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Research Article

The Impact of *Kaempferia galanga* L. Extract on Nerve Growth Factor Expression in A Rat Model of Traumatic Brain Injury

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ABSTRACT

Introduction: Head injuries contribute significantly to morbidity and mortality globally, and there is need of effective treatment. This study focuses on evaluating NGF (Nerve Growth Factor) expression in a rat model of traumatic brain injury, exploring the potential therapeutic effects of *Kaempferia galanga* L. extract. **Methods:** Male Wistar rats were used in the experiment, and traumatic brain injury was induced using Marmarou's weight drop model. Four groups of rats were studied: a negative control group, a group with traumatic brain injury without *Kaempferia galanga* L. extract, and two groups with traumatic brain injury treated with different doses of *Kaempferia galanga* L. extract. Rats were divided further based on the time of decapitation, either 24 or 48 hours post-injury. NGF expression was assessed using immunohistochemistry. **Results:** The study confirmed NGF expression variations among groups, with stronger expression observed 48 hours post-injury in rats receiving 1200 mg/kgbb of *Kaempferia galanga* L. extract. This suggests a potential impact of the extract on NGF expression, likely attributed to its anti-inflammatory and antioxidant properties. **Discussion:** *Kaempferia galanga* L. extract has known anti-inflammatory and antioxidant effects, which may contribute to increased NGF expression observed in this study. **Conclusion:** This study sheds light on the potential benefits of *Kaempferia galanga* L. extract in promoting NGF expression and improving outcomes in traumatic brain injury, emphasizing the need for further investigation to translate these findings into clinical practice.
Key words: Traumatic Brain Injury, Nerve Growth Factor, kaempferia galanga.

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INTRODUCTION

The occurrence of head injuries is a significant contributor to morbidity and mortality across all age groups. Understanding the epidemiology of head injuries is crucial for preventive measures, population-based primary prevention strategies, and enhancing effective and efficient management, including rehabilitation for those affected by head injuries [1].

Neuropathological changes associated with head injuries are influenced by various factors, including the type and severity of the injury and the damage it can cause, whether blunt or sharp, diffuse or local. The pathology of head injuries is also affected by factors such as age, comorbidities, alcohol, hypoxia, sepsis, and the speed of treatment [2].

The highest incidence of Traumatic Brain Injury (TBI) is reported in the Americas - USA and Canada (1299 cases per 100,000 people, 95% CI 650–1947) and in Europe (1012 cases per 100,000 people, 95% CI 911–1113), while the lowest is in Africa (801 cases per 100,000 people, 95% CI 732–871). The largest volume of TBIs is observed annually in Southeast Asia (18.3 million) and the Western Pacific (17.3 million). The estimated global incidence of TBI of all causes and severities is 939 cases per 100,000 people (95% CI 874–1005), with an estimated 69.0 million (95% CI 64.2–73.8 million) people worldwide suffering from TBI each year. Mild TBI affects approximately 740 cases per 100,000 people, totaling 55.9 million individuals annually, while an estimated 5.48 million people suffer from severe TBI each year (73 cases per 100,000 people) [3].

In developing countries like Indonesia, the frequency of brain injuries tends to increase alongside technological advancements and development. It rise from 14.5% in 2007 to 14.9% in 2013. The number of deaths increased from 6 per 100,000 population in 2000 to 9 per 100,000 population in 2009. Brain injuries contribute to nearly half of all trauma-related deaths, given that the head is the most frequently and vulnerable part involved in accidents. Brain injury cases primarily involve the productive age group, between 15-44 years, and are more dominated by males than females. The most common causes are traffic accidents, followed by falls, especially in the pediatric age group [3].

At RSUD Dr. Soetomo Surabaya, from January 2002 to December 2013, the highest number of brain injury cases was reported in 2002 with 2005 cases, and the lowest number was reported in 2010 with 916 cases. The reported highest percentage of deaths due to brain injuries occurred at 11.22% of all brain injury cases, and the lowest was 6.17%. This rate is higher than the international literature standard, which ranges from 3 to 8% [4].

