

The Effect of *Indigofera tinctoria* Extract on Cdk5 Expression and Signs of Inflammation in A Chronic Pain Rat Model

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Abstract: Chronic pain is a challenging case that requires efforts to find effective drugs with minimal side effects. In the last decade, Cdk5 has become a potential target of chronic pain treatment. *Indigofera tinctoria* is a plant that grows abundantly in Indonesia and has been used for various purposes. This plant is proven to contain indirubin which was proven as a Cdk5 (Cyclin dependent kinases 5) inhibitor. The objective of this research is to analyze the effect of *Indigofera tinctoria* in a chronic pain model. This research was conducted on 35 rats divided into 5 groups. CFA (Complete Freud's Adjuvant) solution was injected into the right plantar area to make a chronic pain model. *Indigofera tinctoria* extract was given orally in 200 mg/KgBW (D1 group), 300 mg/KgBW (D2 group), and 400 mg/KgBW (D3 group) doses. Treatment was given for 7 days after the sign of chronic inflammation were detected (28 days after CFA induction). The analgesic effect was determined by pain latency on the hotplate, while the anti-inflammatory effect was evaluated by measuring the hind paw volume. The Cdk5 expression was detected at the dorsal root ganglia by immunohistochemistry method. The mean pain latency value of the D2 and D3 groups was greater than that of the other groups. The inflammatory sign in the treatment group was smaller and differs significantly from the positive control group. Cdk5 expression was not statistically different, however, the treatment group showed a lower Cdk5 expression. *Indigofera* extract has potential analgesic, anti-inflammatory, and Cdk5 inhibitory effects.

Keywords: *Indigofera tinctoria*; Cdk5; analgesic, anti-inflammatory

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Introduction

Chronic pain is a very disturbing condition and affects various aspects such as the economy and social relations (Mills et al., 2019). Research conducted in 42 countries shows that the incidence of chronic pain is high (Gobina et al., 2019). This may be due to difficulty in chronic pain treatment (Lynch, 2016). Some patients even need additional drugs such as antianxiety, antidepressants, anticonvulsants, and opioids (Lynch, 2016; Schultz & Licciardone, 2022). These additional drugs pose a risk of serious side effects (Schwan et al., 2019). Therefore, new, safe, and potent drug discovery is emerging. The factor believed to be the key to chronic pain and becoming the target of treatment is Cdk5

(Gomez et al., 2020; Utreras et al., 2011). Many research have been conducted to develop a potent Cdk5 inhibitor, but finding selective and safe Cdk5 inhibitor is still a challenging issue (Liu et al., 2017; Utreras et al., 2011). This protein kinase plays an important role in the activity of TRPV-1 which is responsible for pain induction and transmission. Cdk5 plays a role in the phosphorylation and insertion of TRPV-1. Cdk5 also has a role in other important receptors, such as N-methyl-D-aspartate (NMDA), P/Q type voltage-dependent calcium channel, and ATP-gate P2X receptors (Kumar Pareek, 2012). Activation of these receptors by Cdk5 will cause sensory nerves to be more sensitive to various stimuli. This condition is related to a chronic pain

condition. On the other hand, Cdk5 also has an important role in the inflammatory process by activating proinflammatory factors (Utreras et al., 2011). Zhu *et al* (2021) proves that Cdk5 expression is increased in chronic pain. One substance that has been proved to be effective as a Cdk5 inhibitor is indirubin (Blažević et al., 2015; Tan et al., 2007). The plant that is proven to contain indirubin is *Indigofera tinctoria* or tarum. Extracts from this plant have been proven to contain flavonoids, alkaloids, glycosides, indigotin, indirubin, and rotenoids (Laitonjam & Wangkheirakpam, 2011; Wahyuningsih et al., 2017). Indonesian people have known it since the days of the Tarumanegara Kingdom and used it as a natural dye and animal feed (Al-Rasyid, MYA; Saade & I, 2019; Muzayyinah, 2014; Wahyuningsih et al., 2017). Health research proves that this plant can be used for various treatments, including as a laxative, expectorant, anthelmintic, antidiabetic, anti-inflammatory, antiepileptic, and to treat neuropathy (Keziah et al., 2016; Wahyuningsih et al., 2017). Based on the results of several studies mentioned, we hypothesize that *Indigofera tinctoria* plant extracts are potential candidates for chronic pain treatment. In this study we would like to examine the effect of *Indigofera tinctoria* extract on Cdk5 expression regarding to its analgesic and anti-inflammatory effect. The development of this plant as a chronic pain medicine will be very helpful because this plant is known to be very easy to cultivate.

