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Antiinflammation activity of *Muntingia calabura* L. leaves ethanol, ethylacetate and chloroform extracts

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Abstract

Muntingia calabura L. leaves extract are reported rich in flavonoidal compounds which shows a potential activity such as antidiabetic, antipyretic, analgesic and anti-inflammatory. Several subgroups of flavonoid compounds include flavones, flavanones, flavans and biflavans. This study aimed to analyze the anti-inflammatory activity of ethanolic, ethylacetate, and chloroform extracts of *M. calabura* L. leaves. The extraction process was carried out by maceration, where the yield of the three solvents were 19.14%; 8.41% and 6.59% w/w, respectively. The anti-inflammatory activity was carried out by observing a decrease in edema due to 1% carrageenan induction after 4 hours. The statistical results of anti-inflammatory activity showed that the ethanol extract had a significant difference with the positive control of p value < 0.05 in which a dose of 240 mg showed a decrease in edema volume (IEV) that was closed to the positive control (acetosal = 52.12%) which was 62.51% compared to the dose 60 mg and 120 mg. Furthermore, the % IEV of ethyl acetate extract of *M. calabura* L. leaves at a dose of 60, 120, and 240 mg were 36.30%, 26.83%, 24.24%, respectively. These results demonstrated that an average level of anti-inflammatory activity was 22.89%, and was closed to the dose of 500 mg/kg of acetosal-induced mice. Finally, the chloroform extract with the dose of 60 mg, 120 mg, 240 mg had a significant value of p < 0.05 with a positive control (acetosal). It could be concluded that the ethanol, ethylacetate and chloroform extracts of *M. calabura* L. leaves may have anti-inflammatory potential with acetosal as a comparison.

INTRODUCTION

Inflammation is one of the defense mechanism caused by the physical trauma, infection in open wounds such as pus or antigen reactions from a certain disease causing pain, temperature changes, physiological changes and even interfere with activities (Yuliati *et al*. 2010). The inflammatory response is characterized by the presence of red color, heat, but also swelling or edema (Dyatmiko *et al*. 2003). Some well-known anti-inflammatory drugs are NSAIDs or non-steroidal anti-inflammatory drugs, which have the most frequent effect on gastrointestinal bleeding, so they need to be used with caution (Tjay and Tjoa. 2018). It could be the reason for encouraging the development of anti-inflammatory drugs that comes from nature, one example is *Muntingia calabura* L. leaves.

Muntingia calabura leaves are reported to be rich in flavonoidal compounds which can be efficacious as antidiabetic, antipyretic, analgesic, anti-inflammatory and other activities. The kinds of flavonoid compounds includes flavones, flavanones, flavans and biflavans. *Muntingia calabura* also contains alkaloids, saponins and

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tannins (Bait *et al*. 2021). Extracts of water, chloroform and methanol were investigated in vitro by Bait *et al*. (2021) as an inhibitor of the proliferative process or the propagation of cancer cells as well as being used as antioxidants. This was also in line with research conducted Bait *et al*. (2021) which stated that the methanol extract of dried *M. calabura* leaves could significantly reduce blood glucose levels in rats. Bait *et al*. (2021) proved that quinone reductase activity from the ethyl acetate extract was induced in cell culture measurements, indicating its antidiabetic activity. *Muntingia calabura* leaves extract had potential as an antipyretic and analgesic. The ethanol extract of *M. calabura* leaves contains alkaloids, anthraquinones, polyphenols, saponins and flavonoids (Widyaningrum *et al*. 2016), the ethylacetate extract of *M. calabura* leaves contains alkaloids, anthraquinones and flavonoids (Widyaningrum *et al*. 2019) while the chloroform extract contains alkaloids, anthraquinones, saponins, polyphenols and flavonoids (Widyaningrum *et al*. 2020).

The anti-inflammatory activity of *M. calabura* leaves presumably because it inhibits the release of inflammatory mediators such as histamine and prostaglandins (Paulita *et al*. 2021). This examination was carried out using a phytochemical screening method equipped with thin layer chromatography. The difference in the results of this secondary metabolite content causes differences in the potency of *M. calabura* leaves as both antipyretic and analgesic. The greatest analgesic and antipyretic potential exhibited in ethanol extract with a capacity of 52.43% at the highest dose compared to ethyl acetate and chloroform extracts with the same dose (Widyaningrum *et al*. 2016). Amongst the aforementioned description, the researchers aimed to study and analyze the anti-inflammatory activity using three extraction solvents with different levels of polarity, ranging from nonpolar, semipolar and polar as well as to improve previous research on the *M. calabura* leaves activity as analgesic and antipyretic agent.

MATERIALS AND METHODS

This research use *M. calabura*, ethanol 70%, etylacetate, chloroform, carrageenin 1%, diclofenac natrium, aquadest and fried oil.

Extraction by Ethanol, Ethylacetate and Chloroform

1. Drying and Powder Making

The *M. calabura* leaves were washed with water and sorted from impurities, the leaves were dried under direct sunlight for 2 days, then aerated for one week, then the leaves were dried in an oven at 50ºC for 24 hours. The dried simplicia was powdered using a powder machine.

2. Extraction by ethanol, ethylacetate and chloroform

One kg of *M. calabura* leaf powder was macerated with 70% ethanol, 1 kg with ethyl acetate and 1 kg with 10 liters of chloroform, then filtered through a Buchner funnel. Then remaceration was carried out with the same ratio of simplicia and solvent as during the initial maceration. The filtrate was evaporated with a rotary evaporator and then aerated until each solvent evaporates and a thick filtrate was obtained. The viscous extract was placed in a desiccator for further drying.