In nerve cells affected by injury, initially, Schwann cells regulate cytokine secretion to facilitate the destruction and phagocytosis of axons distal to the injury site. After the destruction process is complete, Schwann cells stimulate axon regeneration and temporarily cease myelination until the axon is reformed. Schwann cells located distally from the injury express p75NTR, which plays a vital role in maintaining neurons and inhibiting NFkB activation. Increased NFkB activation correlates with the

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absence of apoptosis in distal neurons after injury, and NGF can trigger proliferation and reactions in Schwann cells, marked by increased NGF expression. The administration of NGF can enhance myelination of regenerating axons, but inflammatory cells also increase. This leads to side effects on peripheral nerve growth during demyelination, which is treated with NGF [5].

Extract from Kampferia galanga L. possesses potent anti-inflammatory, analgesic, vasorelaxant, and pro-apoptotic activities. The anti-inflammatory effect is attributed to ethyl-p-methoxycinnamate (EPMC), which inhibits enzymatic activity in COX-1 and COX-2, making it a pain reliever during inflammation. Additionally, kaempferol found in galangal extract can have neuroprotective effects and reduce inflammation[7]. Several studies also indicate that galangal extract can penetrate the blood-brain barrier [2]. Galangal extract also has antioxidant potential, with phenolic and flavonoid content playing a role in antioxidant activity [6]. The administration of antioxidants can modulate NGF levels [8].

This study evaluates NGF expression in the brains of experimental animals at 24 and 48 hours post-traumatic brain injury. These time points are reported to be ideal for evaluating brain edema after experimental brain injury and its relationship with NGF expression. Both time points represent the peak of traumatic brain inflammation in experimental animals [9].

MATERIALS AND METHODS

This study is experimental analytics using simple random sampling. The treatment for all samples was conducted simultaneously, and observations were made using a posttest-only control group design. We examined the effects of *Kaempferia galanga* L. extract on NGF expression in the rat brain as an indicator of brain injury.

Animal Study

The experimental unit consisted of male Wistar rats (Rattus norvegicus), aged 2.5-3 months, with a body weight of 250 - 300 grams, and they were in good health. The selection of rats as experimental animals was based on the consideration that Wistar rats (Rattus norvegicus) genetically resemble humans and have the ability to adapt to the laboratory environment. Sample grouping was done using simple random sampling by assigning a number to each rat, thus ensuring that the experimental animals, experimental conditions, and research materials were homogeneous [10].

TBI Animal Model

The Marmarou's weight drop model caused traumatic brain injury in male Wistar rats (Ratus novergicus). The weight was dropped freely from 100 cm onto the rat's head, touching the calvaria bone [11].

The research conducted at ITD (Institute of Tropical Disease) Universitas Airlangga laboratory, rats that have undergone traumatic brain injury using Marmarou's weight drop model and received *Kaempferia galanga* L. extract treatment are placed in separate cages for post-treatment care. Isolating rats reduces external factors that could affect their post-treatment condition and NGF expression after immunohistochemical staining.

To retrieve brain tissue, grouped rats are euthanized by cervical dislocation at 24 and 48 hours after traumatic brain injury treatment, under ketamine and xylazine anesthesia. The brain is removed surgically.

Grouping of the rats are as follows:

1. Group A: Negative control group with no traumatic brain injury treatment and no administration of *Kaempferia galanga* L. extract.

2. Group B: Rats with traumatic brain injury treatment without *Kaempferia galanga* L. extract.

3. Group C: Treatment group with traumatic brain injury followed by the administration of *Kaempferia galanga* L. extract at a dose of 600 mg/kg body weight.

4. Group D: Treatment group with traumatic brain injury followed by the administration of *Kaempferia galanga* L. extract at a dose of 1200 mg/kg body weight.

For Paraffin Block Preparation, brain tissue is fixed in 10% formalin for 15-24 hours. After that, graded alcohol (30%, 50%, 70%, 80%, 96%, and absolute) dehydrates it for 60 minutes. Two 60-minute xylene treatments clear the tissue. A softener is then added for 60 minutes at 48°C. Leaving the tissue in a mold for 24 hours hardens it. It's mounted on a poly-L-lysine-coated glass slide.