Materials and Methods

This is an experimental laboratory research with a post-test-only control group design. This experiment involved white rats, 3-month-old, male, Wistar strain. The experimental unit was divided into 5 groups, those are negative control, positive control, D1 (200mg/KgBW of *Indigofera tinctoria* extract), D2 (300 mg/KgBW of *Indigofera tinctoria* extract), and D3 (400 mg/KgBW of *Indigofera tinctoria* extract). The number of replications for each group was 5. The negative control group consists of normal rats. The positive control group consists of chronic pain model rat without any treatment.

Indigofera tinctoria extraction

Indigofera tinctoria plant (tanaman tarum) was collected from Gunung Kidul and identified by Balai Materia Medica, Batu, Malang, East Java (identification certificate no. 074/567/102.20-A/2022). Three kilograms of *Indigofera* leaves and twigs were dried at room temperature, and powdered using a fruit blender. The powder was then soaked in ethanol for about 24 hours. The soaked solution was then put into a beaker glass and immersed in a vessel filled with water at a temperature of 50° C and then aerated until a paste

was formed at the bottom of the beaker glass. This paste was formulated as the *Indigofera* solution.

Animal treatment

The procedure used in this study has been approved by the Ethics Committee of the Faculty of Veterinary Medicine, Airlangga University (No: 2.KEH.065.06.2022). Rats were adapted in the Pharmacology Animal Laboratory of the Medical Faculty, Airlangga University for 7 days and kept in solitary cages using soft mats. The dark and light cycles are carried out normally without manipulation. Rats were fed using standard feed produced by PT Charoen Pokphand Indonesia, and given water *ad libitum*. The cages were cleaned every morning. Chronic pain induction was done by injecting 0.1 ml of CFA (Sigma Aldrich) in the plantar area (right hind paw). CFA injections were carried out weekly. This procedure was repeated 4 times (28 days). *Indigofera* extract was given orally using a sonde. The doses of *Indigofera tinctoria* extract given were 200 mg/kg BW, 300 mg/kg BW, and 400 mg/kg BW according to their respective groups. The *Indigofera* extracts were given every morning before the rats were fed. This treatment started on the 29th day after CFA induction and given for 7 days. The control positive group was only injected by CFA without *Indigofera* administration.

Pain assessment

The pain threshold was assessed on the 36th day by using a hot plate. The hot plate temperature setup was 51° C (Yang et al., 2007). Each rat was assessed 3 times and the average value was taken. The pain threshold was assessed based on the latency period of the rat to stay at the hot plate (counted in seconds). The latency test was stopped when the rats showed one or more of the following behaviors: jumping from the hot plate, licking their hind paw, or wagging their paw.

Inflammation assessment

The inflammation sign was assessed based on the hind paw volume (ml). The volume of leg was measured using a plethysmometer. Signs of inflammation in the legs were determined based on the difference in paw volume between normal and affected limbs.

Cdk5 expression

Cdk5 expression were determined by the immunohistochemical method. Cdk5 expression were evaluated in the dorsal root ganglia. Rats were sacrificed after all of the procedure had finished. Dorsal ganglion

was taken from each rat by following the sciatic nerve as a marker. The dorsal ganglion was then used in the histological preparation. The Intensity of the Cdk5 expression was determined using the IRS scoring system and the IRS value are shown in table 3.

Result and Discussion

Result

The average pain latency values of the three repeated assessments are shown in table 1. These results were then analyzed using the SPSS program. The data shows a normal distribution. One Way ANOVA test shows a significant difference between groups (p value: 0.00). The mean latency value of the treatment groups (D1, D2, and D3) were higher than the control groups, however, the mean latency of D2 (20.35 ± 2.14) and D3 (16.58 ± 1.21) groups were statistically significant based on Fisher's LSD test. Higher pain latency showing the analgesic effect of the tested rat.

