Jurnal Pijar MIPA
https://jurnalfkip.unram.ac.id/index.php/JPM

ISSN 1907-1744 (Print)
ISSN 2460-1500 (Online)

Comparison of the Effects of a Combination of *Centella asiatica* and *Mentha piperita* Leaf Extracts on the Number of Normal Brain Cells in Male Mice (*Mus musculus*)

Anak Agung Istri Mas Padmiswari[1*], Ni Wayan Sukma Antari[2], Ida Bagus Maha Gandamayu[3], I Nengah Adiana[3]

[1]Bachelor of Applied Acupuncture and Herbal Medicine Study Program, Faculty of Health, Institute of Technology and Health Bali, Bali, Indonesia
[2]Bachelor of Clinical and Community Pharmacy, Faculty of Health, Institute of Technology and Health Bali, Bali, Indonesia
[3]Bachelor of Applied Nursing in Anesthesiology, Faculty of Health, Institute of Technology and Health Bali, Bali, Indonesia
[4]Bachelor of Nursing, Faculty of Health, Institute of Technology and Health Bali, Bali, Indonesia
[*]e-mail: anakagungpadmi@gmail.com

Received: August 16, 2025. Accepted: September 2, 2025. Published: October 1, 2025

Abstract: Chronic stress is known to cause neuronal damage in the hippocampus, particularly in the CA1 and CA3 areas, which play a crucial role in memory and learning. One way to prevent this damage is by using natural ingredients with neuroprotective properties. This study aimed to compare the effects of *Centella asiatica* and *Mentha piperita* leaf extracts, either singly or in combination, on the number of normal neurons in the hippocampus of male mice exposed to stress. Twenty-five male mice were divided into five treatment groups: K− (control without treatment), K+ (stress without extract), P1 (stress + *Centella asiatica* extract), P2 (stress + *Mentha piperita* extract), and P3 (stress + combination of *Centella asiatica* and *Mentha piperita*). Stress was induced using a restraint stress method, while the extracts were administered orally at the prescribed dosage. After the treatment period, the hippocampus was removed, and histological slides were prepared with Hematoxylin-Eosin staining to count the number of normal neurons in the CA1 and CA3 areas. The results showed that the K+ group experienced a significant decrease in the number of normal neurons compared to the K− group. Administration of gotu kola (P1) and mint (P2) extracts increased the number of neurons compared to K+, but not to the same extent as K−. The combination of *Centella asiatica* and *Mentha piperita* extracts (P3) showed the most optimal effect, with the number of normal neurons approaching that of the negative control group. These findings indicate that the combination of *Centella asiatica* and *Mentha piperita* has synergistic potential in protecting hippocampal neurons from stress-induced damage. Further research is needed to explore the molecular mechanisms underlying this neuroprotective effect.

Keywords: *Centella asiatica*; Hippocampus; *Mentha piperita*; Normal Neurons; Stress.

Introduction

Cognitive decline and brain cell degeneration are significant health concerns, particularly in the context of neurodegenerative diseases such as Alzheimer's and dementia. Therefore, efforts to find safe and effective neuroprotective agents are crucial. Medicinal plants have become a potential alternative source for the development of such agents due to their high bioactive content and minimal side effects compared to synthetic drugs. One plant known to have neuroprotective potential is gotu kola (*Centella asiatica*). Gotu kola has long been used in traditional Asian medicine for its ability to enhance brain function and repair nerve tissue. *Centella asiatica* extract can increase the proliferation and differentiation of nerve cells by increasing the expression of neurotrophic factors [1]. Triterpenoids such as asiaticoside and madecassoside play a major role in this mechanism [2]. Triterpenoid compounds from *Centella asiatica* act as neuroprotective agents against Alzheimer's, including mechanisms that reduce ROS production and improve mitochondrial function [3]. Gotu kola leaves also contain high levels of antioxidants, which can have a positive effect on brain blood circulation [4]. Research by demonstrated the ability of gotu kola leaves to improve memory and learning impaired by systemic inflammation [5]. Furthermore, *Centella asiatica* leaves can also improve cognition and memory in offspring of mice with perinatal hypothyroidism [6].