Examinations of NGF expression by immunohistochemistry

Immunohistochemistry (IHC) staining in this study followed the method developed by Hsu et al., using the avidin-biotin peroxidase complex (ABC) method[12]. The primary antibody used was a polyclonal NGF antibody for rats (Sigma-Aldrich Inc., Germany). Tissue sections were blocked with endogenous peroxidase activated by 0.3% H2O2 for 30 minutes at room temperature. Non-specific binding sites were blocked with 1% fetal bovine serum in PBS for 30 minutes at room temperature. The tissue sections were then incubated with the primary NGF antibody (1:500) overnight at 4°C. After being washed with PBS 3 times, the slides were incubated for 30 minutes at room temperature with a biotinylated secondary antibody, followed by a 30-minute incubation with streptavidin-HRP at room temperature and the addition of the chromogenic substrate 3,3'-diaminobenzidine (DAB). The slides were rinsed with running water and counterstained with hematoxylin, followed by rehydration and covering with Entellan. Tissue samples were observed under a light microscope with a 40x objective lens in five fields of view.

Data analysis

The assessment of NGF expression intensity was conducted using a microscope with a 400x magnification by comparing the strength of expression intensity in the research sample groups with the control. The

Table 1: ANOVA test after 24 hours.

Group	Amount of NGF		
	Average	Standard Deviation	P
24 hours negative control	10,640	1,862	
24 hours positive control	28,440	11,843	
Treatment + galangal 600 mg 24 hours	21,040	5,009	0.094
Treatment + galangal 1200 mg 24 hours	22,800	16,349	

Table 2. ANOVA test after 48 hours.

Group	Amount of NGF		
	Average	Standard Deviation	P
48 hours Negative control	9,800	08,12	
48 hours Positive control	18,920	6,318	
Treatment + galangal 600 mg 48 hours	25,880	7,884	0.013
Treatment + galangal 1200 mg 48 hours	22,800	16,349	

Table 3. Post Hoc Turkey Analysis.

Group	Average	Standard Deviation	Group	Average	Standard Deviation	p
			Positive control 48 hours	18,920	6,318	0.211
Negative control 48 hours	9,800	0.812	Treatment + Galangal 600 mg 48 hours	25,880	7,884	0.011*
			Treatment + Galangal 1200 mg 24 hours	22,960	9,743	0.041*
Positive control 48 hours	18,920	6,318	Treatment + Galangal 600 mg 48 hours	25,880	7,884	0.424
			Treatment + Galangal 1200 mg 48 hours	22,960	9,743	0.801
Treatment + Galangal 600 mg 48 hours	25,880	7,884	Treatment + Galangal 1200 mg 48 hours	22,960	9,743	0.912

Table 4. IHC NGF expression in 24 and 48 hours post treatment.

GROUP	SAMPLE				
	1	2	3	4	5
24 hour group					
Treatment without extract *Kaempferia galanga* L	+	++	++	+	+
Treatment + Extract *Kaempferia galanga* L 600 mg/ kgBB	+	++	++	++	+
Treatment + Extract *Kaempferia galanga* L 1200 mg/ kgBB	+	+++	++	++	++
48h group					
Treatment without extract *Kaempferia galanga* L	++	++	+	+	+
Treatment + Extract *Kaempferia galanga* L 600 mg/ kgBB	++	++	++	+++	++
Treatment + Extract *Kaempferia galanga* L 1200 mg/ kgBB	++	++	+++	+++	+++
Without treatment and without extracts *Kaempferia galanga* L	+	++	+	+	+
Control	+				

Expression from NGF is performed semi - quantitatively with Description : +, intensity weak ; ++, intensity medium ; +++, intensity strong

assessment of NGF expression intensity was done semi-quantitatively, with the positive control serving as a reference for strong or positive intensity, indicated as three plus signs (+++), while moderate and weak intensities were compared to the positive control. Observations and assessments were carried out in the cortical and subcortical areas of the cerebrum by a neuropathologist.

The assessment of NGF was also performed quantitatively. The collected data were coded, tabulated, and entered into a computer. Data analysis included descriptive analysis and hypothesis testing. Data that were ratio-scaled were expressed in terms of mean, standard deviation, frequency distribution, and percentages in the descriptive analysis and frequency analysis. Data analysis was conducted using the SPSS statistical software.

Normality testing of the data was performed using the Shapiro-Wilk statistical test because the sample size was less than 50. Statistical tests were conducted using one-way ANOVA and path analysis. Test results were considered significant if they had a p-value of <0.05.

RESULTS

The study was conducted on male Wistar rats (Rattus norvegicus), weighing 200-250 grams, which had undergone adaptation processes in the laboratory and were then subjected to random sample selection.

