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*Research Articles*

# Studi Perbandingan Imunohistokimia NeuN dan NSE untuk Penilaian Neuroprotektif Kombinasi Centella asiatica, Kayu Manis, dan Spirulina pada Cedera Otak Traumatis

## *Comparative Study of NeuN and NSE Immunohistochemistry for Neuroprotective Assessment of a Combination of Centella asiatica, Cinnamon, and Spirulina in Traumatic Brain Injury*

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## ABSTRAK

Latar Belakang: Cedera otak traumatis (traumatic brain injury/TBI) merupakan penyebab utama morbiditas dan mortalitas secara global, yang sering mengakibatkan gangguan kognitif dan fungsional. Strategi neuroprotektif yang efektif diperlukan untuk mengurangi cedera sekunder pasca-TBI. Penelitian ini bertujuan mengevaluasi efek neuroprotektif kombinasi Centella asiatica, kayu manis, dan spirulina menggunakan pewarnaan imunohistokimia NeuN dan neuron-specific enolase (NSE) pada model tikus TBI. Metode: Sepuluh ekor tikus jantan Sprague-Dawley (200–400 g, usia 10–12 minggu) diberikan TBI menggunakan model Marmarou yang dimodifikasi, kemudian diberikan ekstrak kombinasi Centella asiatica, kayu manis, dan spirulina secara oral selama 10 hari. Pada hari ke-11, jaringan otak dikumpulkan untuk pemeriksaan histopatologi dan pewarnaan imunohistokimia menggunakan penanda NeuN dan NSE untuk mengevaluasi neurogenesis dan kelangsungan hidup neuron. Hasil: Analisis imunohistokimia menunjukkan ekspresi positif rata-rata sebesar 71,66% pada kelompok NeuN, yang mengindikasikan peningkatan kelangsungan hidup neuron dan neurogenesis, sedangkan kelompok NSE menunjukkan 0% pewarnaan positif pada semua sampel. Pewarnaan NeuN memberikan gambaran yang jelas mengenai preservasi neuron dalam jaringan otak, sedangkan NSE tidak menunjukkan neurogenesis atau perlindungan neuron yang terdeteksi pada model ini. Kesimpulan: Pemberian ekstrak kombinasi Centella asiatica, kayu manis, dan spirulina menunjukkan efek neuroprotektif pada model tikus TBI subakut, yang ditunjukkan oleh peningkatan ekspresi imunohistokimia NeuN, mencerminkan peningkatan neurogenesis dan kelangsungan hidup neuron. Pewarnaan NeuN memberikan gambaran yang lebih jelas dan sensitif dibandingkan NSE, mendukung keandalannya sebagai penanda imunohistokimia untuk evaluasi neuroproteksi dan neurogenesis selama fase subakut TBI pada model yang diberikan intervensi berbasis herbal.

**Kata kunci** : NSE, NeuN, Cedera otak traumatis, Centella asiatica, Kayu manis, Spirulina

## ABSTRACT

Background: Traumatic brain injury (TBI) is a major cause of morbidity and mortality globally, often resulting in cognitive and functional impairment. Effective neuroprotective strategies are needed to mitigate secondary injuries following TBI. This study evaluated the neuroprotective effects of combined Centella asiatica, cinnamon, and spirulina using NeuN and neuron-specific enolase (NSE) immunohistochemical staining in a rat model of TBI. Methods: Ten male Sprague-Dawley rats (200–400 g, 10–12 weeks) were subjected to TBI using the modified Marmarou model and treated orally with a combination extract of Centella asiatica, cinnamon, and spirulina for 10 days. On day 11, brain tissues were collected for histopathological examination and immunohistochemical staining using NeuN and NSE markers to evaluate neurogenesis and neuronal survival. Results: Immunohistochemical analysis revealed a mean positive expression of 71.66% in the NeuN group, indicating increased neuronal survival and neurogenesis, while the NSE group consistently showed 0% positive staining across all samples. The NeuN staining provided a clear depiction of neuronal preservation in the brain tissue, whereas NSE did not demonstrate detectable neurogenesis or neuronal protection in this model. Conclusion: The administration of a combined extract of Centella asiatica, cinnamon, and spirulina demonstrated neuroprotective effects in a rat model of subacute traumatic brain injury, as indicated by increased NeuN immunohistochemical expression, reflecting enhanced neurogenesis and neuronal survival. NeuN staining provided a clearer and more sensitive depiction than NSE, supporting its reliability as an immunohistochemical marker for evaluating neuroprotection and neurogenesis during the subacute phase of TBI in models treated with herbal-based interventions.


