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Analgesic activity test of Inggu leaf (*Ruta angustifolia* [L.] Pers) with Tail Flick and Writhing Test method

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ABSTRACT

Pain is a sensory and emotional experience related to tissue damage. This study aims to determine the analgesic effect of ethanol extract from Inggu leaves and the effective dose of ethanol extract of Inggu leaves by tail flick and writhing test methods. The tail flick and writhing test methods have been widely used to develop non-steroidal anti-inflammatory drugs. Steroid and flavonoid compounds contained in Inggu leaves are thought to have an effect as analgesics. The powder of Inggu leaves was extracted using the maceration method with 96% ethanol solvent. A total of 25 Wistar male white rats were divided into 5 groups, namely positive control mefenamic acid 9 mg/200 g BB, negative control CMC Na 1%, ethanol extract of Inggu leaves doses of 5 mg/200 g BB, 10 mg/200 g BB and 20 mg/200 g BB. The data obtained were analyzed using the ANOVA test, and then the LSD test was used to determine the differences between groups. The results showed that the 5 mg/200 g BW extract doses, 10 mg/200 g BW, 20 mg/200 g BW, and positive control significantly differed from the negative control group. The extract dose of 20 mg/200 g BB is comparable to the positive control, indicating that the extract dose of 20 mg/200 g BB has the most effective analgesic activity.

Keywords:
Analgesic; Inggu leaves; Tail Flick; Writhing Test.

Introduction

Pain is the most complex and complex human experience, influenced by interactions between emotions, behavior, and cognitive and physiological sensory factors. Pain is a subjective sensory and emotional experience, an unpleasant one related to actual tissue damage or potential or perceived in the events described by the term damage (Kemenkes, 2021). The cause of pain is mechanical or chemical, heat or electrical stimuli, which can cause tissue damage and release substances called pain mediators. The most important pain mediators are histamine, serotonin (5-HT), prostaglandins, bradykinin, and potassium ions. These substances then stimulate pain receptors located in free nerve endings in the skin, mucous membranes, and other tissues. From the free nerve endings of the skin and tissue, stimulation is transmitted via sensory nerves to the central nervous system (CNS), through the spinal cord to the thalamus (opticus) then to the pain center in the cerebrum, where the stimulation is felt as pain (Muhsinah, 2020).

An analgesic is a substance that can relieve or suppress pain in therapeutic doses. Based on potential mechanisms of action and effects, side drugs that have analgesic effects are divided into 2 groups, namely pain relievers such as opioids with intense action and central drugs, also called narcotic analgesics or hypnoanalgesics. Pain relievers such as non-opioid which has weak to moderate effects that work peripherally without antipyretic and antiphlogistic effect. Therapy using chemicals is still not satisfactory, which can cause side effects such as nausea, vomiting, stomach ache, constipation, and poisoning (Agus, 2023). Traditional medicine is not much different in treating various diseases; even though much science has advanced or developed, especially in the health sector, the lack of knowledge and information regarding the types of

plants that can be used as traditional medicine and how to make them is a problem and difficulty for the community, enthusiasts of traditional medicine (Tone *et al*., 2013).

One of the plants Indonesian people use as a medicinal ingredient with analgesic properties Inggu leaves (*Ruta angustifolia* [L.] Pers). Inggu is a type of plant from the *Rutaceae* family. Inggu leaves can treat hypertension, topical treatment for earaches and headaches, and external treatment in skin antiseptic and mosquito repellent (Permatasari, 2013). According to Noer & Pratiwi (2016), from the results of qualitative phytochemical tests (phytochemical screening) of Inggu leaves (Ruta angustifolia [L.] Pers), there are chemical compounds containing flavonoids, steroids, alkaloids, saponins, tannins, quinones, and triterpenoid. Based on previous research by Luhurningtyas *et al*. (2013), flavonoids act as analgesics with a mechanism of action that inhibits the action of the cyclooxygenase enzyme, which will reduce the production of arachidonic acid so that it can reduce the pain experienced (Robinson, 1995). It will reduce the production of prostaglandins by arachidonic acid so that it can reduce pain (Gunawan *et al*., 2008). Steroids are efficacious as analgesics and can inhibit the phospholipase enzyme, causing pain, while saponins in analgesics inhibit prostaglandins, which act as causes of inflammation, and saponins can dissolve in water but not in ether (Robinson, 1995).