Rats in groups B, C, and D were divided into two subgroups based on the time of decapitation, which was either 24 hours or 48 hours post-traumatic brain injury. The rat brains were then collected, fixed with formalin, and subjected to IHC (Immunohistochemistry) examination to evaluate NGF expression.

The NGF expression assessment was performed semi-quantitatively by a pathologist in a blind manner, without knowledge of the treatment groups. NGF expression in the brain parenchyma was compared with the control. The cortical area was used as the positive control for NGF IHC expression.

Shapiro Wilk test was done and showed that the data was normally distributed. Lavene test was done and showed that the data is homogeneous. Further ANOVA test showed that there was no difference in NGF expression in rat without *Kaempferia galanga* L extract treatment and rat with *Kaempferia galanga* L extract treatment 24 hours after treatment. But in 48 after treatment, ANOVA test showed there was a statistically significant difference in NGF expression in rat without *Kaempferia galanga* L extract treatment and rat with *Kaempferia galanga* L extract treatment.

Further test with Post Hoc Tukey showed that there was statistically significant difference between negative control group and treatment group with 600 mg *Kaempferia galanga* L extract. There was also statistically significant difference between negative control and treatment group with 1200 mg *Kaempferia galanga* L extract

Immunohistochemistry NGF expression

This study used IHC NGF expression in the parietal subcortical as a positive control. The parietal subcortical area shows IHC NGF expression with strong intensity (+++) marked with dark brown color. The expression of IHC NGF in the hippocampus area in the group of mice without brain injury treatment and without administration of galangal extract is a weak control for the expression of IHC NGF (+), as shown in Figure 1

Figure 2 represents the IHC NGF expression in groups B, C and D which underwent decapitation 24 hours after treatment. IHC NGF expression in groups B, C and D which underwent decapitation 48 hours after treatment is shown in Figure 3.

Comparison of NGF Immunohistochemistry Expression

IHC NGF expression in group B which underwent decapitation 24 hours after treatment showed homogeneous results with weak intensity (+) in 60% of samples, moderate intensity (++) in 40% of samples. Group C showed 40% of samples with weak intensity (+) IHC NGF expression, 60% of samples showed moderate intensity (++) at 24 hours post-treatment. 24 hour IHC NGF expression in group D showed expression with weak intensity (+) in 20% of samples, moderate intensity (++) in 60% of samples and 20% of samples showed strong intensity. IHC NGF expression 48 hours after treatment in group B showed expression with

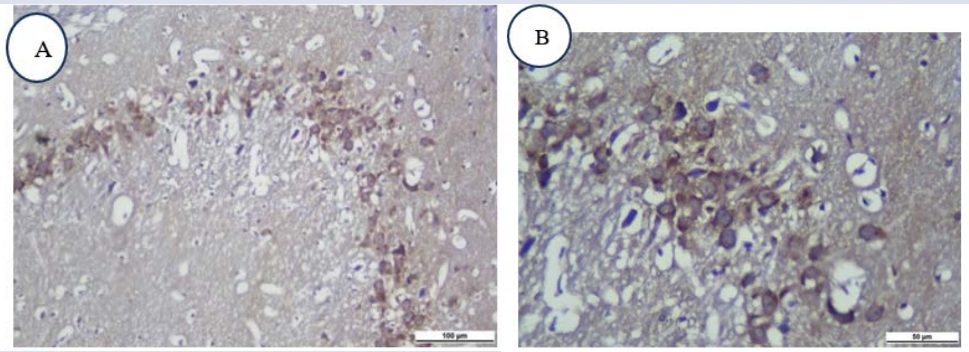

Figure 1. NGF *immunohistochemistry* expression in the hippocampus as a positive control (medium intensity: ++) 20 times magnification (A), 40 times magnification (B).

