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Analgesic Effect of Ethanol Extract Mangrove (*Avicennia alba)* and Soursop (*Annona muricata)* Leaves in Mice

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Abstract
Analgesic drugs are drugs that can reduce pain due to tissue damage. Efforts are made to relieve pain by using non-steroidal anti-inflammatory drugs (NSAIDs). Long-term use of NSAIDs has side effects such as digestive tract disorders, kidney and liver damage, and skin allergies, so alternative herbal medicines are needed that have lower side effects, one of which is using mangrove Secondary metabolite compounds such as flavonoids and tannins from ethanol extract leaves of mangrove (*Avicennia alba)* and soursop *(Annona muricata)* function as analgesics that inhibit prostaglandin biosynthesis. This study uses the writhing test method to determine the analgesic activity of ethanol extract from mangrove (Avicennia alba) and soursop (Annona muricata) leaves in male mice. This research was experimental using a *posttest-only control research design* using 25 mice with 5 treatment groups, namely the negative control group Na-CMC 0.5%, the positive control group mefenamic acid, the *Avicennia alba* leaf extract group 250 mg/kgBW, the *Annona muricata* leaf extract group 250 mg/kgBW and a combination of mangrove (*Avicennia alba)* leaves and soursop *(Annona muricata)* leaves extract 1:1, all treatments were induced by 1% acetic acid intraperitoneally, the mice's writhing was observed every 5 minutes for 30 minutes and analyzed using ANOVA and LSD tests. The results showed that the combination of ethanol extracts *from* mangrove (*Avicennia alba)* leaves and soursop *(Annona muricata)* leaves provided the same analgesic effect as the positive control (mefenamic acid).

Keywords: Analgesic, *Avicennia alba*, *Annona muricata.*

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INTRODUCTION

The definition of pain is a sensory and emotional experience resulting from actual potential damage or describing the condition of tissue damage (Kurniawan, 2018). One method of treating pain involves NSAIDs (*Non-Steroidal Anti-Inflammatory Drugs).* NSAIDs are drugs that have antipyretic, analgesic, and anti-inflammatory properties. In Indonesia, as many as 52.1 % of patients received NSAID drug therapy, including mefenamic acid (fenamates group), which is often used as an analgesic and anti-inflammatory (Kemenkes RI, 2018). Mefenamic acid is an example of a non-narcotic analgesic and belongs to the NSAID class of drugs. Mefenamic acid is used for mild to moderate pain, including headaches, toothache, postoperative and *postpartum pain,* dysmenorrhea, musculoskeletal and joint disorders such as osteoarthritis and rheumatoid arthritis, and menorrhagia (Tjay *et al.*, 2007).

Side effects from long-term use of NSAIDs occur in the gastrointestinal, hematological, and renal areas, so their use must be limited (Singh *et al.,* 2008). Regarding the side effects of using NSAIDs, efforts are needed to minimize them by developing alternative analgesics that have fewer side effects. Alternative herbal medicine has been known since ancient times in Indonesia, and many medicinal plants have been reported to have therapeutic effects on diseases (Auliyah *et al.,* 2019).

One plant that has potential as an analgesic is the mangrove plant. The mangrove plant that is often used is the leaves. According to research by Guntara *et al.* (2019), mangrove leaves are rich in steroid compounds, saponins, flavonoids, and tannins. Flavonoids and saponins have analgesic and anti-inflammatory abilities because these two compounds inhibit enzymes that induce inflammation,

especially the arachidonic acid metabolism pathway and the prostaglandin synthesis pathway. In research using the acetic acid induction method, it was found that the methanolic extract of *Avicennia alba leaves* at a dose of 500 mg/kgBW significantly (p=0.01<0.05) could inhibit peripheral and central pain in the soles of the hind feet of albino mice using the *hot plate* test method and the *writhing test* method (Rahman *et al.*, 2011). The stretching method is a chemical method used to induce pain from the periphery by injecting irritants such as phenylquinone and acetic acid into test animals (Gawade, 2012). The writhing test was chosen because it is the most widely used test to measure the response to analgesic activity in peripheral nerves using chemical stimulation in the form of an intraperitoneal injection of acetic acid. Acetic acid is used because it can cause temporary damage to surrounding tissue so that the stimulus will be carried to the brain and interpreted as peripheral pain by type C nerve fibers (Guyton and Hall, 2011). Meanwhile, in research by Purmata *et al. (2020),* ethanol extracts *of* mangrove (*Avicennia alba)* leaves at doses of 250 mg/kgBW and 500 mg/kgBW had an analgesic effect on male mice induced by 0.7% acetic acid pain.