In addition to gotu kola leaves, mint leaves (*Mentha piperita*) are also known to have effects on the central nervous system [7]. Mint leaves contain menthol and other phenolic compounds with antioxidant and anti-inflammatory properties. The use of mint leaf extract has been shown to improve short-term memory performance and reduce mental fatigue in humans [8]. This demonstrates the potential of mint leaves to support brain health, although studies at the cellular level and in animal models are still limited. Other research also indicates that mint leaves can help alleviate stress [9]. Mint leaves have potential neuroprotective effects through various mechanisms, particularly those related to antioxidant activity and modulation of apoptosis [10]. Mint leaves in gel form exhibit topical analgesic activity and have the potential to be developed as a topical analgesic gel formulation to reduce pain and stress [11]. Although these

How to Cite:
A. A. I. M. Padmiswari, N. W. S. Antari, I. B. M. Gandamayu, and I. N. Adiana, "Comparison of the Effects of a Combination of Centella asiatica and Mentha piperita Leaf Extracts on the Number of Normal Brain Cells in Male Mice (Mus musculus)", *J. Pijar.MIPA*, vol. 20, no. 6, pp. 1087–1091, Oct. 2025. https://doi.org/10.29303/jpm.v20i6.9998

two plants demonstrate neuroprotective potential, research comparing their combined effects on brain cells, particularly in experimental animals such as mice, is still rare. Previous studies have tended to evaluate the effects of each plant individually. Given the potential synergistic effect between the active compounds of both plants, it is essential to evaluate whether the combination of Centella asiatica and Mentha piperita yields superior effects compared to single administration.

The current literature lacks scientific data exploring the combined effects of Centella asiatica and Mentha piperita extracts on normal brain cells, particularly in terms of increasing brain cell number as an indicator of cellular regeneration or protection. However, in herbal medicine approaches, combinations of several plants are often used to achieve optimal therapeutic effects. Furthermore, most existing studies focus solely on disease models or oxidative stress, while the effects of these plants on the normal brain are still understudied. Therefore, it is crucial to understand the effects of this combination of these two plants on normal brain tissue, particularly in the context of preventing early neurological damage. This research may also contribute to the development of herbal-based phytopharmaceuticals for brain health. Based on the description above, this study aims to compare the effects of a combination of *Centella asiatica* and *Mentha piperita* leaf extracts on the number of normal brain cells in male mice. The results of this study are expected to fill the gap in scientific data regarding the effects of combining these two plants and serve as a basis for further research into the development of herbal therapies for brain health**.**

Research Methods

Research Design

This research was a laboratory experimental study using a completely randomized design (CRD) to evaluate the effects of gotu kola (*Centella asiatica*) and mint (*Mentha piperita)* leaf extract, and their combination, on the number of normal brain cells in male mice with ethical permit letter number: 04.0261.KEP ITEKES-BALI/VII/2024 . The study involved five treatment groups, each consisting of five randomly selected mice. The treatment groups consisted of:
K− (negative control): not given stress treatment or extract.
K+ (positive control): given stress treatment without extract.
P1: given stress and *Centella asiatica* leaf extract at a dose of 300 mg/kg bw.
P2: given stress and *Mentha piperita* leaf extract at a dose of 300 mg/kg bw.
P3: given stress and a combination of *Centella asiatica* and *Mentha piperita* leaf extract (1:1) at a dose of 300 mg/kg bw.

Stress induction was carried out using an immobilization method, which involved placing the mice in a narrow tube for 2 hours daily for 14 days [2]. After the stress period, mice in the treatment group were given the extract orally using a gastric tube at the appropriate dose for 28 days [4].