**Keywords**: NSE, NeuN, Traumatic brain injury, Centella asiatica, Cinnamon, Spirulina

## INTRODUCTION

Traumatic brain injury (TBI) remains one of the leading causes of death and neurological disability worldwide, resulting from mechanical processes affecting cerebral structures and leading to distortion and damage at the initial phase of trauma [5]. The mechanical insult causes disruption of neuronal and vascular integrity, initiating a cascade of biochemical and molecular responses that culminate in both immediate and delayed damage to brain tissues. Patients with TBI often experience cerebral edema, which occurs in more than 25% of cases, leading to increased intracranial pressure and requiring immediate intervention to prevent disability or death [8]. The accumulation of excess fluid within the brain parenchyma not only contributes to structural compression but also impairs cerebral perfusion and oxygenation, exacerbating secondary injury.

TBI does not merely affect physical structures but also results in a wide spectrum of clinical manifestations. Cognitive, physical, emotional, and psychosocial dysfunctions may occur temporarily or permanently after TBI, with severity influenced by the initial injury mechanism [27]. These dysfunctions can profoundly impair an individual's ability to reintegrate into daily activities, work, and social life, often resulting in long-term disability. TBI is classified based on the Glasgow Coma Scale into mild (GCS 13–15), moderate (GCS 9–13), and severe (GCS 3–8) injuries, with mild TBI accounting for 70–90% of cases, and 15–20% of individuals experiencing long-term dysfunction if not managed properly [7]. Despite being labeled as “mild,” such cases are not benign, and a substantial proportion of patients continue to suffer from persistent neurobehavioral and cognitive impairments.

Globally, TBI affects 69 million people annually, highlighting its status as a critical public health concern [3]. Among its most severe complications is intracranial hemorrhage, which significantly increases the risk of mortality and disability. In the United States, more than 1.7 million people suffer from TBI each year, with an individual experiencing a brain injury every 21 seconds [1]. Similarly, TBI is a major cause of mortality and disability in individuals under 40 in the UK [13]. In Indonesia, the prevalence of head injuries is reported to be around 11.9%, with an increasing trend over the years [12]. Contributing factors include traffic accidents, occupational hazards, and falls—particularly in developing countries where safety regulations are inconsistently enforced. TBI often leads to primary effects at the time of

trauma, such as direct neuronal disruption, and secondary injuries due to complications such as cerebral edema, blood-brain barrier disruption, tissue necrosis, and hyperthermia [14,17]. The evolution of secondary brain injury involves complex pathophysiological processes, including oxidative stress, excitotoxicity, mitochondrial dysfunction, and neuroinflammation. These secondary injuries, mediated by inflammatory responses, oxidative stress, and cellular apoptosis, significantly contribute to clinical implications and poor outcomes in TBI patients [23].

The concept of neuroprotection in TBI refers to pharmacological therapies that interrupt the pathophysiological cascade to enhance recovery by protecting neurons, maintaining cerebral perfusion, and reducing secondary injuries [21,28]. Neuroprotection is particularly important during the acute and subacute phases of TBI, as it aims to preserve neuronal viability and prevent further deterioration of neurological function. However, despite advancements in neurosurgical techniques and neurointensive care, long-term disabilities remain prevalent, highlighting the need for more effective and adjunctive neuroprotective interventions [22]. Recent trends emphasize pharmacological strategies, focusing on stabilizing edema, reducing oxidative stress, and improving cognitive and neurobehavioral functions post-TBI through antioxidant-based therapies [7]. In this context, the use of immunohistochemical markers such as NeuN and neuron-specific enolase (NSE) has become increasingly important in evaluating neuronal integrity and damage following TBI. These markers provide valuable insights into neuronal survival, neurogenesis, and the overall histopathological landscape of injured brain tissue [23].

