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Review

Analgesic Effect of Ethanol Extract of *Dillenia ochreata* (Miq.) Teijsm. & Binn. Ex Martelli in Wistar Rats

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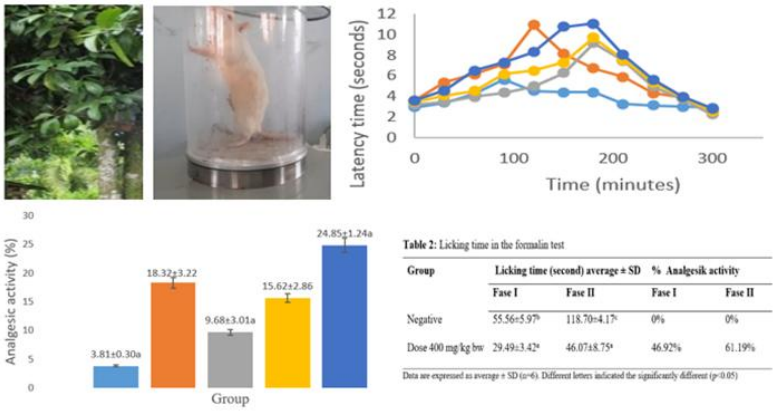
Abstract

Dillenia ochreata is a traditional medicine used to treat wounds and scabies. In wound healing, one of the treatments is to reduce pain (analgesic). Some compounds of triterpenoid groups have been known to be active as analgesic compounds. The leaf of *D. ochrea* was reported to contain secondary metabolites triterpenoid centulic acid and 3β-glucopyranosyl-lup-20(29)-en-28 oat. This study aimed to evaluate the analgesic activity and standardization of the ethanol extract of *D. ochreata* leaf and determine the mechanism of action. Analgesic activity was determined by a hot plate method and formalin test, and the mechanism of action was through muscarinic, dopamine, and opiate receptors, standardization of extract using the method issued by the Indonesian Ministry of Health. The ethanol extract of *D. ochreata* leaf at a 400 mg/kg bw dose has higher analgesic activity (24.85%) than the positive control (18.32%). Statistical analysis showed a significant difference in analgesic activity percentage between the positive group and those with a 400 mg/kg bw group dose. The 400 mg/kg bw dose also showed a significant difference ($p<0.05$) between neurogenic pain (46.92%) and inflammation (61.19%) in the formalin test caused by opioid receptors. The evaluation of the analgesic mechanism showed the ethanol extract of *D. ochreata* leaf works through opioid receptors. The extract meets the requirements of the standard parameters. The leaf extract of *D. ochreata* can be developed as an anti-analgesic from natural medicine.

Keywords: *Analgesic; Dillenia ochreata; formalin test; hot plate test; receptors.*

Graphical Abstract



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Introduction

Pain is a sensory and emotional feeling related to tissue damage, such as inflammation, infection or muscle spasms. The drugs most widely used for the treatment of pain are non-steroidal anti-inflammatory drugs (NSAIDs). The use of NSAIDs for a long period can cause side effects such as gastrointestinal, heart and kidney effects [1]. Therefore, a relatively safe medicinal substance, such as traditional medicinal plants, is needed for pain (analgesic) treatment. *Dillenia ochreata* is a traditional medicine plant that is used for the treatment of wounds and scabies. In Indonesia, this plant is known by the local names simpur and simpor [2]. The phytochemical analysis reported that *D. ochreata* leaves contain triterpenoid, steroid, phenolic, and flavonoid compounds. *D. ochreata* leaf ethanol extract is active in healing burns and incision wounds [3]. A subchronic toxicity test of the ethanol extract of *D. ochreata* leaves up to 400 mg/kgBW showed no toxicity [4]. The leaf of *D. ochreata* contained triterpenoid centulic acid and 3β-glucopyranosyl-lup-20(29) -en-28 oats, which are active as an antibacterial with MIC value to *Escherichia coli* and *Staphylococcus aureus* 60 μ g/ml and 120 μ g/ml respectively for centulic acid and 15 μg/ml and 60 μg/ml for 3β-glucopyranosyl-lup-20(29)-en-28 oat [5, 6]. Centulic acid can potentially treat inflammatory diseases by suppressing proinflammatory cytokines such as TNF-α, INFγ, IL-6, and IL-12 [7]. Some compounds of triterpenoid groups have been known to be active as analgesic compounds. Two triterpenes, betulonic acid and cabraleone, isolated from the leaves of *Combretum glutinosum*, have been reported to be active as an analgesic [8]. The analgesic activity mechanisms of triterpenoid involve the inhibition of phospholipase enzymes and the cyclooxygenase and lipooxygenase pathway that involves modulating the activity of inhibitory neurotransmitters, as well as interaction with the central nervous system. This compound can reduce the production of pain mediators such as prostaglandins and leukotrienes, and modulate synaptic transmission in the brain [9]. Analgesic activity of the extract was related to its potential to modulate the release of inflammatory mediators responsible for pain. This study aims to determine the analgesic effects of *D. ochreata* leaves in rats, determine the analgesic mechanism of action and the standardization of extract

