[7]. Our previous research demonstrated the significant antioxidant potential of *Piper cubeba* L. f. extract, suggesting its promise as a neuroprotective agent[8]. However, the previously identified effective dose was prohibitively high, necessitating optimization. Consequently, this study aimed to formulate the *P. cubeba* extract into a nanoemulsion (NE) and evaluate its efficacy as a brain-specific antioxidant.

MATERIALS AND METHODS

Materials

All excipients utilized in this research were of pharmaceutical grade, ensuring suitability for formulation. The initial selection of the oil phase involved a solubility screening process, where the extract was dissolved in various oils at a 1:10 ratio (extract:oil, w/v) and visually observed for clarity. The oils evaluated included sunflower oil, oleum ricini, virgin coconut oil, olive oil, almond oil, corn oil, oleic acid, and Capryol 90 (Gattefosse, Saint-Priest, France). The oil that yielded a clear solution without any visible precipitation was subsequently chosen for further formulation development. Following oil selection, the most suitable surfactant was identified. This involved mixing 15% v/v of each surfactant (in deionized water) with 10 µL of the pre-selected oil. The resulting mixture was vortexed, and additional oil was incrementally added until a cloudy liquid formed. The surfactant capable of producing the clearest solution with the largest volume of oil was selected. Surfactants assessed in this stage included Tween 20, Tween 60, and Tween 80. Finally, a co-surfactant was chosen through the construction of pseudo-ternary phase diagrams, maintaining a fixed surfactant-to-co-surfactant (Smix) ratio of 1:1. Polyethylene glycol (PEG) 400 and propylene glycol were evaluated as potential co-surfactants in this study[9].

This study commenced with the meticulous selection of excipients and the subsequent formulation of NE. The prepared NE underwent comprehensive characterization, including analyses of globule size, polydispersity index (PDI), thermodynamic stability, zeta potential, pH, and viscosity. Furthermore, the morphology of the globules was precisely determined using transmission electron microscopy (TEM). For *in vivo* evaluation, NE was orally administered to rats at a dose of 200 mg/kg BW daily for seven consecutive days. Following this period, the antioxidant activity of NE was assessed by measuring catalase and lipid peroxidase activity in the rat brains.

Methods

Smix ratio selection

The selection of appropriate Smix ratios is critical for the successful formation of stable NE. In this study, the chosen Smix were systematically prepared at various ratios: 1:1, 2:1, 1:2, and 1:3. To comprehensively map the emulsion region and identify optimal formulation compositions, each Smix ratio was then blended with the oil phase across a wide range of oil-to-Smix proportions, specifically from 1:9 to 9:1 (oil: Smix).

Piper cubeba nanoemulsion preparation

The *P. cubeba* NE was meticulously prepared using a high-speed stirring method. Initially, the plant extract was precisely measured and combined with the designated oil phase. This mixture was then stirred at 1000 rpm and maintained at a controlled temperature of 25°C using a magnetic stirrer until a homogeneous solution was achieved. Subsequently, the Smix was gradually incorporated into the oil-extract blend, with continuous stirring to ensure complete miscibility and homogeneity. Finally, deionized water was added dropwise to the mixture while maintaining constant stirring for an additional 10 minutes.

Particle size, polydispersity index, and zeta potential evaluation

The particle size, PDI, and zeta potential of the samples were determined using a Particle Size Analyzer (DelsaMax PRO, Beckman Coulter, US). Prior to measurement, each sample was prepared by diluting it with the appropriate solvent or diluent to achieve an optimal concentration for analysis, ensuring accurate and reproducible results.

Viscosity determination

The viscosity of the prepared formulations was determined through single-point measurement using a ViscoQC 300 viscometer (Anton Paar, Austria).

pH determination

The pH of the nanoemulgel preparation was precisely measured using a calibrated digital pH meter at a controlled room temperature of 25±1°C.