Centella asiatica, cinnamon, and spirulina have been recognized for their potential neuroprotective effects due to their antioxidant, anti-inflammatory, and neurogenic properties. These natural compounds offer a promising complementary approach to conventional therapies. Centella asiatica has been traditionally used for neurological disorders and vascular protection, with studies indicating its effectiveness in cognitive improvement post-stroke and neuroprotection in various brain injury models. Its bioactive components, such as asiaticoside and madecassoside, are known to enhance neuroplasticity and reduce oxidative damage. Cinnamon, on the other hand, exhibits anti-inflammatory effects on the central nervous system and modulates immune responses, potentially through the regulation of microglial activity and cytokine expression. Spirulina, known as a superfood, provides comprehensive nutrients, essential amino acids, and powerful antioxidants such as phycocyanin, which may enhance immune function and facilitate neuronal recovery in neurological injuries [23,25]. Based on the significant impact of TBI and the potential neuroprotective roles of these natural agents, this study aims to evaluate the neuroprotective effects of combined Centella asiatica, cinnamon, and spirulina using NeuN and NSE immunohistochemical markers in a rat model of TBI. Through this investigation, it is expected that effective therapeutic strategies can be identified to enhance neurogenesis, promote neuronal survival, and reduce overall neuronal damage following traumatic brain injury.

## RESEARCH METHOD

This study investigated the effects of administering a combined extract of Centella asiatica, cinnamon, and spirulina on NeuN and NSE immunohistochemical expressions in brain tissue following traumatic brain injury (TBI). The responses were then compared between two groups: the group evaluated using the NeuN marker and the group evaluated using the NSE marker after receiving the combined extract treatment.

### *Animals*

The study utilized ten Sprague-Dawley rats, each aged between 10 and 12 weeks with a body weight ranging from 200 to 400 grams. Throughout the experiment, the animals were maintained on a standard diet (Comfeed AD-2) with free access to water. The rats were allocated into two groups: (1) a group subjected to traumatic brain injury and treated with a combination extract of Centella asiatica, cinnamon, and spirulina, followed by histological analysis using the NSE marker; and (2) a group subjected to traumatic brain injury and treated with the same combination extract, followed by histological analysis using the NeuN marker.

*Treatment of brain injury and sampling*

Ten Sprague-Dawley rats were randomly divided into NeuN and NSE groups. Under ketamine anesthesia (10 mg/kg IM), a craniotomy was performed at 1 mm posterior and 2 mm lateral to the bregma. TBI was induced using the Modified Marmarou Model by dropping a 40 g weight from a height of 20 cm onto the exposed dura mater [2,18]. This mrthod offers a straightforward method to induce diffuse brain injury in animal studies, simulating trauma from falls or traffic accidents in humans while minimizing mortality and research costs [6,19]. At 30 minutes post-injury, all rats received an oral combined extract of Centella asiatica, cinnamon, and spirulina at 1 mL/200 g BW/day for 10 days [24]. On day 11, the rats were euthanized with ketamine (20 mg/kg intracardially), and brain tissues were extracted and frozen at -80°C [15,20]. The brains were processed into paraffin blocks, sectioned (4–6 μm), and stained with H&E and immunohistochemically using NeuN and NSE markers. Neuronal counts were assessed in hot spot areas to evaluate neuroprotection.

*Equipment resources*

The study was conducted on male Sprague-Dawley rats weighing 200–400 g, aged 10–12 weeks, housed in standard cages at room temperature. Equipment used included diluted ketamine for anesthesia, microsurgical instruments for craniotomy, a 40 g metal ball dropped from 20 cm through a guiding tube using the modified Marmarou model, and tools for brain extraction and immunohistochemical processing.

## RESLUT AND DISCUSSION

This study investigates the neuroprotective effects of combined Centella asiatica, cinnamon, and spirulina extracts in a rat model of traumatic brain injury (TBI), assessed through NeuN and NSE immunohistochemical markers. Table 1 presents the positive expression percentages, showing a mean level of 71.66% in the NeuN group, while the NSE group consistently demonstrated 0% across all samples. These differences in positive expression levels between groups are clearly illustrated in Figure 1, showing a significantly higher positive expression in the NeuN group after treatment. The histopathological appearance of neuronal cells assessed using NeuN immunohistochemical staining is displayed in Figure 3A, demonstrating positively stained neurons as evidence of neurogenesis following the administration of the combined extracts after traumatic brain injury.