Materials and Methods

Chemicals and Eqipments

Materials used in research: the leaves of *Dillenia ochreata*, Ethanol 96% (Brataco®), Na CMC (Brataco®), diclofenac sodium (Aarti Drugs®), Formalin 2.5%, atropine sulfate (Ethica industry pharmacy®), metoclopramide (Pharma chemistry®), naloxone hydrochloride (Indo pharma®), Tool used in this study: *rotary evaporator* (IKA® HB 10), Hot Plate (DLAB®).

Test Animals.

The experiment used 73 Male albino rats from the Animal Laboratory Centre at Palembang, South Sumatra, Indonesia. The protocol experiment of the research has been approved by the ethics committee from Ahmad Dahlan University with register No. 022210064. The test animals were acclimatized for seven days, and the test animals were provided standard food and drinks.

Sample preparation

Dillenia ochreata leaves fresh (1 kg) were collected from the Ngulak I Village, Sekayu, Musi Banyuasin district in South Sumatera, Indonesia. The sample was identified as *Dillenia ochreata* (Miq) Teijsm. & Binn.ex Martelli at Herbarium Bogoriense as Research Center for Biology, Indonesian Institute of Science Cibinong, Indonesia, with register number B-82/IV/D1.01/i/2021.

Extraction

The fresh *D. ochreata* leaves were dried and ground in a grinding mill into a powder. The *D. ochreata* leaves powder (200 g) was extracted using the maceration method using ethanol 96% (1 L) for 72 hours. After that, the Whatman filter paper was used for filtration. The maceration was carried out with three repetitions [6]. The filtrate was concentrated to dryness using a rotary evaporator and water bath at 60 ^{0}C at a speed of

80 rpm until a concentrated extract was obtained at a constant weight. Then, the crude extract was determined as the yield percentage.

Hot Plate Test

The test was conducted using the hot plate method described by Raveendran et al. (2019) [10]. Wistar rats (25 male, weighing 150-250 g) were divided into five groups: negative (1% NaCMC). Positive (diclofenac sodium 4.5 mg/kg body weight (BW) and three treatment groups at doses 100, 200 and 400 mg/kg bw. A preliminary animal test was conducted to determine the initial response. The animal is placed on the hot plate at a temperature of 50 ^{0}C. The response time was measured, such as jumping, withdrawing, pawing, and feet licking. After the preliminary test, the animal was treated with ethanol extract of *D. ochreata* with doses of 100, 200, and 400 mg/kg bw while the positive control group was given diclofenac sodium. Hot plate tests were conducted sixty minutes after treatment, and the test was performed every 30 minutes for 5 hours. The analgesic activity was determined based on response time compared to the control negative and expressed as latency time [11].

Formalin Test

The twelve rats were divided into two treatment groups comprising six male rats. Group 1 was treated with Na CMC 1%, while group 2 was treated with ethanol extract, which provided the greatest analgesic activity in the hot plate test (400 mg/kg bw). Two hours after treatment, each rat was injected on the surface of its left hind paw with 0.05 ml formalin solution (2.5%). Then, the animals were hot plate tested and observed response time for such as jumping or withdrawal of the paws 1 hour was recorded. The initial phase lasts 0 to 5 minutes, and the final lasts 20 to 60 minutes. The percentage of analgesic power was calculated [12].