Soursop leaves contain steroid/terpenoid compounds, flavonoids, coumarins, alkaloids, and tannins (Puspitasari *et al*., 2016). Flavonoid and alkaloid compounds are responsible for providing analgesic effects by inhibiting the cyclooxygenase and lipoxygenase enzymes in prostaglandin biosynthesis in the arachidonic acid metabolism pathway, thereby inhibiting pain mediators (Wemay, 2013). Based on research by Dju *et al.*, 2021, a single dose of 250 mg/kgBW of soursop leaf ethanol extract (Annona muricata L.) had analgesic activity in male white rats induced by acetic acid with a writhing protection percentage of 48.16% and writhing effectiveness of 75.71%. Meanwhile, research by Kuswinarti *et al*. (2018) using the *hot plate method* showed that 400mg/kgBW at the 4th-hour induction provided the highest analgesic effect.

Therefore, researchers use the writhing test method to investigate the single and combined analgesic effect of ethanol extract from mangrove and soursop leaves as an analgesic.

MATERIALS AND METHODS

Tools used in the research include sondes, 1 mL syringes, dropper pipettes, glass beakers, stirring rods, water baths, filters, analytical scales, stopwatches, ovens, porcelain crucibles, mortars and pestles, buncher funnels*, rotary evaporator,* measuring cup, *water bath, and* scissors.

The materials used in the research were mangrove leaves (*Avicennia alba*), soursop leaves (*Annona muricata),* test animals in the form of 25 male mice, 1% acetic acid, 70% ethanol, mefenamic acid in Sodium carboxymethyl cellulose (Na – CMC) 0 .5%, 0.5% Na–CMC solution.

This research used a laboratory experimental design using male mice (*Mus musculus)* as test animals. Male mice are generally used as test animals because male mice have more stable hormonal conditions than female mice (Indarwati *et al.,* 2015). Twenty-five mice were 3-4 months old with a body weight of ±20-30 grams, then divided into 5 treatment groups. Before being given treatment, the mice were adapted to the environment for ± 2 weeks. The treatment group was divided into 5 groups, namely the negative control was given Na-CMC 0.5% the positive control was given mefenamic acid at a dose of 1.3 mg/20gBW, determination of the dose of mangrove leaf extract refers to the research of Rahman *et al.,* (2011) namely a dose of 250 mg/kgBW has analgesic activity in male white mice induced by 0.7% acetic acid. While the dose of soursop leaf extract refers to the research of Dju *et al*. (2021), a dose of 250 mg/kgBW has analgesic activity in male white mice induced by acetic acid. Furthermore, the dose is based on comparing mangrove leaf extract and soursop leaf with a comparison group of 1:1 (250 mg/kgBW: 250 mg/kgBW). After 30 minutes, the test substance was injected intraperitoneally (IP) into the 1% acetic acid solution as much as 0.5 ml/20gBW. Induction of 1% acetic acid intraperitoneally, namely the animal's back is held so that the abdominal skin becomes tense. At the time of injection, the position of the mouse's head is lower than its abdomen. The injection method is by tilting the needle at the edge of the abdomen from the midline to avoid the bladder and not too high so as not to hit the liver (Sinata and Lutfi, 2020). Acetic acid is used because it can damage tissue locally, causing pain in the abdominal cavity during intraperitoneal administration (Stanos, 2020).

The determination of mangrove leaves (*Avicennia alba*) and soursop leaves (*Annona muricata*) was carried out at the Botany Laboratory, Biology Department, Faculty of Mathematics and Natural Sciences, University of Lampung, to prove the correctness of the mangrove leaf and soursop leaf materials. The results of plant determination showed that the test results used were mangrove (*Avicennia alba*) and soursop (*Annona muricata*).