Research Preparation Stages

Research Materials

This study used 8–10-week-old male mice (Mus musculus) weighing 25–30 grams, obtained from a standardized animal laboratory. The animals were housed in a controlled environment with a 12:12-hour light–dark cycle, a temperature of 22–25°C, and were provided with food and water ad libitum. The main ingredients were ethanol extracts of gotu kola *(Centella asiatica)* and mint *(Mentha piperita)* leaves, obtained from dried crude drugs and extracted using a maceration method using 70% ethanol. The extracts were stored at low temperature until use. Additional materials included 0.5% NaCMC solution as an oral suspension, 0.9% physiological NaCl solution, and materials and equipment for preparing histological preparations of brain tissue, such as 10% formalin, graded alcohol, xylene, paraffin, a microtome, slides, coverslips, and Cresyl violet dye for Nissl staining [4].

Extract Preparation Process

Centella asiatica and *Mentha piperita* leaf extracts were prepared separately using a maceration method. Fresh leaves were thoroughly washed, air-dried in the shade, and then ground into a fine powder. 100 grams of the powder was weighed and soaked in 500 mL of 70% ethanol for three 24-hour periods at room temperature with periodic stirring. The filtrate was filtered, and the maceration process was repeated twice using fresh solvent. Afterwards, all filtrates were combined. The solvent was evaporated using a rotary evaporator at 40–50°C to obtain a thick extract, which was then further dried in a low-temperature oven. For the combination treatment, the *extracts of Centella asiatica and Mentha piperita* were mixed in a 1:1 ratio according to the prescribed dosage. The dried extract was stored in a tightly closed dark bottle at 4°C until used [4].

Brain Tissue Isolation

On the 29th day after extract administration, mice were sacrificed by cervical dislocation. The brain was removed through a craniotomy and then washed with a physiological NaCl solution to remove any remaining blood. The brain was then fixed in 10% formalin for 24–48 hours before being processed into paraffin blocks. Tissue sections were made using a microtome at a thickness of 5 µm across the hippocampus (CA1 and CA3). The slides were then used for histopathological analysis, employing Nissl staining with Cresyl violet [4].

Nissl Staining Procedure

The tissue sections mounted on slides were first deparaffinized in xylene twice for 5 minutes, followed by gradual rehydration using absolute, 95%, and 70% alcohol, each for 2 minutes. After rinsing with running water, the slides were stained with Cresyl violet solution for 10 minutes. Subsequently, they were briefly washed with distilled water, re-dehydrated using graded alcohols, cleared with xylene, and mounted on a coverslip using permanent adhesive (Permount) [4].

Research Implementation Stage

Analysis of Normal Brain Cell Counts

Observations were made in the hippocampal area (CA1 and CA3) under a light microscope at 400× magnification. Normal neurons were identified based on the following criteria: intact soma, round nuclei with clear nucleoli, and basophilic cytoplasm without signs of degeneration. The number of cells was counted in five random fields per preparation, then the average was calculated for each animal and each group [4].

Data Analysis

Data were analyzed quantitatively using SPSS version 22.0 for Windows. The Kolmogorov–Smirnov test was used to assess the normality of the data distribution. If the data were normally distributed, analysis was continued with a One-Way ANOVA. If significant differences were found, analysis was continued with a Duncan's Multiple Range Test (DMRT) at a 5% significance level ($P < 0.05$). For data not normally distributed, the Kruskal–Wallis test was used.

Results and Discussion

Histological observations of the hippocampus (CA1 and CA3) revealed differences in the number of normal brain cells between treatment groups. Normal brain cells were identified based on the criteria of intact soma, round nuclei with distinct nucleoli, and basophilic cytoplasm without signs of degeneration.