Research on the analgesic effect of ethanol extract from Inggu leaves has not been carried out much, seen from the pain population in Indonesia, which is increasing, especially in osteoarthritis disease by consuming analgesic drugs in the long term, so in this research, a study will be carried out on ethanol extract of Inggu leaves regarding analgesic activity which will be tested on male white Wistar rats using two methods, namely the tail flick method and the writhing test (chemical stimulation). The principle of the tail flick method is a movement response by flicking the tail (Voigt, 1995), while the writhing test method is a writhing response with glacial acetic acid as a pain inducer. This method provides a rapid evaluation of types of peripheral analgesics (Gupta *et al*., 2013). The extraction method used is maceration because maceration is a simple way of extracting by immersing the simplistic powder in the liquid and is suitable for initial extraction (Depkes, 2000). The distiller used in this extraction process is 96% ethanol because 96% ethanol is stable, does not affect efficacious substances, is not volatile, and the heat required for concentration is less. It can be mixed with water in any ratio.

Methods

Materials

The tools used in the study were a blender or sample grinder, sieve number 40, oven, *beaker glass,* measuring cup, separatory funnel, Erlenmeyer, stirring rod, dropper pipette, measuring pipette, maceration bottle, rotary evaporator, filter paper, moisture balance, flannel cloth, analytical balance, injection syringe, sonde needle, gloves, stopwatch, a set of tail flick analgesic-meter tools. The test animals used in this research were male white Wistar rats, 2-3 months old, weighing 150-200 grams. The material used in this research was fresh green Inggu leaves, which did not change color, obtained from the Tawangmangu area, Central Java. Mefenamic acid as a positive control was obtained from PT Dexamedica Palembang, CMC Na, distilled water or distilled water, 96% ethanol, and 0.5% glacial acetic acid.

Plant determination

Plant determination was carried out at the MIPA Biology Laboratory, Sebelas Maret University, Surakarta. This determination aims to identify Inggu plants by determining the correctness of Inggu leaf samples based on the plant's morphological characteristics.

Determination of drying loss of Inggu leaf powder and extract

Determination of the drying loss of powder and Inggu leaf extract was carried out at the Pharmaceutical Technology Laboratory of Setia Budi University using a moisture balance tool. Inggu leaf powder and extract were weighed at 2 grams each, put into a moisture balance apparatus at a temperature of 105°C and waited until the number (%) appeared, then weighed, replicated three times. The numbers listed on the moisture balance tool are the results (%) of

drying loss produced by powder or extract from Inggu leaves; the moisture content in simplicial powder is not more than 10%.

Preparation of ethanol extract of Inggu leaf powder

Inggu leaf ethanol extract was made using the maceration method. Inggu leaf powder weighed as much as 150 grams, then put into a maceration bottle, and 1125 ml of 96% ethanol was added. The maceration bottle is stored at room temperature, avoided from direct sunlight, and shaken constantly 3 times a day. After 5 days, the yield was filtered using flannel cloth and filter paper, then the dregs were added with 375 ml of 96% ethanol and then filtered using flannel cloth and filter paper. The liquid extract was concentrated using a flat evaporator at 40°C until a thick extract was obtained, then the yield (%) was calculated (Depkes 2000).