Thermodynamic stability characteristics

The thermodynamic stability of the formulations was rigorously evaluated using a combination of centrifugal, heating-cooling cycle, and freeze-thaw cycle tests. First, the centrifugal test was performed by subjecting samples to 5000 rpm for 30 minutes at a controlled temperature of 25°C. This rapid stress test aimed to detect immediate signs of instability, such as phase separation, turbidity, or precipitation. Formulations demonstrating stability after centrifugation proceeded to the heating-cooling cycle test. This involved exposing samples to alternating temperatures of 40°C and 4°C, with each cycle lasting 24 hours, repeated for a total of three cycles. Subsequently, the stable formulations underwent a freeze-thaw cycle test. This involved three cycles of alternating temperatures, holding samples at -20°C and 25°C for 48 hours each. Throughout all stability assessments, key parameters including globule size, PDI, phase separation, turbidity, and precipitation were meticulously observed to determine the overall thermodynamic stability of the formulations[10].

Transmission electron microscopy analysis

Nanoemulsion morphology was determined using TEM. The sample was diluted with distilled water and examined with JEOL JEM-1400.

Brain sample preparation

Male Sprague Dawley rats, weighing 190-220 g, were utilized to evaluate antioxidant activity. The experimental protocol received ethical approval from the Universitas Muhammadiyah Prof. DR. HAMKA ethics committee in June 2023 (No: 02/23.06/02632). Prior to the study, all rats underwent a one-week acclimatization period, during which they had *ad libitum* access to standard laboratory chow and water.

For the experiment, rats were randomly assigned to two groups: NE of *P. cubeba* group, which received 200 mg/kg orally, and a control group, administered the vehicle orally, both for a duration of seven days. On the eighth day, animals were humanely anesthetized and subsequently decapitated. Brains were immediately isolated, rinsed thoroughly with cold Phosphate-Buffered Saline (PBS) (pH 7.4), and carefully blotted dry with absorbent paper. The prefrontal cortex and hippocampus were then precisely dissected on an ice tray. Each brain region was weighed, and a 10% (w/v) homogenate was prepared by adding a 0.1 M phenylmethylsulfonyl fluoride (PMSF)-PBS (pH 7.4) cocktail. Homogenization was performed at 4000 rpm for 20 minutes at 4°C.

Rat's brain catalase activity

Catalase activity in rat brain tissue was determined by measuring the decomposition of H_2O_2. Briefly, 100 µL of each brain tissue supernatant was incubated with 200 µL of 10 mM H_2O_2 at 37°C for 2 minutes. The reaction was then quenched by adding 1.2 mL of a working solution containing cobalt (II) chloride, sodium hexametaphosphate, and sodium bicarbonate (1:1:18 ratio). Samples were subsequently incubated in the dark for 10 minutes at room temperature. For the standard curve, phosphate-buffered saline (PBS) was used in place of the supernatant. The absorbance of each sample was measured spectrophotometrically at 450 nm. Catalase activity was calculated using the following **Equation 1**, where t is incubation time (2 minutes), S_0 is the absorbance of the standard, S is the absorbance of the sample, and f is dilution factor of the sample[11].

$$\text{Catalase activity (U/g)} = \frac{(2.393/t)\cdot\log(S0/S)}{f} \qquad [1]$$

Determination of rat's brain malondialdehyde

Malondialdehyde (MDA) levels in rat brain homogenates, indicative of lipid peroxidation, were quantified using the thiobarbituric acid reactive substances (TBARS) assay. Specifically, brain homogenate was combined with 20% (w/v) trichloroacetic acid (TCA) and 0.67% (w/v) thiobarbituric acid (TBA) in a 1:1:2 (homogenate:TCA:TBA) volumetric ratio.