Table 1. Average Number of Normal Brain Cells (neurons/mm²) in the Hippocampus of Male Mice (Mean ± SD)

Group	CA1 (neuron/mm²)	CA3 (neuron/mm²)	P
K−	145.6 ± 5.2	152.8 ± 6.1	0.001
K+	112.4 ± 4.9	118.6 ± 5.3	0.001
P1	135.8 ± 5.0	142.2 ± 5.7	0.001
P2	129.6 ± 4.7	137.4 ± 5.1	0.001
P3	141.2 ± 5.4	148.9 ± 5.6	0.001

Note: a value of p<0.05 indicates a significant difference

Histological observations of the mouse hippocampus revealed differences in the number of normal neurons in the CA1 and CA3 areas among the various treatment groups. In the negative control group (K−), the highest number of normal neurons was recorded in the CA1 area at 145.6 ± 5.2 neurons/mm² and in the CA3 area at 152.8 ± 6.1 neurons/mm². In contrast, the positive control group (K+) given stress treatment without extract showed the lowest number of normal neurons, specifically 112.4 ± 4.9 neurons/mm² in CA1 and 118.6 ± 5.3 neurons/mm² in CA3. Administration of gotu kola leaf extract (P1) increased the number of normal neurons to 135.8 ± 5.0 neurons/mm² in CA1 and 142.2 ± 5.7 neurons/mm² in CA3, while administration of mint leaf extract (P2) resulted in the number of normal neurons of 129.6 ± 4.7 neurons/mm² in CA1 and 137.4 ± 5.1 neurons/mm² in CA3. The highest effect was found in the administration of a combination of gotu kola leaf extract and mint leaves (P3), with the number of normal neurons reaching 141.2 ± 5.4 neurons/mm² in CA1 and 148.9 ± 5.6 neurons/mm² in CA3. Statistical analysis revealed significant differences between the treatment groups ($p < 0.05$), both in the CA1 and CA3 areas, with a tendency to increase the number of normal neurons in the group receiving the extract, particularly in the combination of *Centella asiatica* and *Mentha piperita*.

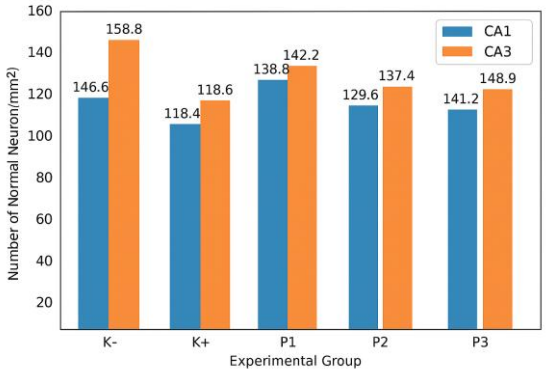

Figure 1. Comparison of the Number of Normal Brain Cells in CA1 and CA3 of the Hippocampus

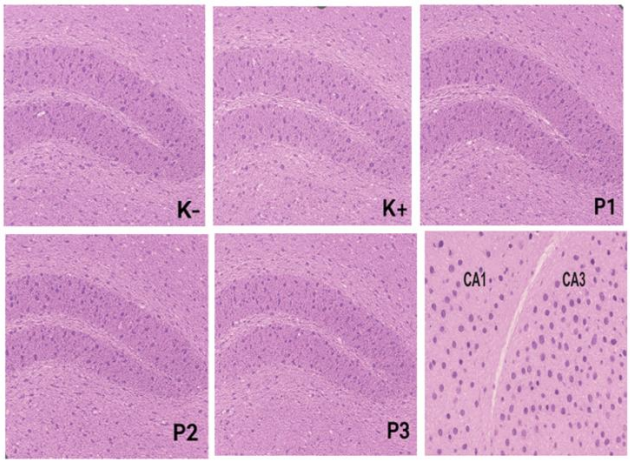

Figure 2. Comparison of Histological Images of Normal Brain Cells in CA1 and CA3 of the Hippocampus

The results of this study showed a significant difference in the number of normal neurons in the CA1 and CA3 areas of the hippocampus of mice between treatment groups. The negative control group (K−) had a relatively high and stable number of neurons, indicating normal physiological conditions in the absence of stress exposure. In contrast, the positive control group (K+) treated with stress exhibited a significant decrease in the number of normal neurons in both the CA1 and CA3 areas, consistent with previous research indicating that chronic stress exposure can induce apoptosis and neuronal degeneration in the hippocampus [13]. Administration of gotu kola (*Centella asiatica*) extract to the P1 group was shown to increase the number of normal neurons compared to the K+ group [14]. This finding is consistent with the results that triterpenoid compounds and caffeoylquinic acid from gotu kola leaves can enhance dendritic arborization and promote neuroplasticity [15]. In addition, the antioxidant effect of gotu kola has also been reported to play a role in reducing

oxidative stress, one of the mechanisms by which neuronal damage occurs due to stress [16].