Test the analgesic effect of the tail flick method

Mice acclimatized for ± 18 hours were grouped into five groups of 5 mice. The test groups are as follows:

Group 1 Negative control was given orally 1% CMC-Na solution.
Group 2 Positive control was given orally a solution of 9 mg mefenamic acid/200 g BW.
Group 3 Giving a dose of 5 mg/200 g BW of Inggu leaf extract given orally to mice.
Group 4 Give mice a dose of 10 mg/200 g BW of Inggu leaf extract orally.
Group 5 Give mice a dose of 20 mg/200 g BW of Inggu leaf extract orally.

The test animals were given a test solution; their t_0 was calculated first, then the test animals were given the test solution according to their group, 30 minutes later they were given thermal in the form of heat at a temperature of 70°C which was obtained from an electric current in the animal, then the animal was tested using a tail. flick analgesy-meter, then record the time the test animal pulls or flicks its tail. Tests were carried out at 30, 60, 120, 180 and 240 minutes.

The procedure for testing the analgesic effect is the writhing test method

Twenty-five mice were randomly divided into 5 groups according to each method and fasted for 10 hours while still being given water.

Group I CMC Na (negative control)
Group II mefenamic acid (positive control) dose 9 mg/200 g BW
Group III ethanol extract of Inggu leaves at a dose of 5 mg/ 200 g BW
Group IV, ethanol extract of Inggu leaves, dose of 10 mg/ 200 g BW
Group V ethanol extract of Inggu leaves dose 20 mg/ 200 g BW

After being treated with a single oral dose, 30 minutes later the mice were given a pain stimulant in the form of 0.5% glacial acetic acid in the amount of 0.6 ml by intraperitoneal injection (i.p.) and placed in an observation area. The number of writhing was counted in each treatment group. One writhing is characterized by the rat's legs and arms being pulled forward and backward and the abdomen touching the floor. Then, observe and record the number of wriggles shown by the test animal every 10 minutes for 90 minutes.

Calculation of percent analgesic power

Tail flick method. According to Budiati *et al*. (2010), Calculation of the percent analgesic power of the tail flick method is expressed by percent pain resistance (PHN), which is calculated using the formula:

$$\text{PHN} = \frac{(\text{T1} - \text{T2})}{\text{T1}} \times 100\% \qquad (1)$$

where T1 is average response time (seconds) in the control group without drug, and T2 is average response time (seconds) when administering the test substance.

Writing test method. The effect of administering the extract on the analgesic effect was carried out by calculating the average number of writhing responses. After obtaining the average number of writhing responses, a curve was created comparing the average writhing response

versus test time. Then, the AUC (Area under the curve) is calculated, namely the average area under the curve, which is the relationship between the average stretching response per unit of time. With Formula:

$$AUC^{n}_{n-1} = \frac{Wt_{n-1} + Wt_{n}}{2}\left(t_{n} - t_{n-1}\right) \quad (2)$$

where Wt_{n-1} is the average response to stretching on, and Wt_{n} : the average response to stretching on.

The amount of inhibition of the amount of stretching is calculated using the Handerson and Forsaith equation, namely:

$$\%\text{analgesic power} = \left(100 - \left[\frac{P}{K} \times 100\right]\right)\% \quad (3)$$

where P is the cumulative average number of writhing test animals in the treatment group, and K is the cumulative average number of wriggles of negative control test animals

Data analysis

Analyzed using the Kolmogorov-Smirnov test to determine the normal distribution of the data, and the Lavene test to determine the homogeneity of the data. If the data is normally distributed and homogeneous, then it can be continued with statistical tests using One Way ANOVA and Post Hoc tests. If the data is not homogeneous, then proceed with the Kruskal-Wallis test. If there is a difference, proceed with the Man-Whitney test to identify the differences between the groups.