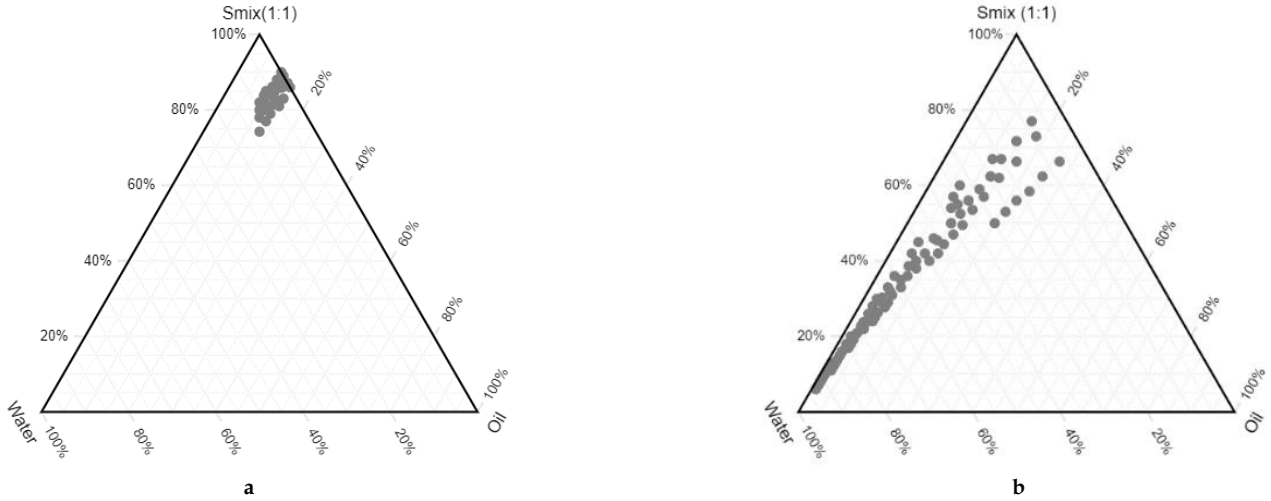
The resulting mixture was then heated at 90°C for 15 minutes to facilitate the formation of the pink MDA-TBA adduct. Following heating, the samples were centrifuged, and the absorbance of the supernatant was measured spectrophotometrically at 532 nm. Quantification of MDA was achieved by generating a standard curve using tetraethoxypropane (TEP) as the primary standard.

Data analysis

Statistical analysis was performed after assessing the normality and homogeneity of variance for all collected data. Subsequently, one-way Analysis of Variance (ANOVA) was conducted to determine significant differences between experimental groups.

RESULTS AND DISCUSSION

This study focused on developing *P. cubeba*-loaded NE preparations, commencing with the meticulous selection of excipients. Capryol 90 was identified as the optimal oil phase, and Tween 20 was chosen as the primary surfactant, based on their established compatibility and emulsifying properties[12]. Subsequently, a critical step involved the selection of an appropriate co-surfactant to enhance NE stability and formation. As depicted in **Figure 1**, propylene glycol demonstrated superior performance, yielding a larger and more stable NE region in the pseudo-ternary phase diagram compared to PEG 400.

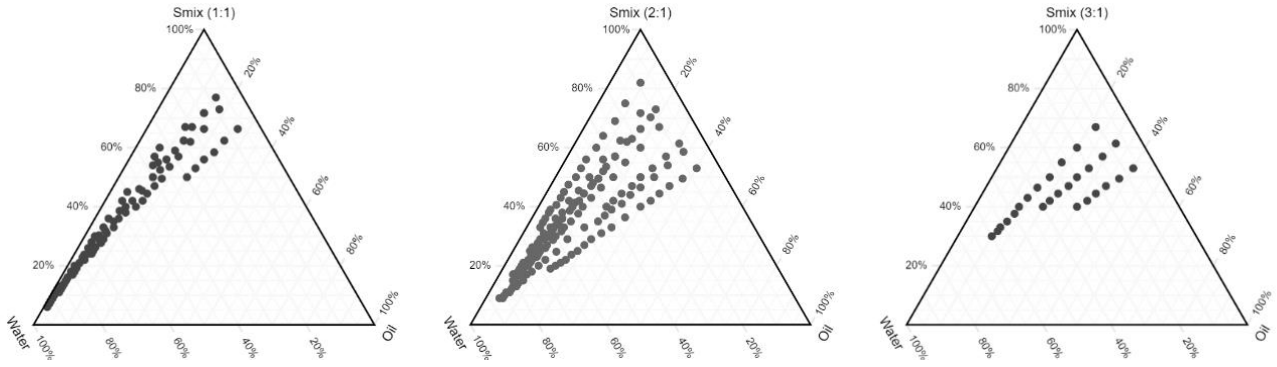


a **b**

Figure 1. Pseudo-ternary phase diagram of the NE composed of Capryol 90 (oil), Tween 20 (surfactant), water, and cosurfactant: (**a**) PEG 400 and (**b**) propylene glycol.