Traumatic brain injury (TBI) is recognized as one of the major causes of long-term disability and mortality on a global scale. This neurological condition is especially prevalent among individuals in the productive age group, thereby posing significant socio-economic burdens. TBI occurs as a result of an external mechanical impact, which leads to structural disruptions in the brain, such as axonal shearing, contusions, or hematomas, and functional impairments at the cellular and molecular levels. The primary insult initiates a cascade of biochemical and physiological events leading to secondary brain injuries. These secondary injuries manifest in the form of oxidative stress, neuroinflammation, blood-brain barrier

breakdown, and programmed cell death mechanisms such as apoptosis, which, if not promptly and effectively managed, exacerbate neuronal damage and worsen patient prognosis [5,7,23].

To mitigate these detrimental outcomes, various therapeutic approaches have been explored, ranging from surgical interventions and pharmacotherapy to neurorehabilitation and the use of bioactive compounds from natural products. The present study aims to evaluate a novel strategy involving a combination of natural neuroprotective agents—Centella asiatica, cinnamon, and spirulina—known for their antioxidant, anti-inflammatory, and neurogenic properties. This combination was administered to assess its impact on neuronal survival and neurogenesis post-TBI. To evaluate these effects, immunohistochemical staining using two neuronal markers—NeuN (neuronal nuclei) and NSE (neuron-specific enolase)—was performed. NeuN is widely employed in neuroscience research as a reliable indicator of mature neurons, while NSE is often used to gauge the severity of neuronal injury [23,11].

The experimental model employed Sprague-Dawley rats, a standard laboratory animal for neuroscience research due to their well-characterized neuroanatomy and predictable response to experimental manipulations. These rats, weighing between 200 and 400 grams and aged between 10 to 12 weeks, were subjected to controlled cortical impact using a modified Marmarou weight-drop model to induce TBI. All rats received the same dose of the combined herbal extracts following injury induction. The goal was to simulate a consistent injury pattern and evaluate the extent of neuronal recovery or protection facilitated by the treatment. Histopathological analysis performed on the brain tissues revealed that the NeuN-stained group demonstrated a mean positive expression of 71.66%, indicating a high density of surviving or newly differentiated neurons. In contrast, the group stained with NSE showed a consistent 0% expression, suggesting an absence of detectable acute neuronal injury.

NeuN protein is a specific neuronal marker widely used in immunohistochemical studies to evaluate neuronal cell differentiation under both normal and pathological conditions [9]. NeuN is a protein encoded by the Rbfox3 gene and belongs to the RNA-binding Fox (Rbfox) family, which is known to modulate alternative splicing and influence neuronal development, differentiation, and synaptic plasticity [16]. As such, NeuN is not merely a structural marker but also has functional relevance in neural physiology. Its expression is localized within the nucleus and perinuclear cytoplasm of post-mitotic, mature neurons and is most prominently observed in brain regions such as the cerebral cortex, hippocampus, and cerebellum—areas commonly affected in TBI [4]. This localization facilitates the accurate identification of mature neurons, making NeuN a highly specific and sensitive tool for neurohistological studies.

Unlike other neuronal markers such as MAP-2 or βIII-tubulin, which may also be present in non-neuronal tissues such as skeletal muscle, NeuN's specificity to neurons minimizes the risk of false-positive results and enhances the validity of quantitative histological assessments [10]. Furthermore, NeuN is absent in neural progenitor cells and glial cells, particularly during their undifferentiated or proliferative stages. This absence ensures that NeuN staining reflects only mature neuronal populations, thereby making it particularly suitable for evaluating neurogenesis or neuronal survival in pathological conditions like TBI.

An additional advantage of using NeuN is its nuclear and perinuclear localization, which supports automated image analysis techniques such as binary image processing. This enables objective and reproducible quantification of neuronal density in defined regions of interest (hotspots) in the brain tissue. Such automated counting reduces human error and observer bias, contributing to more reliable outcomes in experimental neuropathology [10]. In this study, the significantly high NeuN positivity in treated rats suggests that the combination of Centella asiatica, cinnamon, and spirulina may exert protective or regenerative effects on neurons. This neuroprotective potential may be attributed to the antioxidant compounds in Centella asiatica (e.g., asiaticoside), cinnamaldehyde in cinnamon, and phycocyanin in spirulina, all of which

have demonstrated the ability to scavenge reactive oxygen species and modulate inflammatory pathways.