Results and Discussions

Table 1. Average time (seconds) of pain inhibitory response

Group	Mean± SD (seconds) pain inhibitory response				
	Minutes to-30	**Minutes to-60**	**Minutes to-120**	**Minutes to-180**	**Minutes to-240**
CMC- Na	0,94±0,67	1,92±0,73	2,68±0,98	3,30±1,66	1,32±1,28
Mefenamic acid	1,98±1,67	3,50±1,17	5,10±1,60	5,22±2,19	1,82±1,26
Extract dose 5 mg/ 200 g BW	1,06±0,72	2,16±0,93	4,10±1,45	5,26±1,62	1,86±1,21
Extract dose 10 mg/ 200 g BW	1,40±0,76	3,13±1,54	5,05±2,72	4,15±1,32	1,94±1,27
Extract dose 20 mg/ 200 g BW	1,73±1,10	3,29±2,23	4,27±1,73	4,71±1,93	2,97±1,83

This test was carried out to determine the analgesic activity of Inggu leaf ethanol extract by measuring the ability of the test compound to overcome pain sensations. The tail flick method is a method that uses an analgesic meter with the parameter used is the reaction time, which causes a pain response in the tail of the test animal after being placed in a beam with a hot infrared lamp so that the tail gets maximum heat (Canon 2007). The analgesic activity of Inggu leaf extract was tested on male white mice aged 2-3 months with a body weight of 150-200 grams. The test materials used were CMC Na solution, mefenamic acid suspension solution, and Inggu leaf ethanol extract solution. The solution makes a suspension because it cannot dissolve completely in water, so CMC Na is added as an emulsifier. Mefenamic acid is a positive control because it can be used as an analgesic; besides that, it also has mild side effects compared to other NSAID drugs (Tjay & Rahadja 2002). The test animal used was a male white rat. The choice of male gender is due to more stable biological conditions and less prone to stress and hormonal influences (Harmita 2005). Test animals were grouped into 5 groups, each consisting of 5 mice. Groups I to group 5 were given oral treatment in succession, then the analgesic effect was tested using a tail flick analgesic meter until the tail flick was treated. Testing analgesic activity obtained quantitative

data on the average time (seconds) the test animals could withstand pain and SD stimuli; the results can be seen in Table 1 and Figure 1.