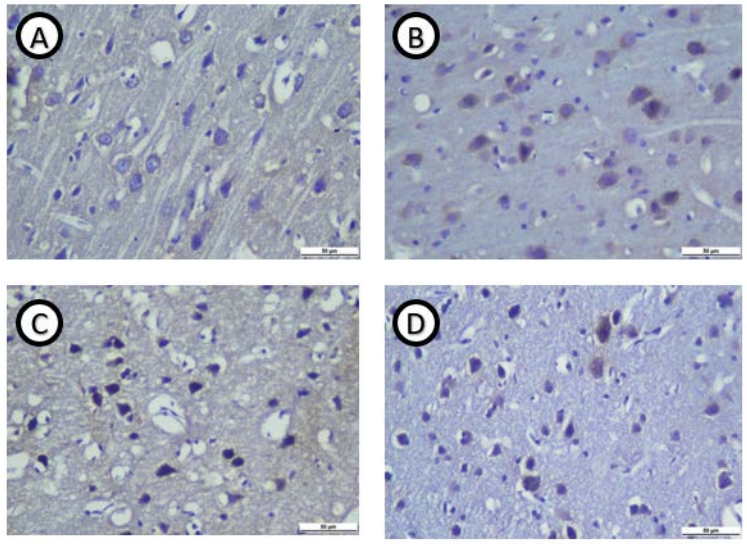

Figure 2. Expression NGF *immunohistochemistry 24 hours* post treatment in the group without treatment (weak intensity : +) (A) treatment group without *Kaempferia galanga* L extract (medium intensity: ++) (B) treatment group and received 600 mg/kgbb *Kaempferia galanga* L extract (medium intensity: ++) (C) group treatment and received 1200 mg/kgbb *Kaempferia galanga* L extract (strong intensity: +++) (D) (40 times magnification).

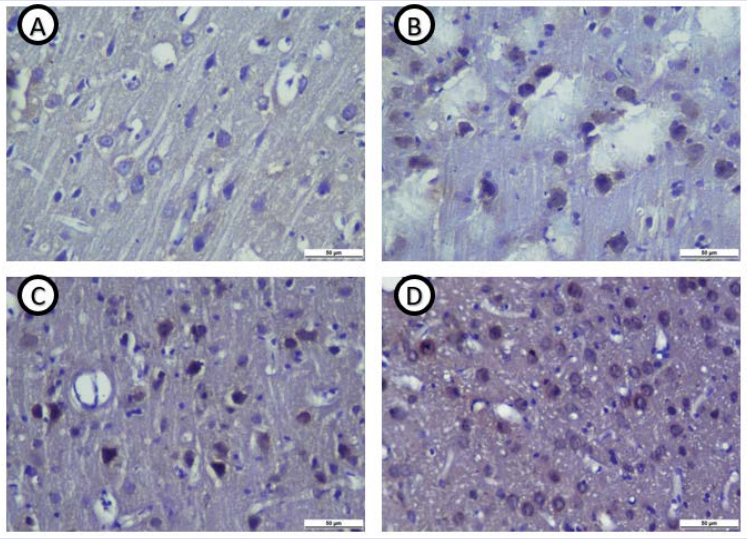

Figure 3. Expression NGF *immunohistochemistry* 48 hours post treatment in the group without treatment (medium intensity : ++) (A) treatment group without *Kaempferia galanga* L extract (medium intensity: ++) (B) treatment group and received 600 mg/kgbb *Kaempferia galanga* L extract (strong intensity: +++) (C) treatment group and received 1200 mg/kgbb *Kaempferia galanga* L extract (strong intensity: +++) (D) (40 times magnification).