Evaluation Mechanism of Analgesic Action.

The evaluation mechanism of the analgesic effect was determined by examining the muscarinic, dopamine and opiate receptors. Thirty-six rats were divided into six groups. Evaluate the mechanism of muscarinic receptors using two groups (I and II), evaluate the mechanism of dopamine receptors using two groups (III and IV) and evaluate the mechanism of opiate receptors using two groups (V and VI). The treatment in each group is shown in Table 1. All rats have conducted a hot plate test for 1 hour before administration of all receptor antagonists. One hour later, all groups were treated with the 2 ml ethanol extract at the dose with the highest analgesic effect determined by the hot plate test (400 mg/kg bw), then 2 hours after the ethanol extract was administered, and all rats conducted a hot plate test [13].

Standardization of the Extract

Standardization of the extract was carried out by measuring specific parameters (organoleptic properties, the water and ethanol soluble extractive content) and non-specific parameter analysis (water content, total ash content, acid soluble ash content, metal contamination and microbial contamination) using the method issued by Indonesian Ministry of Health [14].

Statistical Analysis

The data were analyzed statistically using the Shapiro-Wilk normality test. If the data is normally distributed (p-value > 0.05), continue to test with the One-way ANOVA test analysis and Paired samples t-test using the SPSS®.

Result and Discussion

Hot plate test. D. ochreata leaf simplicia (200 g), after Extraction with 96% ethanol and concentration, obtained a crude extract of 29.62 g with a yield percentage of 14.81%. The hot plate method determined the analgesic effect with heat stimulation based on latency time. Analgesic activity was observed every 30 minutes for 5 hours, as shown in Figure 1. The negative control group showed a decrease in the average latency time, with peak analgesia occurring at 90 minutes; meanwhile, the positive control group was given diclofenac sodium, effective as an analgesic and an NSAID class. The diclofenac sodium contained analgesic effectiveness of 4-6 hours [15]. The maximum analgesic effect of diclofenac sodium occurred at 120 minutes [16].

After the 120th minute, there was a decrease in the analgesic effect. The treatment group doses of 100, 200, and 400 mg/kg bw showed the peak analgesic time at the 180th minute, and the maximum peak was obtained at a dose of 400 mg/kg bw (Figure 1).

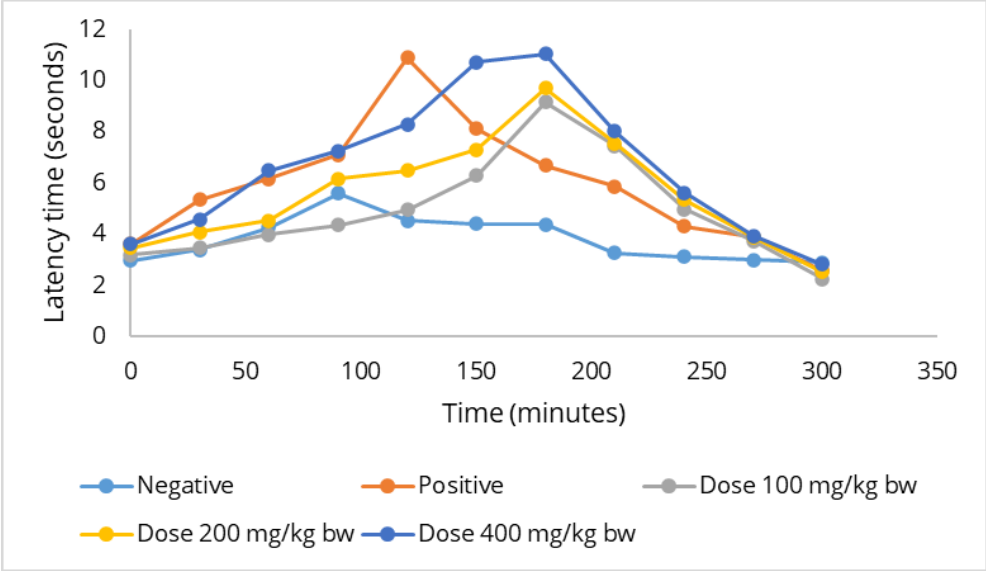
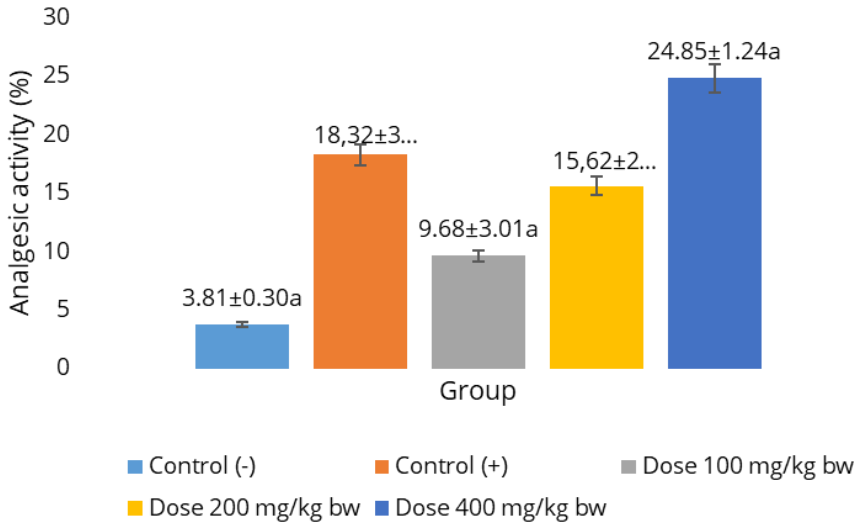