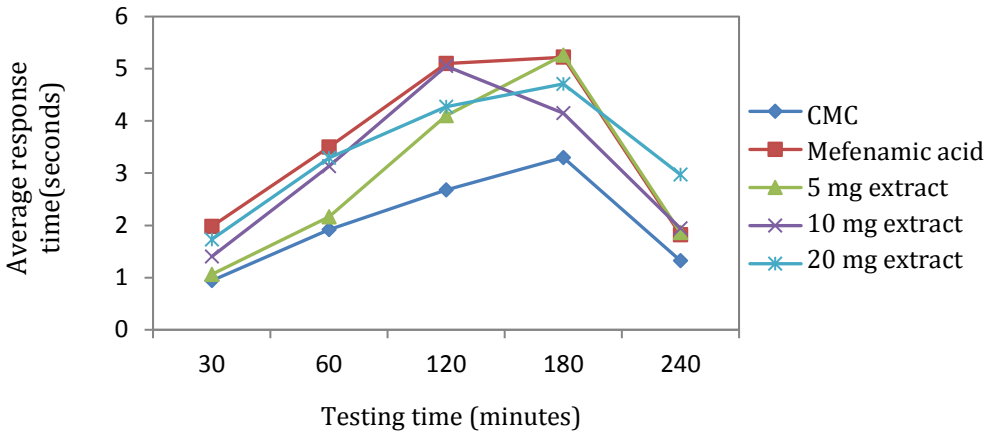

Figure 1. Average time (seconds) of pain inhibitory response

The overall results in the treatment group were an increase in pain barriers. The negative control group produced the lowest average pain inhibition response value compared to the other control groups. It is aligns with research by Wagh et al. (2006), which showed that CMC Na as a control group could not increase pain inhibition compared to other control groups. Because CMC Na does not contain active substances inhibiting pain, it cannot withstand it longer. The treatment control group that was given the ethanol extract test preparation of Inggu leaves showed an average increase in pain inhibition starting to appear at the 30th minute. However, when compared with the negative control, according to the results of the statistical test, LSD extract at a dose of 5 mg/ 200 g BW was not able to inhibit pain in the first minute. 30th, but the extract dose of 10 mg/ 200 g BW and 20 mg/ 200 g BW had an analgesic effect at the 30th minute, which means both doses were able to inhibit pain at that minute. The extract dose of 5 mg/ 200 g BB has an analgesic effect at the 120th minute because it increases the pain threshold to withstand greater pain. It is due to the inhibition of pain stimulation at the 120th minute, and then a decrease in the pain threshold occurs at the 120th minute, 180th to the final 240th minute. In contrast to the extract dose of 10 mg/200 g which has a fast analgesic onset time and visible analgesic effect in the 30th minute, but has a short duration as seen from the decrease in pain threshold which occurs from the 60th minute to the 180th minute with the result The average pain barrier produced was smaller than the average pain barrier produced at extract doses of 5 mg/ 200 g BW and 20 mg/ 200 g BW at the same minute. The increase in pain resistance that occurs and the analgesic effect that appears is different for each dose of extract, this indicates that there is also a different analgesic effect. Analgesic activity is expressed in percent pain resistance based on the formula, according to Budiati *et al*. (2010), to obtain data on pain inhibition reaction time with results in the treatment group. The results can be seen in Table 8, and the percentage of pain resistance is calculated

Table 2. Percentage of Pain Barriers (PHN)

Test group	**Pain barrier percentage % (mean± SD)**
Negative control (CMC Na 1%)	-
Positive control (Mefenamic acid)	74,910±12,87
Extract dose 5 mg/ 200 g BW	42,458±15,56*
Extract dose 10 mg/ 200 g BW	52,774±9,31*
Extract dose 20 mg/ 200 g BW	68,912±18,54

Information : * = Significantly different from the positive control using the LSD test

According to Sirait *et al*. (1993), the analgesic activity of the test preparation is indicated by the percentage of pain inhibition provided that is greater than or equal to 50% of the negative control group, so it is considered effective as an analgesic. The statistical test results showed that the percentage of pain barriers was normally distributed with a significant value of 0.651 (> 0.05) and homogeneous with a significant value of 0.152 (> 0.05). The test results from One Way ANOVA show that there are significant differences between treatment groups with a significant value of 0.000 (<0.05) followed by the LSD test results show that there are significant differences between treatment groups. The statistical test results showed that the ethanol extract of Inggu leaves at doses of 10 mg and 20 mg / 200 g BW significantly differed from the negative control CMC Na, thus proving that Inggu leaf extract has an analgesic effect. The ethanol extract group of Inggu leaves at a dose of 20 mg/ 200 g BW was comparable to the positive control group of mefenamic acid. It can be assumed that at a dose of 20 mg/ 200 g BB contains more active compounds.

The writing test method is used to test non-narcotic analgesics. The principle of this method is to observe the decrease in the number of writhing that occurs as a result of administering the test substance to mice given a 0.5% v/v acetic acid solution. Acetic acid was chosen because it can provide a fairly good pain stimulus to test animals by triggering a local inflammatory response resulting from the release of free arachidonic acid from phospholipid tissue via cyclooxygenase (COX), and prostaglandin biosynthesis, an increase in prostaglandin levels from acetic acid induction increases inflammatory pain by increasing capillary permeability in the peritoneal cavity. The pain response given is characterized by writhing in the form of pulling the test animal's hands and feet forward and backward and the abdomen touching the floor; In this test, a test preparation of ethanol extract of Inggu leaves was given with three dose variations as carried out in the tail flick method, namely with an extract dose of 5 mg, 10 mg and 20 mg/ 200 g BW. The positive control used in this test was mefenamic acid at a dose of 9 mg/ 200 g BW, while the negative control used was CMC Na 1%. Observations were carried out for 90 minutes at intervals of every 10 minutes. Then note the number of squirms caused. One writhing is characterized by the test animal pulling both arms and legs forward and backward, and the abdomen touching the floor. The results of the observations provide data in the form of the number of writhes which are then processed to determine the pain threshold percentage value. The results of the average number of writhes in the treatment group at each time can be seen in Table 2 and Figure 2.