Table 1. Pain latency in hotplate examination (in second)

Groups	Means	p-value (one-way ANOVA)
Negative control	9.17 ± 1.32	0.00
Positive control	9.40 ± 1.85	
D 1	10.46 ± 1.08	
D 2	20.35 ± 2.14 ^a	
D 3	16.58 ± 1.21 ^a	

^a sign shows significantly different. The pain latency of the treatment group is higher than the control group. Higher pain latency indicating higher analgesic effect.

The mean difference in hindlimb volume between normal and affected hindlimb (edema) is shown in Table 2. These values indicates the inflammation sign and severity of inflammation. All groups that had CFA induction have a greater volume and are significantly different from the normal rats, thus indicating chronic inflammation. The volume of edema in the treatment group were higher than the negative control group (normal rats), but lower than the positive control group. The smallest mean volume of edema was found in the group that received a dose of 300 mg/kg BW *Indigofera tinctoria* extract (D2 group).

Table 2. Hindlimb volume as inflammation sign

Treatment group	Means ± SD	p-value (one-way ANOVA)
Negative control	0.12 ± 0.03	0.00
Positive control	1.47 ± 0.09 ^a	
D 1	1.03 ± 0.14 ^{ab}	
D 2	0.58 ± 0.15 ^{abc}	
D 3	0.97 ± 0.05 ^{ab}	

^{a, b, c} sign shows significantly different when compared to no superscript number. The ^b sign shows significantly different compared to no superscript number and ^a. The ^c sign shows significantly different to all groups. Hindlimb volume of the treatment group is smaller than the positive control group. A reduction in the hindlimb volume indicated a reduction in the inflammatory process.

Cdk5 expression in the dorsal root ganglia can be seen in figure 1. Cdk5 expression is not statistically different, however, the treatment group showed lower Cdk5 expressions compared to the positive control group. The Cdk5 expression is shown as brown color in the IHC examination (figure 1).

Table 3. Cdk5 expression in the dorsal root ganglia

Treatment group	IRS Means ± SD	p value (Kruskal Wallis)
Negative control	1.6 ± 0.98	0.56
Positive control	3.3 ± 1.06	
D 1	3.12 ± 0.85	
D 2	1.7 ± 0.3	
D 3	1.89 ± 0.11	

Cdk5 expression is not significantly different, however, the treatment groups show a lower expression than that in the positive control group. High Cdk5 expression indicates that the pain transmission and the inflammatory process is still going on. The results that comparing these three parameter (pain latency, inflammation, Cdk5 expression) are presented as a bar chart in figure 2.

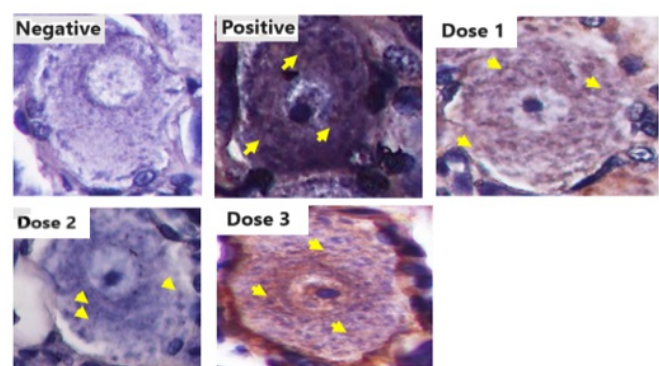


Figure 1. The brown color pointed by the arrow shows the expression of Cdk5. The higher the brown color intensity the higher the Cdk5 expression. The positive control group shows the highest intensity

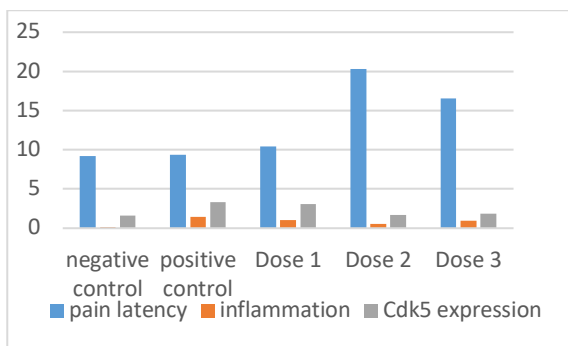


Figure 2. Pain latency, inflammation sign, and Cdk5 expression. The treatment groups showed higher pain latency, lower signs of inflammation, and lower Cdk5 expression