Figure 1: Latency time to heat stimulation

The decrease in the analgesic response of the positive control and the treatment group was caused by the metabolism of compounds into an inactive form, thereby reducing the effect [17]. The decrease in response to analgesic effects was also caused by repeated exposure to painful stimuli, which can cause an increase in pain [18]. The difference in individual thresholds makes pain more subjective and challenging to categorize within a specific range. Statistical analysis showed a significant difference ($p < 0.05$) in latency time between treatment groups. There was also a significant difference ($p < 0.05$) between the positive control (diclofenac sodium) and the treatment group dose of 400 mg/kg bw. Percentage analgesic activity showed a decrease in pain response. The 400 mg/kg bw dose group showed the highest percentage of analgesic activity compared to other doses and higher than the positive control group (Figure 2). Therefore, the 400 mg/kg bw analgesic activity was the best dose for suppressing pain. The results of this study supported the wound-healing activity capabilities that have been reported [3]. Other species from *Dillenia*, such as *Dillenia indica* reported that the ethyl acetate extracts of *D. indica* (100 and 300 mg/kg) possessed good central as well as peripheral analgesic activity as compared with pentazocine and indomethacin (10 mg/kg) respectively]19].

Some compounds that act as analgesics include triterpenoids, steroids, phenolics and flavonoids. Previous studies have shown that *D. ochreata* contained triterpenoids, such as centulic acid. Triterpenoids serve as analgesics by suppressing proinflammatory cytokines. including TNF-, INFγ, IL-6, and IL-12 [11]. The compound also inhibits the production of prostaglandin E2 (PGE2), thereby producing an analgesic effect [20]. Steroid compounds can stimulate the biosynthesis of lipomodulin protein, which can inhibit the enzymatic phospholipase action. This enzyme releases arachidonic acid and its metabolites, thereby possessing analgesic and anti-inflammatory effects [21]. Phenolic compounds act as analgesics by suppressing the formation of free radicals, which cause tissue damage. The free radicals trigger the biosynthesis of arachidonic acid, a mediator of inflammation and pain [22]. Furthermore, flavonoids act as analgesics by inhibiting the action of the cyclooxygenase (COX) enzyme, which causes a decrease in the amount of prostaglandin production, thereby reducing pain.



Data are expressed as mean ± SD (n=5). Letters indicate a significant difference (p<0.05).

Figure 2. Percentage analgesic activity

Formalin Test

Analgesic activity was determined using the formalin method by observing the length of time the rat licks its paw. Licking time was a pain parameter in the formalin test, and a higher observed rate in rats correlated with more significant pain perception [23]. The licking time in the negative control group for each phase was more valuable because 1% Na CMC does not have properties as an analgesic. The treatment group had an analgesic activity percentage of 46.92% and 61.19% for Phase I and II, respectively (Table 2).