Table 2. Data on the average number of stretches

Group	Average number of movements per minute (X±SD)								
	10’	20’	30’	40’	50’	60’	70’	80’	90’
CMC Na 1%	15±5,10	15,6±3,05	14,4±4,10	13±4,53	12,6±5,59	10,4±5,32	7 2±2,59	6,6±6,31	4,6±3,91
Mefenamic acid 9 mg/ 200 g BW	4,8±1,30	4,4±2,30	4,2±0,84	2,8±1,92	2,0±2,00	2,0±1,58	0,4±0,89	0,6±1,34	0,2±0,45
Extract dosage 5 mg/ 200 g BW	9,6±3,13	6,8±3,42	6,4±1,82	6,0±2,00	5,4±2,19	5,4±3,51	4,2±2,28	3,0±1,87	2,0±1,92
Extract dosage 10 mg/ 200 g BW	7,2±2,39	7,0±2,00	6,4±2,70	3,0±2,45	3,2±2,17	5,0±0,71	2,2±1,79	2,2±1,64	2,0±1,58
Extract dosage 20 mg/ 200 g BW	6,8±3,27	4,6±0,55	3,4±2,61	3,4±1,14	2,4±1,34	2,8±0,84	2,6±2,07	1,6±1,14	0,4±0,55

The results showed decreased writhing in all treatment groups, and measurements started at 10 minutes. This is because acetic acid has a swift onset, around 5 minutes (Sujono *et al.* 2007). The negative control group, which was only given 1% CMC Na, could not treat pain because it did not contain active substances, as evidenced by the highest average number of writhes produced compared to the positive control group and the treatment control group. According to Syamsul *et al*. (2016), testing using a negative control group aimed to compare the presence or absence of analgesic activity in the positive control group and the treatment control group and ensure that the decrease in the number of writhing was only caused by the administration of the test preparation. The treatment group was given ethanol extract of Inggu

leaves with three different dosage variations, a writhing response was elicited in the 10th minute. The three different dose variations experienced a decrease in the average writhing response. Inggu leaf ethanol extract at a dose of 5 mg/200 g BW experienced a decrease in the average writhing response until the 50th minute. It increased at the 60th minute, and after that, the analgesic effect was visible again until the final minute. In contrast to the extract dose of 10 mg/ 200 g BW, the analgesic effect was visible when the 50th minute until there was an increase in the writhing response at the 60th minute. In the control group treated with ethanol extract of Inggu leaves with a dose of 20 mg/ 200 g BW, there was a decrease in the average writhing response until the 50th minute, then an increase in the average writhing response occurred at the 60th minute and the analgesic effect was visible again from the 50th minute. 80 to 90 minutes because there was a decrease in the average writhing response. A decrease in the writhing response indicates an inhibition of pain stimuli.