In contrast to NeuN, NSE is a cytoplasmic enzyme involved in glycolysis, primarily found in mature neurons and neuroendocrine cells. Its physiological role includes energy production through glucose metabolism, but its clinical significance lies in its utility as a biomarker for neuronal damage. During acute brain injury, compromised neuronal membranes lead to the release of cytoplasmic contents, including NSE, into the extracellular space and systemic circulation. Consequently, elevated serum NSE levels serve as an indirect indicator of the extent of neuronal membrane disruption [10,11].

However, NSE's expression dynamics limit its application in histological studies, especially those aiming to assess neuroregeneration or delayed neuroprotection. As supported by prior research, serum NSE concentrations typically rise within 12 hours following TBI and return to baseline levels within a few days, depending on the severity of injury and subsequent cellular recovery [26]. The temporal nature of NSE expression underscores its utility in acute-phase diagnostics but makes it less suitable for evaluating longer-term outcomes, such as those targeted by neuroprotective therapies. In the current study, brain tissue sampling was performed on day 11 post-TBI. By this time, acute-phase markers like NSE are likely to have subsided, especially if no ongoing neuronal injury is present. The absence of detectable NSE in the immunohistochemical analysis supports this hypothesis and reinforces the conclusion that the herbal treatment may have prevented the continuation or exacerbation of acute neuronal injury. It also highlights the specificity of NSE as a damage marker rather than a marker of neuronal survival or differentiation [10,11].

This discrepancy between NeuN and NSE results further emphasizes the importance of selecting appropriate biomarkers based on the timing and objectives of a given study. NeuN is advantageous for evaluating long-term neuroprotective and neuroregenerative outcomes, as it identifies the preservation or re-emergence of mature neuronal populations. On the other hand, NSE provides insight into the immediate pathological responses following TBI, particularly membrane integrity and cytoplasmic leakage. Therefore, although both markers are valuable, they serve distinct roles within different phases of TBI pathology [22].

Given these findings, the data strongly suggest that NeuN is the more appropriate marker for assessing the therapeutic efficacy of natural compounds in the subacute to subacute phases of TBI. Its ability to reflect neuronal survival aligns well with the goals of neuroprotective and neuroregenerative strategies [16]. Furthermore, the combination of Centella asiatica, cinnamon, and spirulina appears to support neuronal viability, possibly through mechanisms involving free radical scavenging, anti-inflammatory effects, and enhancement of synaptic integrity [23]. These mechanisms not only prevent further neuronal loss but may also stimulate endogenous repair pathways, such as the proliferation and differentiation of neural progenitor cells into mature neurons, ultimately contributing to tissue recovery [4].

Meanwhile, although NSE remains a useful marker in clinical and research settings, especially for early TBI diagnosis and prognosis, its application in histological contexts must be carefully timed. In future studies, a multimodal approach combining early serum NSE evaluation with late-stage NeuN immunostaining could provide a comprehensive picture of both injury progression and recovery.

In conclusion, this study demonstrates that the administration of a combined extract of Centella asiatica, cinnamon, and spirulina exerts beneficial effects on neuronal preservation and potential neurogenesis in a rat model of TBI. The findings, as indicated by the high NeuN expression and absent NSE staining, underscore the utility of NeuN as a robust marker for assessing long-term neuronal outcomes in brain tissue. The results also highlight the importance of selecting appropriate markers based on the phase of injury being studied. NeuN and NSE are not interchangeable but rather complementary, and their judicious use can

enhance the accuracy and interpretability of neuropathological research outcomes. These insights contribute to the growing body of evidence supporting the therapeutic potential of herbal-based interventions in mitigating the deleterious effects of TBI and promoting neural recovery.

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## CONCLUSION

From the results and explanations above, it can be concluded that the administration of a combined extract of Centella asiatica, cinnamon, and spirulina demonstrated neuroprotective effects in a rat model of subacute traumatic brain injury, as indicated by increased NeuN immunohistochemical staining reflecting enhanced neurogenesis. In contrast, NSE staining showed no expression across all samples. These findings suggest that NeuN serves as a more sensitive and reliable immunohistochemical marker than NSE for assessing neurogenesis and neuroprotection during the subacute phase of traumatic brain injury, particularly in models treated with herbal-based interventions.

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