Following co-surfactant selection, the investigation proceeded to optimize Smix ratio. Initial explorations indicated that increasing the proportion of co-surfactant beyond a certain point did not expand NE area. Consequently, the focus shifted to varying the surfactant content within the Smix. As illustrated in **Figure 2**, a Smix ratio of 2:1 yielded the most extensive NE region, while a further increase to a 3:1 ratio resulted in a discernible reduction in NE area. Based on these findings, the Smix ratio of 2:1 was selected for the subsequent development of the *P. cubeba* NE formulations, as it provided the most favorable conditions for NE formation.

Initial formulation trials involved varying concentrations of Smix, oil, and extract; however, only three specific formulations consistently yielded a stable, transparent NE, as detailed in **Table I**. These three promising formulations were subsequently subjected to rigorous thermodynamic stability testing. All selected formulations demonstrated excellent stability following centrifugation, with no significant alterations observed in globule diameter or PDI across three cycles of both heating-cooling and freeze-thaw stress tests (**Table II**). Microscopic examination further confirmed the successful formation of a clear NE, characterized by uniformly spherical globules, as depicted in **Figures 3** and **4**. On the other hand, the *P. cubeba* NE exhibited a Zeta potential of -10.76 mV at pH 4.16, with a viscosity of 56.8 mPa·s.



a **b** **c**

Figure 2. Pseudo-ternary phase diagram of NE composed of Capryol 90 (oil), Tween 20 (surfactant), propylene glycol (cosurfactant) and water with the variation of Smix ratio of (**a**) 1:1, (**b**) 2:1, and (**c**) 3:1.

Table I. Diameter and PDI of *P. cubeba* NE.

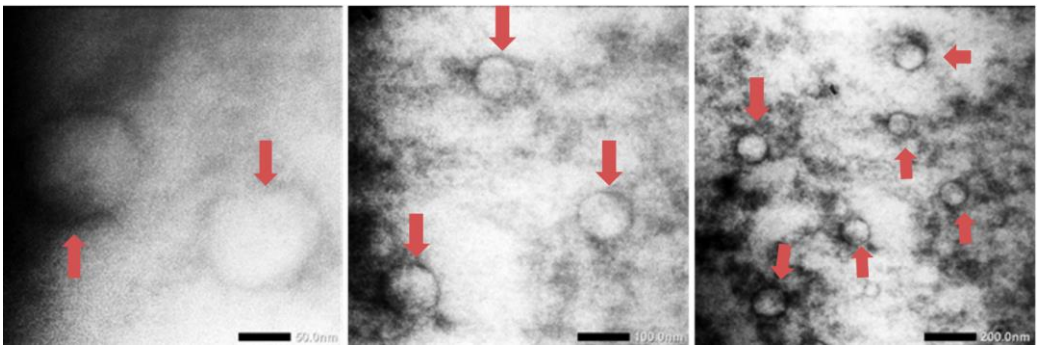
Formula	Smix (2:1)	Oil	*Piper cubeba* extract	Diameter of globules (nm)	PDI
1	30	5	2.5	9.63 ± 0.05	0.07 ± 0.026
2	30	2.5	2.5	10.83 ± 0.05	0.084 ± 0.011
3	40	2.5	5	10 ± 0.08	0.056 ± 0.015

Table II. Diameter and PDI of *P. cubeba* NE after thermodynamical study.