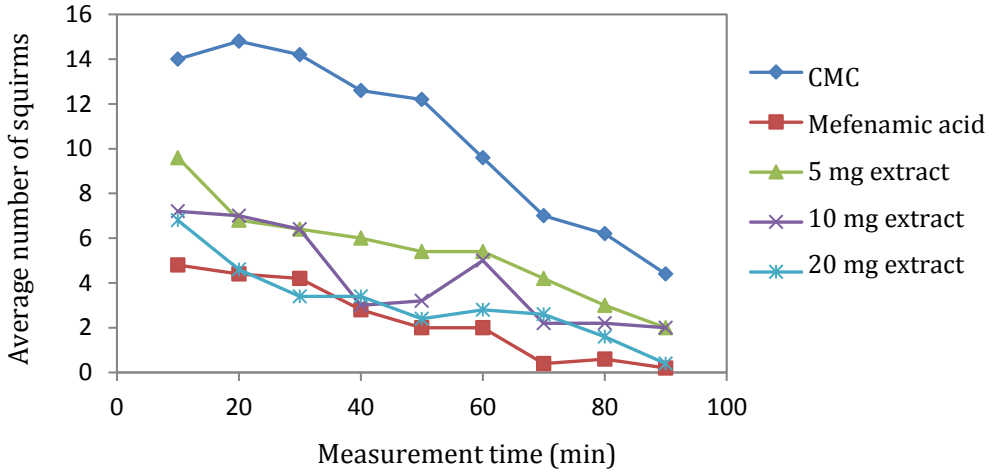

Figure 2. Data graph of the average number of stretches

The observations using the writhing test method showed that all doses of the extract produced an average writhing response comparable to the positive control. The average writhing response power is used to calculate the AUC and the percentage of writhing inhibition as analgesic power, which can be seen in Table 3.

Table 3. Percentage of analgesic power

Test group	Average total AUC	Percentage % analgesic power (mean± SD)
CMC Na 1%	896,00	-
Mefenamic acid 9 mg/200g BW	189,00	77,81±5,36[a]
Extract dose 5 mg/ 200 g BW	427,00	48,40±8,32[ab]
Extract dose 10 mg/ 200 g BW	336,00	58,92±8,63[ab]
Extract dose 20 mg/ 200 g BW	245,00	70,34±6,02[a]

Information :
a = Significantly different from the negative control using the LSD test
b = Significantly different from the positive control using the LSD test

Table 3 shows the total AUC of each treatment and the drug levels in the blood administered after treatment. The processes of absorption, distribution, metabolism, and excretion influence AUC. The absorption process is related to the solubility of a drug. The distribution process is related to the amount of drug given so it will affect the amount of drug carried by the bloodstream throughout the body. Metabolic processes are related to the intensity of action of drugs that bind to plasma proteins. The excretion process is related to the amount of a drug excreted through urine. The higher the dose of Inggu leaf ethanol extract, the percentage of analgesic power decreases. The percentage of analgesic power is the coactivity of increasing the pain threshold with the ability of the test compound to inhibit pain induced by acetic acid, so

that large doses produce a small writhing response caused by the test animal. Based on the compound identification test for the tail-flick and writhing test methods, positive results were obtained that Inggu leaves contain steroid, flavonoid, and tannin compounds; this is in accordance with research by Noer & Pratiwi (2016). According to research by Shuib et al. (2015), the flavonoids contained in Inggu leaves also have an important anti-inflammatory role and most likely have an analgesic effect. Among these compounds, namely steroids, flavonoids, and tannins, have various effects: antitumor, antioxidant, analgesic, anti-inflammatory, antiviral, antibacterial, antifungal, and anti-diarrheal (Syukri, 2008; Soeksmanto, 2006; Hosseinzadeh, 2002). Steroids work by inhibiting phospholipase and preventing the release of arachidonic acid as well as blocking the cyclooxygenase and lipooxygenase pathways so that the formation of prostaglandins and leukotrienes is inhibited (Katzung, 2002; Tjay and Rahardja, 2007). The results of plant extracts using the writhing test method show that the reduction in pain with a writhing response may occur due to the analgesic properties of the extract through inhibition of prostaglandin synthesis (Ferdous *et al*., 2008). According to Deb *et al*. (2010) in their research stated that flavonoids and tannins work by inhibiting the synthesis of prostaglandins.

Conclusion

Based on the research conducted, it can be concluded that First, the ethanol extract of Inggu leaves at a dose of 5 mg/ 200 g BW, 10 mg/ 200 g BW and 20 mg/ 200 g BW has analgesic activity using the tail flick and writhing test methods. Second, the extract dose of 20 mg/ 200 g BW has optimal analgesic activity and is equivalent to the positive control.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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