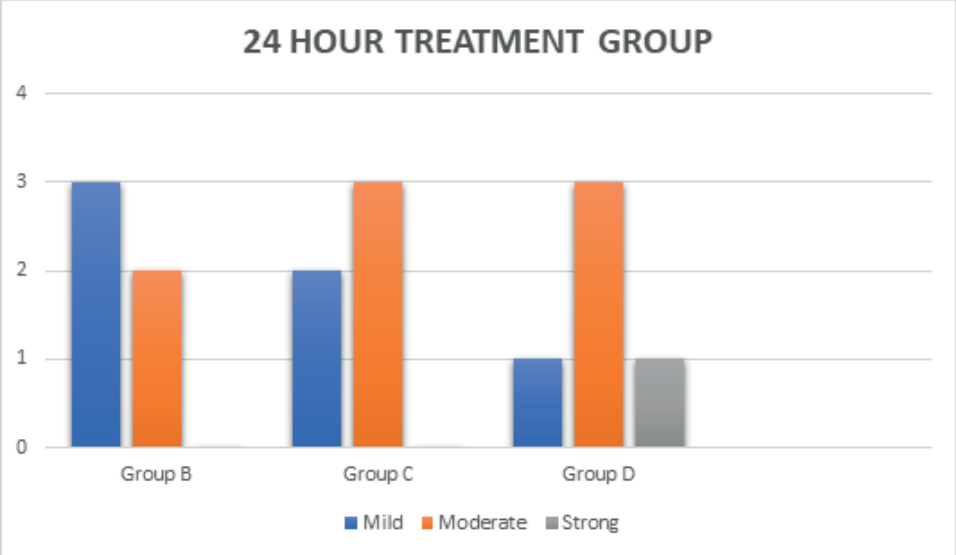

Figure 4. Comparison graph of NGF expression 24 hours after treatment. (Group B: Brain injury treatment without administration of *Kaempferia galanga* L extract; Group C: brain injury treatment group + *Kaempferia galanga* L extract 600 mg/kgbb; Group D: brain injury treatment group + *Kaempferia galanga* L extract 1200 mg/kgbb).

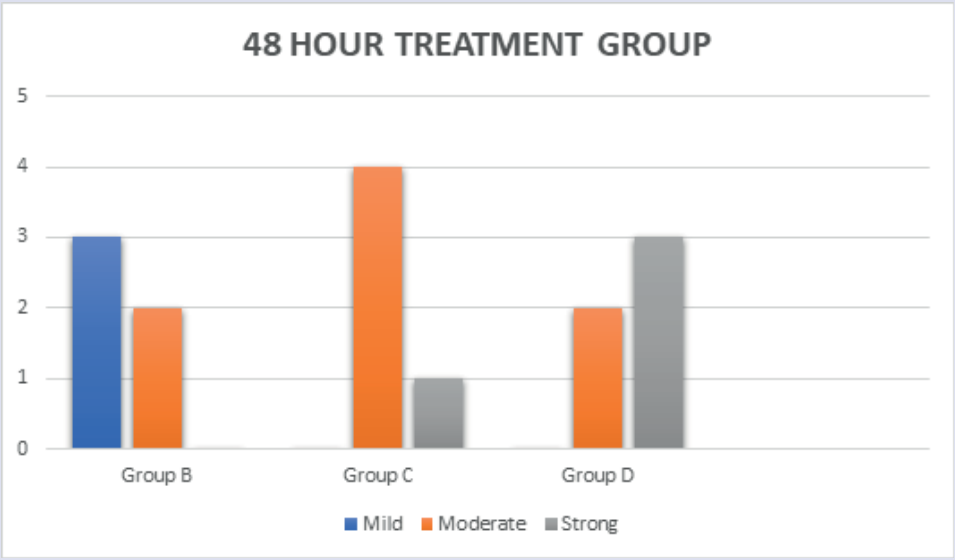

Figure 5. Comparison graph of NGF expression 48 hours after treatment. (Group B: Brain injury treatment without administration of *Kaempferia galanga* L extract; Group C: brain injury treatment group + *Kaempferia galanga* L extract 600 mg/kgbb; Group D: brain injury treatment group + *Kaempferia galanga* L extract 1200 mg/kgbb).

mild intensity in 60% of samples and moderate intensity (++) in 40% of samples. Group C showed moderate intensity IHC NGF expression in 80% of samples and one sample or 20% with strong intensity. IHC NGF expression in group D showed moderate intensity (+) in 40% of samples and 600% of samples with strong intensity.

Of the mice in group B, both groups that underwent 24 hour decapitation, 60% showed NGF expression with mild intensity and 40% showed moderate intensity, while at 48 hours it showed the same intensity. Group C in samples that underwent 24 hour decapitation showed 40% weak intensity and 60% showed moderate intensity, while at 48 hours showed 80% showed intensity and 20% showed strong intensity.

The results for group D showed that 24 hours after decapitation, 20% light intensity, 60% moderate intensity and 20% strong intensity, At 48 hours after treatment and administration of the extract, there was a difference in intensity compared to 24 hours, namely 40% moderate intensity and 60% strong intensity.

DISCUSSION

The cases of traumatic brain injury represent a major issue in morbidity and mortality. Traumatic brain injury can lead to long-term disabilities that impact socio-economic aspects, particularly in developing countries like Indonesia. Recent therapeutic options are continuously being explored with the aim of improving outcomes for traumatic brain injury patients. Preclinical studies have been conducted extensively using animal models that closely resemble pathological conditions in humans.