Mangrove leaf and soursop leaf extracts were each made at a dose of 7.5 mg/30gBW for mice. The method for making mangrove leaf and soursop leaf extract was to weigh 150 g of each of the

powders. The mangrove leaf and soursop leaf powder samples were macerated using 70% ethanol solvent for 3 days with daily stirring and then filtered. The residue was macerated again with 500 ml of 70% ethanol with stirring every day for 2 days, then the filtrate was filtered and concentrated using *a rotary evaporator* at a temperature of 50 ^{0}C until a thick extract was obtained. For testing, the thick extract was suspended in 0.5% CMC-Na solution.

Na-CMC 0.5% is made by adding 0.5 grams of Na-CMC and then dissolving it in 50 ml of hot distilled water while stirring until everything is dissolved and a thick mass is formed. The solution was then poured into a 100 ml measuring flask, and distilled water was added to a volume of 100 ml to obtain 0.5% Na-CMC. A bottle is used to hold the solution.

The mefenamic acid suspension was made from mefenamic powder, and Na-CMC was added while stirring until a volume of 100 ml was reached and a suspension solution was formed. The dose of mefenamic acid is determined based on the human dose conversion factor. The usual dose of mefenamic acid is 500 mg once. The conversion dose for humans with a weight of 70 kg to mice is 0.0026= 1.3 mg/kgBW.

An acetic acid 1% pain-induced solution was prepared by diluting 1 ml glacial acetic acid in 100 ml distilled water in a volumetric flask. The volume of acetic acid to be given is 0.5ml/20gBW to mice and injected peritoneally into experimental animals according to the mice's body weight.

Mice were adapted to the laboratory environment for approximately ± 2 weeks to get used to living in the environment. Next, the mice were fasted for ± 18-24 hours but were still given water. Each mouse was weighed using a scale, and the results were recorded. Then the mice were grouped into 5 according to the division of each group.

The mice (Mus musculus) in this study were made to experience pain in a pathological condition by using acetic acid as a stimulant for the production of pain. The acetic acid used is a sterile solution of 1% acetic acid, which functions as a stimulant to form prostaglandins and causes pain. This research used the writhing test/stretching method in analgesic testing. This method uses a type of pain inducer in the form of 1% acetic acid at a dose of 0.5 ml/20gBW intraperitoneally, which causes a pain response in the form of writhing. Next, the number of mice stretching was observed every 5 minutes for 1 hour for each treatment. The appearance of writhing characterizes mice; both legs are pulled back and stretched, and the stomach touches the bottom of the floor (Syamsul *et al.*, 2016). The average of writhes produced by each group was calculated and compared between the treatment and control groups.

The percentage of writhing protection and analgesic effectiveness for each dose group was calculated for mice using the formula (Winarti and Wantiyah, 2011):

$$\%protection = 100 - (\frac{The\ average\ amount\ of\ stretching\ of\ the\ test\ material}{The\ average\ number\ of\ negative\ control\ wriggles} x\ 100\%)$$

The percentage of analgesic effectiveness is calculated using the following formula (Winarti and Wantiyah, 2011):

$$\%\ Effectiveness = \frac{\%protection\ of\ the\ test\ material}{\%negative\ control\ protection} x\ 100\%$$

Observation data is presented in tables and graphs, then analyzed statistically using one-way ANOVA to analyze several samples with the same or different data numbers in each sample group. If there are significant differences between the 5 treatment groups, the *Least Significant Difference* (LSD) test is continued to determine the significance of the differences between each treatment group.

RESULTS

Based on observations of the number of writhes produced by each group every 5 minutes for 60 minutes, it was found that on average, the highest number of writhes of mice occurred in the negative control, namely the group that was given acetic acid induction without being given any medication within 60 minutes of observation. A writhing is characterized by the mouse's legs and arms being pulled forward with the abdomen touching the floor. The smallest average number of writhing occurred in the positive control (K+), namely, the group of animals that were given acetic acid induction and given mefenamic acid as a comparison of the treatment group. This can be seen in Figure 1.