In group P2, which was given mint leaf extract (*Mentha piperita*), there was an increase in the number of normal neurons compared to the K+ group, although not as high as the gotu kola group. This supports the research, which showed that phenolic compounds in mint leaves can act as antioxidants and increase the expression of BDNF (Brain-Derived Neurotrophic Factor) in the hippocampus [18]. The protective effect of mint is more closely associated with its anti-inflammatory properties and neurogenesis, although its ability to increase neuron density may not be as strong as that of gotu kola. Group P3, a combination of gotu kola and mint, showed the most optimal results, with the number of normal neurons approaching that of the negative control group. The synergy between gotu kola triterpenoid compounds with polyphenols and flavonoids from mint likely produces a dual protective effect, both through antioxidant mechanisms and increasing neurotrophic factors [19]. This demonstrates the potential of herbal extract combinations in mitigating the effects of chronic stress on cognitive function [20]. In addition to the number of neurons, the hippocampal areas CA1 and CA3 have different sensitivities to stress. The CA1 area is more susceptible to hypoxia and oxidative stress, resulting in more pronounced neuronal decline than the CA3 area in the K+ group [21]. However, administration of the combined extracts was shown to mitigate this difference, demonstrating relatively equal protection across both areas.

This study aligns with previous evidence that the combination of *Centella asiatica* and *Mentha piperita* leaves exhibits neuroprotective activity and could be considered a therapeutic candidate for cognitive impairment, including stress-induced impairment [22]. Meanwhile, mint extract provided additional support in terms of mood improvement, cognitive capacity enhancement, and reduction of neuroinflammation [23]. Therefore, the combination of the two has the potential to provide complementary effects [24]. From a clinical translational perspective, these findings are important for the development of herbal-based supplements to prevent cognitive impairment caused by chronic stress. Although this study was conducted in mice, the molecular mechanisms involved (antioxidant, anti-inflammatory, and neurotrophic enhancement) could form the basis for further studies in humans. However, further research is needed regarding optimal dosage, long-term safety, and bioavailability of the active compounds.

Overall, the results of this study support the hypothesis that chronic stress reduces the number of normal neurons in the hippocampus, and administration of gotu kola, mint, and their combination extracts can provide a protective effect. The combination of the two extracts provided the most effective results, making it a potential alternative in preventing neuronal damage caused by chronic stress.

Conclusion

This study demonstrated that stress reduced the number of normal neurons in the CA1 and CA3 hippocampal regions of male mice. In contrast, treatment with Centella asiatica and Mentha piperita extracts increased neuronal survival, with the P3 combination showing the most optimal effect, closely resembling that of the negative control. These findings contribute to evidence on the neuroprotective role of medicinal plants and suggest potential applications in functional foods, nutraceuticals, or adjunct therapies for stress-related neuronal damage. Further research with larger samples, extended treatment durations, mechanistic exploration, and translational is recommended to confirm efficacy, determine optimal dosage, and assess safety in broader applications..

Author's Contribution

Anak Agung Istri Mas Padmiswari: Designed the research framework, conducted the research, performed data analysis, and prepared the results and discussion. Ni Wayan Sukma Antari: Contributed to data analysis. Ida Bagus Maha Gandamayu: Contributed to data analysis. I Nengah Adiana: Contributed to data analysis.

Acknowledgment

The authors express their gratitude to the Institute of Technology and Health Bali for facilitating and funding this research

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