Table 2: Licking time in the formalin test

Groups	Licking time (second) average ± SD		% analgesic activity	
	Fase I	Fase II	Fase I	Fase II
Negative	55.56±5.97[b]	118.70±4.17[c]	0%	0%
Dose 400 mg/kg bw	29.49±3.42[a]	46.07±8.75[a]	46.92%	61.19%

Data are expressed as average ± SD (n=6). Different letters indicated the significantly different (p<0.05)

Statistical analysis showed a significant difference (p<0.05) in licking time between the negative control and treatment groups. There was a significant difference (p<0.05) in licking time between phases I and II for the negative group. However, there was no significant difference (p>0.05) in the treatment group. Further analysis showed a significant difference (p<0.05) among several phases. In particular, there were significant differences between the negative and positive groups (phase I and II). The codeine (5 mg/kg bw) was reported to have a 54.32% analgesic power in phase I and 61.08% in phase II [24]. The data shows that *D. ochreata* leaf extract had both central and peripheral effects, showing an analgesic power comparable to codeine in suppressing phase I and II. Deraniyagala et al. (2014) [25] reported that another species, *Dillenia retusa*, inhibited the early and late phases of the formalin test by working centrally and peripherally but is more effective in the inflammatory phase. Figure 1 shows that the ethanol extract of *D. ochreata* leaf (400 mg/kg bw) has more analgesic activity than diclofenac sodium. This result shows that the ethanol extract of *D. ochreata* leaf can suppress central and peripheral pain, while diclofenac sodium only works peripherally. In addition, the hot plate method is more suitable for evaluating centrally-acting analgesics [2]. A previous study showed that the advantage of formalin as a pain inductor is the ability to differentiate between mechanisms that occur in central and peripheral pain [23].

Mechanism of Analgesic Effect

The analgesic mechanism of ethanol extract of *D. ochreata* leaf was evaluated by comparing the receptor antagonists of the negative and positive control group (Table 3). Statistical analysis showed no significant differences ($p>0.05$) between muscarinic and dopaminergic receptor groups. There was also no significant difference ($p>0.05$) between before and after treatment in the negative and positive groups. The parametric analysis showed significant differences between opioid receptor groups. Similarly, there was a significant difference ($p<0.005$) between the before and after treatment in the negative group. In the positive group, there was no significant difference between the before and after treatment, as shown by $p>0.05$. The evaluation of the analgesic mechanism of the three methods (Table 3) showed that the ethanol extract of *D. ochreata* leaf works through opioid receptors.

Table 3: Evaluation of the mechanism of analgesic effect.

Groups		Latency time (seconds) ± SD	
		Before treatment	After treatment
Muscarinic receptor			
I.	NaCl Fisiologis + extract dose 400 mg/kg bw (negative)	3.35±0.19[a]	3.70±0.13[a]
II.	II. Atropine Sulfate + extract dose 400 mg/kgBW (positive)	3.44±0.10[b]	3.92±0.49[b]
Dopamine receptor			
III.	NaCMC 1% + extract dose 400 mg/kg bw (negative)	3.22±0.31[a]	3.49±0.38[a]
IV.	Metoclopramide HCl + extract dose 400 mg/kg bw (positive)	3.37±0.14[b]	3.80±0.36[b]
Opioid receptor			
V.	NaCl Fisiologis + extract dose 400 mg/kg bw (negative)	3.32±0.11[a]	3.80±0.38[b]
VI.	Nalokson HCl + extract dose 400 mg/kg bw (positive)	3.53±0.10[c]	3.39±0.08[c]

Data are expressed as mean ± SD (n=6); different letters indicated a significant difference ($p<0.05$).