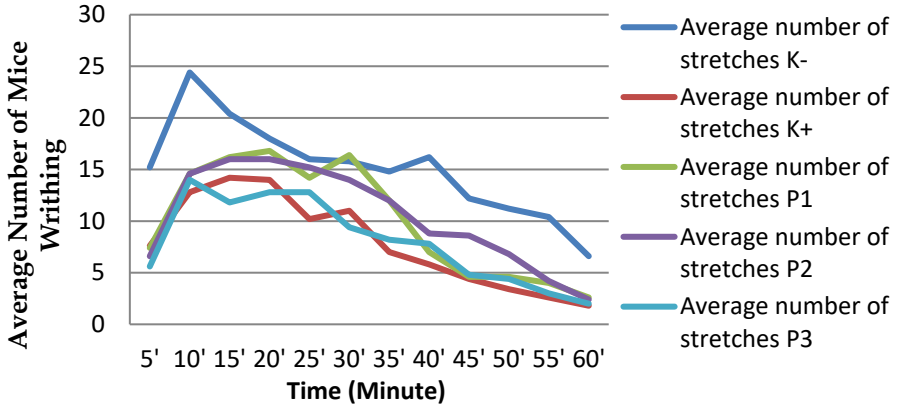

Figure 1. Comparison of the average number of mice writhing

Information:
K-: 1% Na-CMC suspension group and induced by acetic acid
K+: Mefenamic acid suspension group induced by acetic acid
P1: Mangrove leaf extract suspension group 250 mg/kg BW and induced by acetic acid
P2: Soursop leaf extract suspension group 250 mg/kg BW and induced by acetic acid
P3: Suspension group of extracts from a combination of soursop leaves and mangrove leaves 250: 250 mg/kg BW and induced by acetic acid

Based on the data in Figure 1, the average number of mice writhing during 60 minutes in the negative control group was higher. There is no active substance in the negative control group treatment. The extract treatment and positive control groups experienced a decrease in the number of writhing movements compared to the negative control. This shows that the administration of the extract and positive control can reduce writhing in mice, a pain response caused by acetic acid.

Data in the form of the cumulative number of writhes for 60 minutes were then analyzed using the One-Way ANOVA test with a significance level of 95%. This test was carried out to determine significant differences in the average number of writhing data in each treatment group. This can be seen in Table 1.

Table 1. Analgesic activity of ethanol extract of mangrove (*Avicennia alba*) and soursop leaves (*Annona muricata*)

Group	n	Writhing Number	Writhing Protection (%)	Analgesic Effectiveness (%)
K-	5	15.12 ± 2.8 ^{c}	0.0 ± 0.0	0.0 ± 0.0
K+	5	7.9 ± 1.06 ^{a}	46.1 ± 5.1	100 ± 0
P1	5	10.14± 0.22 ^{b}	30.5 ± 7.2	64.1 ± 14.9
P2	5	10.44 ± 0.52 ^{b}	24,0 ± 6.2	50.8 ± 14.2
P3	5	8.06 ± 0.45 ^{a}	40.7 ± 6.67	87.93 ± 17.9

Information:
Superscripts with different lowercase letters in the same line indicate significant difference ($p < 0.05$)
K-: 1% Na-CMC suspension group and induced by acetic acid
K+: Mefenamic acid suspension group induced by acetic acid
P1: Mangrove leaf extract suspension group 250 mg/kg BW induced by acetic acid
P2: Soursop leaf extract suspension group 250 mg/kg BW induced by acetic acid
P3: Suspension group of extracts from a combination of soursop leaves and mangrove leaves 250:250 mg/kg BW and induced by acetic acid

Data in Table 1 shows that the percentage of writhing protection and analgesic effectiveness from the highest to the lowest is in the positive control group, namely 46.1% and 100%. .7% and analgesic effectiveness of 87.93%, mangrove leaf ethanol extract compound showed 30.5% stretching protection and 64.1% analgesic effectiveness, and soursop leaf ethanol extract showed a protection percentage of 23.9% and analgesic effectiveness of 50. 8%.

DISCUSSION

In the analgesic test, the method chosen is the writhing test method because this method is quite sensitive in assessing the pain stimulus given. The principle of this method is to calculate the amount of writing that occurs due to the administration of pain induction, namely 1% acetic acid

intraperitoneally. Writhing can be seen from abdominal contractions characterized by stomach cramps and legs pulled back (Nurhalimah *et al*., 2023). This method was chosen because it has the advantage of not taking a long time, is easy to do and observe, and is fast because it only injects an acetic acid solution as a pain stimulant. However, this method also has disadvantages, which are only used in peripheral analgesic testing (Al-Muqsith, 2015).