The "Marmarou's TBI weight drop" model is one method that can induce diffuse closed head injury conditions in rat experimental models. This model is widely used compared to other traumatic brain injury models because closed head injuries are the most common cases of brain injury [9]. The dropout rate due to the treatment method is lower than other traumatic brain injury models [13].

The study by Vittalrao et al., 2011, involving two doses of *Kaempferia galanga* L. extract, namely 600 mg/kg and 1200 mg/kg, indicated anti-inflammatory properties [14]. The anti-inflammatory mechanism of *Kaempferia galanga* L. is known to work by inhibiting pro-inflammatory cytokines IL-1β and tumor necrosis factor[15].

The experimental results showed stronger NGF expression 48 hours after traumatic brain injury in the rat brains that received 1200 mg/kgbb of *Kaempferia galanga* L. extract compared to the group that received 600 mg/kgbb and higher than the positive control group. These results suggest an influence of *Kaempferia galanga* L. extract that may be related to the dosage and timing. Based on these findings, *Kaempferia galanga* L. extract is hypothesized to enhance NGF expression after traumatic brain injury and has the potential to become a new therapeutic agent for improving nerve recovery post-injury. This effect is likely attributed to the anti-inflammatory and antioxidant effects of *Kaempferia galanga* L. extract and the increased NGF expression [15].

This experimental study revealed variability in NGF expression in each research group based on the dosage of *Kaempferia galanga* L. extract administered. The administration of *Kaempferia galanga* L. extract is believed to contribute to these results. The group of rats receiving 1200 mg/kgbb of *Kaempferia galanga* L. extract showed a stronger NGF expression compared to both the group that did not receive *Kaempferia galanga* L. extract and the group receiving 600 mg/kgbb. A similar trend was observed in the group of rats that were decapitated 48 hours after treatment and received *Kaempferia galanga* L. extract.

Kaempferia galanga L. extract is known to have anti-inflammatory and antioxidant effects (Vittalrao et al., 2011). The anti-inflammatory effect of *Kaempferia galanga* L. is known to work by inhibiting pro-inflammatory cytokines IL-1β and tumor necrosis factor due to the presence of ethyl p-methoxycinnamate (EPMC) (Ahmed et al., 2014). *Kaempferia galanga* L. has lipophilic properties that help it penetrate the Blood-Brain Barrier [6].

Kaempferia galanga L. extract (galangal) contains Kaempferol glycosides, which can provide neuroprotective effects to prevent neurocognitive deficits, cerebral infarction volume, neuronal and axonal damage, and glial cell pathological responses. This mechanism is achieved by inhibiting COX, NO, ROS, and NF-kB. This inhibition of COX and NF-kB content has the potential to be a therapy for neuroinflammatory diseases (6). Kaempferol and its glucoside derivatives have a wide range of pharmacological activities, and studies have shown that these compounds are well absorbed in the intestines and can penetrate the blood-brain barrier. Kaempferol protects nerve cells by preventing mitochondrial membrane potential degradation and inhibiting ROS production, thus increasing cell resistance [17].

In this experimental study, the normality test using the Shapiro-Wilk test indicated that the data followed a normal distribution ($p > 0.05$) with a sample size of less than 50. The Levene test for variance homogeneity showed a significance value of 0.277 ($p > 0.05$), indicating homogeneity in the sample data population. The ANOVA test showed a significance value of 0.094 ($p > 0.05$) for the treatment group compared to the control group at 24 hours, indicating no significant difference in NGF expression in rats with traumatic injuries with or without galangal extract at 24 hours post-treatment. In the 48-hour ANOVA test, the significance value for the treatment group compared to the control group was 0.013 ($p < 0.05$), indicating a significant difference in NGF expression in rats with traumatic injuries with or without galangal extract at 48 hours post-treatment.

The Tukey Post Hoc test was used to determine the difference between two groups with a significance value of $p < 0.05$. The negative control group compared to the positive control group had a significance value of 0.211 ($p > 0.05$), indicating no significant difference between these two groups. The negative control group compared to the treatment group receiving 600 mg of galangal extract showed a significance value of 0.011 ($p < 0.05$), indicating a significant difference. The negative control group compared to the treatment group receiving 1200 mg of galangal extract had a significance value of 0.041 ($p < 0.05$), indicating a significant difference. The treatment group with 600 mg of galangal extract compared to the treatment group with 1200 mg of galangal extract had a significance value of $p > 0.05$, indicating no significant difference between the two groups.