Formula	Before Thermodynamic Stability Test		Centrifugation	Heating-cooling cycles		Freeze-thaw cycle	
	Diameter of globules (nm)	PDI		Diameter of globules (nm)	PDI	Diameter of globules (nm)	PDI
1	9.63 ± 0.05	0.07 ± 0.026	Stable	9.38 ± 0.12	0.049 ± 0.006	10.17 ± 0.05	0.033 ± 0.009
2	10.83 ± 0.05	0.084 ± 0.011	Stable	11.30 ± 0.08	0.114 ± 0.07	11.97 ± 1.01	0.093 ± 0.012
3	10 ± 0.08	0.056 ± 0.015	Stable	10.03 ± 0.05	0.061 ± 0.009	9.8 ± 0.008	0.071 ± 0.005

a **b**

Figure 3. *Piper cubeba* (**a**) nanoemulsion and (**b**) emulsion.



a **b** **c**

Figure 4. Transmission electron microscopy image of *P. cubeba* NE (red arrow) with magnification of (**a**) 80.000x, (**b**) 40.000x, and (**c**) 20.000x.

In **Figure 5A**, the catalase activity data reveals a significant increase in both the hippocampus and prefrontal cortex of rats treated with the *P. cubeba* NE compared to the control group. This is indicated by the statistical significance where control vs NE results in $p < 0.05$, suggesting that the nanoemulsion effectively enhances antioxidant enzyme activity in these brain regions. Conversely, **Figure 5B**, presenting the lipid peroxidation data, shows a statistically significant reduction in lipid peroxidation levels in both the hippocampus and prefrontal cortex of NE-treated rats when compared to the control group, also with $p < 0.05$. This finding suggests that the *P. cubeba* NE effectively mitigates oxidative damage in these crucial brain areas. Collectively, these results indicate that the *P. cubeba* NE exerts an antioxidant effect in the hippocampus and prefrontal cortex of rats by promoting catalase activity and simultaneously reducing lipid peroxidation, thereby suggesting a potential neuroprotective role[13].