Based on the data in Figure 1, it shows that the average number of mice writhing during 60 minutes in the negative control group was higher. This is because there is no active substance in the negative control group treatment. The extract treatment and positive control groups experienced a decrease in the number of writhing movements compared to the negative control. This shows that the administration of the extract and positive control can reduce writhing in mice, which is a pain response caused by intraperitoneal acetic acid. The smaller the average number of writhes shown in a group of mice, the better the analgesic effect on the test material (Nazifah *et al*., 2022).

Based on the results of statistical analysis using the *One-Way ANOVA test*, it shows a significance value of $\rho = 0.000$. This means that data on the average number of mice writhing in each group shows a real difference between the treatment and the negative control ($\rho = <0.05$). Then, to see the differences in the analgesic effect of each group, the analysis continued using the *Least Significance Difference* (LSD), which showed that the best dose of analgesia in mice was the ethanol extract suspension group, a combination of soursop leaves and mangrove leaves, 250: 250 mg/kg BW, which is equivalent to positive control.

Table 1 shows that the percentage of writhing protection and analgesic effectiveness from the highest to the lowest is in the positive control group, namely 46.1% and 100%. 40.7% and analgesic effectiveness of 87.93%, mangrove leaf ethanol extract compound showed 30.5% stretching protection and 64.1% analgesic effectiveness, and soursop leaf ethanol extract showed a protection percentage of 23.9% and analgesic effectiveness of 50. 8%. The combination of ethanol extract from mangrove leaves and soursop leaves (1:1) provides a percentage of protection and analgesic effectiveness that is almost equivalent to the positive control because the chemical content of the two plants combined has been proven to have analgesic activity and there is a synergistic working mechanism when the effects of the active ingredients are combined. The same use will be greater than the effect of each active ingredient separately (Syahrir *et al.,* 2016). These results show that the greater the percent protection and percent analgesic effectiveness, the greater the analgesic effect, and conversely, the lower the percentage of stretch protection and the percentage of analgesic effectiveness, the lower the analgesic effect (Wulandari and Aznam, 2018)

Analgesic power can be seen due to the activity of flavonoid compounds by inhibiting the COX enzyme so that prostaglandin biosynthesis can be inhibited (Sentat, 2018). This is similar to the mechanism of action for tannins, when inhibition of the prostaglandin formation phase in the arachidonic acid metabolism pathway occurs due to the presence of alkaloid compounds (Anshori *et al.,* 2018). The presence of terpenoids and steroids also has analgesic activity by suppressing the phospholipase enzyme so that pain mediators are not formed (Hesturini *et al*., 2017). Inhibition of the pathway of the formation of cyclooxygenase and other pain mediators also inhibits the synthesis of prostacyclin which has the function of protecting the gastric mucosa. So, inhibition of prostaglandin biosynthesis will decrease self-defense by the gastric mucosa against irritants (Hesturini *et al.,* 2022). When a tissue is injured, arachidonic acid will release prostaglandins with the help of the phospholipase enzyme in the cell membrane phospholipids, namely the cyclooxygenase and lipoxygenase enzymes. If these two enzymes are inhibited, it will inhibit the formation of pain mediators (Tia *et al.,* 2017). Thus, the mechanism of high analgesic action can occur due to the combined action and complexity of several compounds with the same action, inhibiting the production of non-specific pain mediators.

From the discussion above, it can be concluded that the ethanol extract of mangrove leaves (*Avicennia alba*) is dosed at 250 mg/kg BW, the ethanol extract of soursop leaves (*Annona muricata* Linn.) is dosed at 250mg/kg BW, and the combination of ethanol extract of mangrove leaves (*Avicennia alba*) and soursop leaves (*Annona muricata* Linn.) dose of 250: 250 mg/kg BW can increase the analgesic activity of mice (*Mus musculus*) induced by acetic acid using the writhing test method. Further research will involve varying doses of combinations of mangrove leaves (*Avicennia alba*) and soursop leaves (*Annona muricata* Linn), as well as acute and chronic toxicity testing to support the safety level of using mangrove and soursop leaves as herbal preparations.

CONCLUSION

The combination of ethanol extract of mangrove leaves (*Avicennia alba*) and soursop leaves (*Annona muricata* Linn.) at a dose of 250:250 mg/kg BW has an analgesic equivalent to the positive control (Mefenamic acid) in male mice induced by acetic acid with a wriggling protection percentage of 40.75% and an analgesic effectiveness percentage of 87.93%.

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CONFLICT OF INTEREST

The authors declare there is no conflict of interest to disclose.

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