The increase in NGF expression may be due to the suppression of pro-inflammatory cytokines IL-1β, TNF, NO, ROS, NF-kB by the anti-inflammatory effect of *Kaempferia galanga* L. The anti-inflammatory effect is attributed to ethyl-p-methoxycinnamate (EPMC), which inhibits COX-1 and COX-2 enzymatic activity. Additionally, Kaempferol found in galangal can have neuroprotective effects and reduce inflammation (Yu et al., 2013). The anti-inflammatory mechanism of *Kaempferia galanga* L. is known to work by inhibiting pro-inflammatory cytokines IL-1β and tumor necrosis factor [15].

This study still has some limitations due to financial constraints. The first limitation is the lack of measurement of the active substance content in *Kaempferia galanga* L. extract. The second limitation is the absence of measurement of NGF in cerebrospinal fluid (CSF). Another limitation is that the primary mechanism of action of the active substance in *Kaempferia galanga* L. extract in influencing brain NGF has not been tested in vitro.

CONCLUSION

In summary, this research sheds light on the promising role of *Kaempferia galanga* L. extract as a potential therapeutic agent to improve outcomes in TBI cases by enhancing NGF expression. However, further research is warranted to comprehensively understand the underlying mechanisms and assess the extract's clinical applicability. By addressing these limitations and conducting additional studies, the potential benefits of this extract could be translated into clinical practice, offering hope for improved outcomes and recovery for TBI patients in the future.

CONFLICT S OF INTEREST

Authors declare there is no conflicts of interest.

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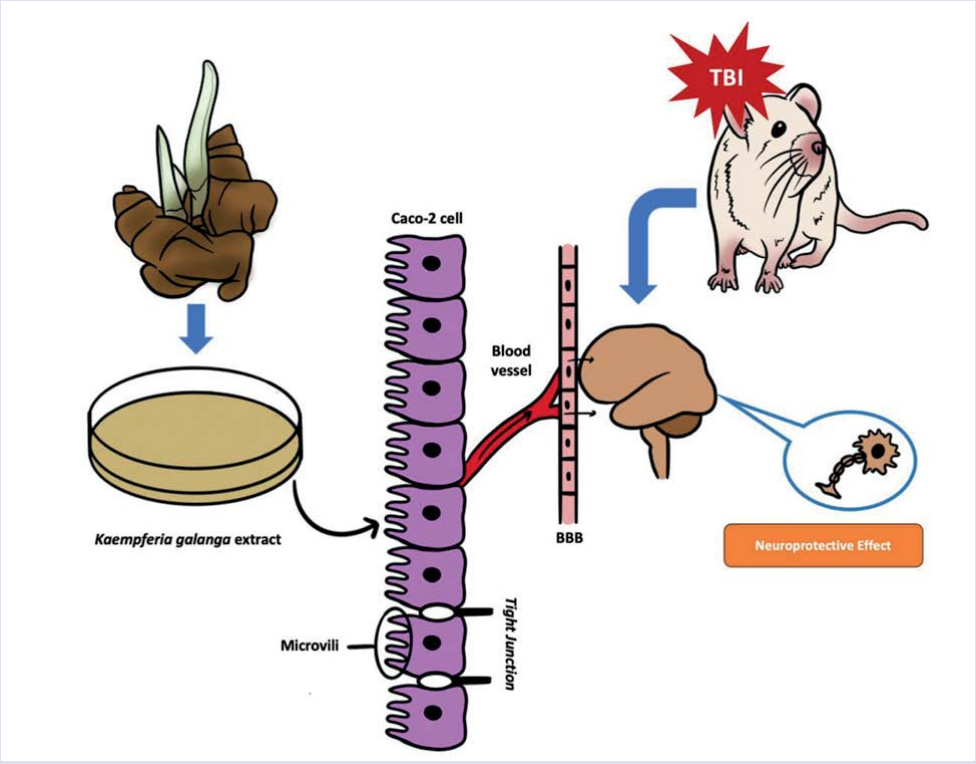
GRAPHICAL ABSTRACT



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