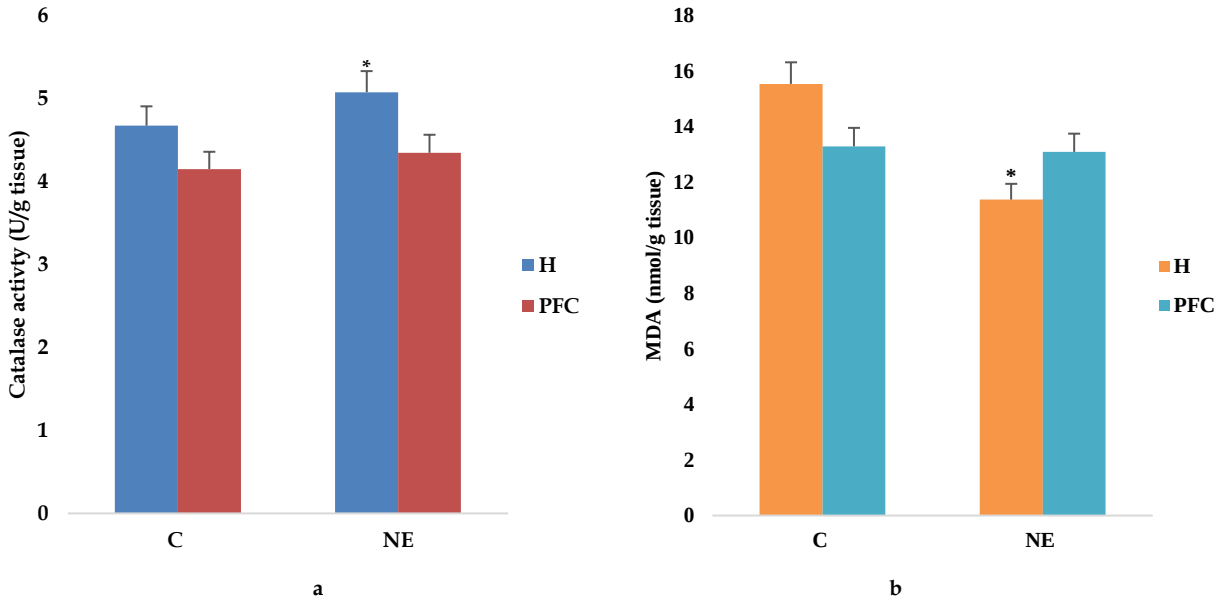


Figure 5. Effect of *P. cubeba* NE on oxidative stress markers in rat brain regions. (**a**) catalase activity and (**b**) lipid peroxidation in the hippocampus (H) and prefrontal cortex (PFC). * statistically significant difference between the control (C) and NE groups ($p < 0.05$).

The brain is particularly susceptible to oxidative stress, a critical factor in the pathogenesis of various neurodegenerative disorders[14]. Within the brain, Reactive Oxygen Species (ROS) are primarily generated through processes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidation, the mitochondrial respiratory chain (MRC), and xanthine oxidase activity[15,16]. An overproduction of ROS frequently correlates with neuroinflammation[17] and predominantly impacts key brain regions, notably the hippocampus and cerebral cortex. Both the hippocampus and prefrontal cortex are instrumental in processing spatial and temporal memories, with hippocampal dysfunction being intimately linked to cognitive decline[18]. Furthermore, sustained oxidative stress has been shown to impair cell proliferation within the cerebral cortex[19].

Given the brain's vulnerability, the development of effective antioxidants for neuroprotection holds significant therapeutic promise for neurodegenerative diseases[20]. Existing research consistently demonstrates that the antioxidant properties of various plants can mitigate neurodegeneration[21,22]. Antioxidant intake is known to bolster the activity of both enzymatic and non-enzymatic endogenous antioxidants[23]. Catalase, as one of the most vital antioxidant enzymes in the body, efficiently converts hydrogen peroxide into oxygen. Deficiencies in catalase activity have been directly implicated in neurodegenerative conditions such as Alzheimer's disease, Parkinson's disease, schizophrenia, and bipolar disorder[24]. Since the hippocampus and prefrontal cortex are strongly associated with cognitive function, enhancing antioxidant levels in these regions could offer therapeutic benefits for patients suffering from Alzheimer's disease and ischemic stroke. These brain areas are also relevant to psychiatric illnesses like schizophrenia[25]. A recognized limitation of the current study is the absence of determined marker levels for *P. cubeba* either within the tested formula or in the target organ, an aspect that will be addressed in future investigations.

CONCLUSION

This study successfully developed a *P. cubeba* extract NE that demonstrated significant neuroprotective potential. Our findings indicate a notable increase in catalase activity and a marked inhibition of lipid peroxidation within brain tissue following treatment. These results collectively suggest that the *P. cubeba* extract NE could be a promising candidate for further development as a neuroprotective agent, particularly in the context of various neurodegenerative diseases. Future research should focus on elucidating the precise mechanisms underlying these neuroprotective effects and conducting *in vivo* studies to validate its therapeutic efficacy and safety profile.

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AUTHORS' CONTRIBUTION

Conceptualization: Lusi Putri Dwita
Data curation: Lusi Putri Dwita
Formal analysis: Lusi Putri Dwita
Funding acquisition: -
Investigation: Lusi Putri Dwita
Methodology: Lusi Putri Dwita, Maria Immaculata Iwo, Rachmat Mauludin, Elfahmi
Project administration: Lusi Putri Dwita
Resources: Lusi Putri Dwita
Software: -
Supervision: Maria Immaculata Iwo, Rachmat Mauludin, Elfahmi
Validation: Maria Immaculata Iwo, Rachmat Mauludin, Elfahmi
Visualization: Lusi Putri Dwita
Writing - original draft: Lusi Putri Dwita, Maria Immaculata Iwo, Rachmat Mauludin, Elfahmi
Writing - review & editing: Lusi Putri Dwita, Maria Immaculata Iwo, Rachmat Mauludin, Elfahmi

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflicts of interest related to this study.

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