Discussion

The results showed that the expression of Cdk5 in the group of rats experiencing chronic pain and inflammation was slightly higher than in normal rats (negative control group), however, this was not statistically significant (Table 3). The results were in accordance with the previous studies about the role of Cdk5 in chronic pain and inflammation. Cdk5 was proven to be the main player in pain pathways (Fang-Hu et al., 2015; Kumar Pareek, 2012). This study showed that the Cdk5 expression in the treatment group that receives *Indigofera tinctoria* extract was lower than the positive control group. This result showed that *Indigofera* extract administration can reduce the Cdk5 activity caused by CFA induction. This effect is related to the indirubin content in the *Indigofera* extract (Leclerc et al., 2001). The Cdk5 expression in the dorsal root ganglia was not statistically significant, however, the sign of pain and inflammation were significantly different. In accordance with Cdk5 expression, the treatment group showed a higher analgesic and anti-inflammatory effect. D2 and D3 groups showed greater pain latency compared to the other group (Table 1). Pain latency of the D1 group was higher than that in the control group, however, this was not statistically significant. These results proved the hypothesis that the *Indigofera* extract have an analgesic effect, and those effect seem to be mediated by its Cdk5 inhibitory effect. This finding strengthens the previous finding that mentioned Cdk5 inhibition would affect the pain transmission and inflammatory stages (Wilkaniec et al., 2018; Wu et al., 2016). Inflammatory signs are characterized by hind paw enlargement or increased hind paw volume. Leg volume in the treatment group was smaller than the positive control group and this difference was statistically significant (Table 2). These results indicate that the administration of *Indigofera* extract can reduce inflammation.

The three results of this study illustrate that in animal models experiencing chronic pain and

inflammation, there was an increase in Cdk5 expression in the dorsal ganglion, although the increase was not significant. These results do not mean that Cdk5 does not have a role in inflammation or pain, but instead illustrate that even a slight increase in Cdk5 can cause symptoms of inflammation and pain. This is based on research conducted by Qorib et al (2021), which proved that Cdk5 expression in the dorsal ganglion has a strong positive correlation with signs of inflammation and pain latency (Qorib et al., 2021). Other studies have also proved the important role of Cdk5 in TRPV1 and NMDA receptors activation which play an important role in the induction and transmission of pain signals in sensory nerve fibers (Xing et al., 2012). Other studies have also proved that giving Cdk5 inhibitors (roscovitine) can increase the anti-inflammatory effect of corticosteroid class drugs (Pfänder et al., 2019). These findings and previous findings indicate the important role of Cdk5 so that Cdk5 becomes an important target in the treatment of chronic pain and inflammation. The indirubin content in the *Indigofera tinctoria* plant used in this study is thought to be a factor causing increased pain latency and improved signs of inflammation. The analgesic effect may be due to the inhibition of Cdk5 by indirubin, which then decrease TRPV1 expression on sensory nerve fibers so that the induction of pain originating from thermal stimulation is decreased (Leclerc et al., 2001). Decreasing the induction in sensory nerve fibers is then seen as a prolonged pain latency in experimental animal tests using a hotplate. Indirubin have been proved as Cdk5 inhibitor through *glycogen synthase kinase-3beta* (GSK-3 beta) binding. GSK-3 is binding site for ATP to activate Cdk5. This mechanism have a similarity to roscovitine and olomoucine (Leclerc et al., 2001). Cdk5 inhibition in the inflammatory model has also been shown to stop the inflammatory process through various pathways including increasing polymorphonuclear cell (PMN) apoptosis, as well as decreasing IL-1 β , IL-6, TNF- α , iNos, and nitrite synthesis (Pfänder et al., 2019). Reduction of the inflammation sign in this study can occur through one of those mechanisms. In this study we cannot make a precise statement about the dose effects relationships because the number of groups involved in this study was less than five. The next study involving a greater group is mandatory to evaluate the dose-effect relationship.

Conclusion

Indigofera tinctoria extract reduces the Cdk5 expression, and shows an analgesic and anti-inflammatory effect. *Indigofera tinctoria* can be developed as potential chronic pain medication.

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