The agonist substances (opioid mimetics) cause the release of endogenous opioids, such as endofins and enkephalins. A previous study showed that opioid peptides mediate analgesia both centrally and peripherally due to the ability to suppress both phases of the formalin test [26]. The endogenous opioids directly inhibit peripheral nociceptive afferent neurons, thereby reducing transmission from the periphery [27]. Administration of an opioid antagonist in the form of Naloxone HCl with with ethanol extract of *D. ochreata* leaf resulted in a decrease in pain reactions when stimulation was administered using a hot plate. Naloxone HCl, acting as an antagonist, blocked the intracellular signalling initiated by the extracted binding to the receptor, thereby eliminating the analgesic effect [28]. These results are consistent with the report of Deraniyagala et al. (2014) [25] that the interaction of naloxone HCl with *D. retusa* fruit extract decreased the pain effects of rats after 2 hours. Triterpenoids suppress visceral pain through the mechanisms of endogenous opioids, nitric oxide, and the opening of K (ATP) channels [29]. Meanwhile, steroids are antinociceptive in the central nervous system [30], producing effects through central receptors or increasing the release of endogenous opioid peptides [31]. The ability of flavonoids to reduce pain stimulation was due to the active participation of μ and δ opioid receptors. Furthermore, flavonoids target δ receptors to reduce pain in rat paw oedema [32, 33].

Standardization of the extract.

Standardization of extract was carried out to determine the quality of the extract through

testing a series of parameters related to quality standard requirements [14]. The result of the standardization ethanol extract of *D. ochreata* leaves can be seen in Table 4.

Table 4: Standardization of the ethanol extract of *D. ochreata* leaves

Parameter	Result	Requirement [14]
Specific parameter		
Organoleptic	Thick, blackish	
	distinctive odour with a slight bitter taste	
Water soluble extractive content (%)	65.33 ± 3.05	> 31
Ethanol soluble extractive content (%)	88.00 ± 2.00	> 70.5
Non spesific parameter		
Water content (%)	6.00 ± 1.00	< 10
Susut Pengeringan (%)	6.38 ± 1.13	< 10
Total ash content (%)	8.18 ± 0.01	< 16.6
Acid insoluble ash content (%)	0.16 ± 0.01	< 0.9
Metal contamination (mg/Kg)		
- Pb content	0.0628 mg/kg	< 10 mg/kg
- Cd content	0.0077 mg/kg	< 0.3 mg/kg
Microbial contamination (cfu/mL)		
- Plate content	0	$< 10^4$
- Yeast content	0	$< 10^3$

The specific parameter's value showed that the extract was thick, blackish, with a distinctive odour and a slightly bitter taste; the water-soluble extractive content was 65.33% ± 3.05, while the ethanol-soluble extractive content was 88.00% ± 2.00. The results of spesific parameter meet the standards parameter was requirement [14]. The non-specific parameters showed the water content value was 6.00% ± 1.00. Its set as viscous extract [34], the drying shrinkage value was 6.38% ± 1.13, the total ash content was 8.18% ± 0.01, and acid insoluble ash content was 0.16% ± 0.01, Pb contamination content was 0.0628 mg/kg, and Cd content was 0.0077 mg/kg. The total plate number and yeast content from the ethanolic extract of *D. ochreata* leaves were not found, indicating secondary metabolites contained in *D. ochreta* leaves extract inhibiting the growth of microorganisms in the extract. The value of non-specific parameters also meets the standard parameter requirement.

Conclusion

The ethanol extract of *Dillenia ochreata* leaf showed higher analgesic activity (24.85%) than the positive control (18.32%) and statistical analysis showed a significant difference ($p<0.05$). This extract exerted pressure on the central (46.92%) and peripheral (61.19%). In addition, the analgesic effect of ethanol extract from *D. chreata* leaf worked through the opioid receptors with a decrease in pain reactions from 3.53±0.10 to 3.39±0.08 seconds. The extract of *D. ochreata* leaves fulfils the standardization parameters of the extract.

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Author Contributions

Conceptualization, M.M. and F.F.; Methodology, F.G. H.Y; Software, F. G. H. Y.; Validation, M.M., F.F. and F.G.; Formal Analysis, M.M., F.G.; Investigation, F.G.; Resources, M.M.; H. Y.; Data Curation, M.M., F.G., F.F. H.Y; Writing – Original Draft Preparation, M.M., F.G.; Writing – Review & Editing, H. Y., F.F.; Visualization, M.M., F.G.;

Supervision, M.M.; Project Administration, F.F. H. Y.; Funding Acquisition, M.M.

Conflict of Interest

The authors declare no conflict of interest

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