3. Anti-inflammatory Activity Testing

The anti-inflammatory test used 30 mice weighing 20-30 mg, aged 6-8 weeks divided into 5 groups, each group consisting of 6 mice. Mice were acclimatized and fasted overnight with water. On the day of testing, the weights were weighed and grouped randomly. Group I was a solvent control (cooking oil), group II was a positive control (acetosal 500mg/kg BW) orally), Group III-V was treated with a dose of ethanol extract, ethylacetate and chloroform 60mg/20gBW orally; 120 mg/20 g body weight orally and 240 mg/20 g body weight orally. After administration of the test and control compounds, the left foot of all mice was injected intraplantar with 0.02 ml of 1% v/v carrageenin suspension. The volume of the left leg was measured by immersing it in a plethysmometer for every 30 minutes for 6 hours after the injection of 1% carrageenin. The volume of edema was determined by the difference in the volume of edema in mice before and after being induced with 1% carrageenin.

Data Analysis

The data obtained in the form of volume of edema before and after 1% carrageenin induced. The difference in the volume of the two legs showed the volume of edema that occurs. Edema volume was analyzed into % increase in edema volume (IEV) by the formula:

$$\%IEV = \frac{(Vt-Vo)}{Vo} \times 100\%$$

Note:
Vo = edema volume before carrageenin induction
Vt = edema volume after carrageenin induction

The percentage of anti-inflammatory power was calculated by comparing the AUC (area under curve) of the treatment to the control. The antiinflammatory power value of the treatment group was compared by the positive control and negative control. The % value of anti-inflammatory power was calculated using the following formula:

$$\%\ \text{Anti-inflammatory power} = \frac{\text{control AUC} - \text{treatment AUC}}{\text{control AUC}} \times 100\%$$

Note: control AUC : AUC without extract giving
Treatment AUC : AUC with extract giving

Then analysed the impact of extract giving to the control used Anova and LSD test by SPSS software.

RESULTS AND DISCUSSION

Ethanol, Ethyl acetate and Chloroform Extract

The method used for the extraction process was maceration. The advantage of this method was that the process was simple, didn't require specific tools and could be used to extract compounds that weren't heat-resistant. (Depkes RI, 1986). Effect of different extraction solvents was investigated on the organoleptic and yield percentage, presented in Table 1. The organoleptic results showed almost the same results in the three extracts, while the highest yield was found in the 70% ethanol extract. This was in line with the secondary metabolite identification research conducted by (Widyaningrum *et al*. (2016) that 70% ethanol extract contained the most secondary metabolites, namely alkaloids, anthraquinones, polyphenols, tannins, saponins and flavonoids.

Table 1. Yield results of ethanol, ethyl acetate and chloroform extract of *M. calabura* leaves

	Ethanol extract	Ethyl acetate extract	Chloroform extract
Consistency	thicky	thicky	thicky
Colour	Blackish green	Blackinsh green	Blackish green
Smell	aromatic	aromatic	aromatic
Weight	19,14%b/b	8,41%b/b	6,59%b/b

Ethanol Extract Anti-inflammatory Activity Testing

The anti-inflammatory activity of the drug was demonstrated by reducing carrageenan-induced edema. As shown in Fig. 1, there was a decrease in the volume of edema between the solvent control, positive control, and three treatment doses. Water as a control solvent showed the least reduction in edema volume when compared to acetosal and the three extract treatments. The extract and acetosal groups experienced a significant decrease in edema volume from the 1st to the 4th hour. The extract dose of 120 mg at the 4th hour showed the highest decrease in edema volume when compared with the administration of extract doses of 60 mg, 240 mg, or acetosal. This could be due to the difference in the size of the feet of the mice initially used in each test, so that the initial volume and the final volume obtained showed such results.

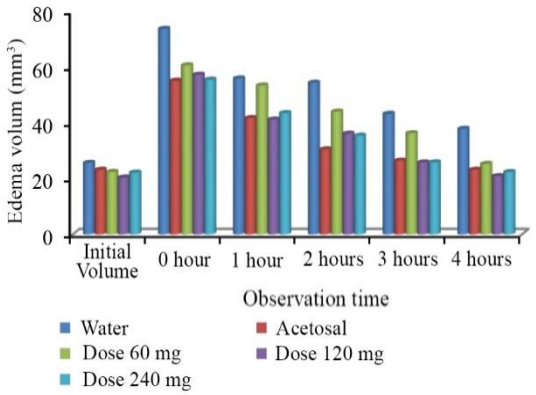

Figure 1. Graph of correlation between edema volume (mm³) and observation time.

Moreover, Fig 2 shows the mean percentage decrease in IEV over the four hours of the experiment. Within four hours there was a decrease in edema in each treatment, but in different volumes. The volume of reduction in edema on water (negative control) showed the least volume of reduction in edema among the other control groups. This was because the negative control did not contain active substances that could reduce the volume of edema. While acetosal (positive control) showed the highest volume reduction of edema because acetosal was a compound that has been tested having anti-inflammatory activity, while for the extract group, a dose of 240 mg had highest effect of reducing edema among other doses. It could be seen at the fourth hour that the highest percentage of IEV in water, namely 55.22%, while the extract group at doses of 60, 120, and 240 mg/kg BW

showed 12.31%, 2.67%, and 1.13%, respectively. From these results, it could be seen that the average percent IEV pattern decreases with increasing of dose. This was because the higher the dose given; the volume of edema also decreases.

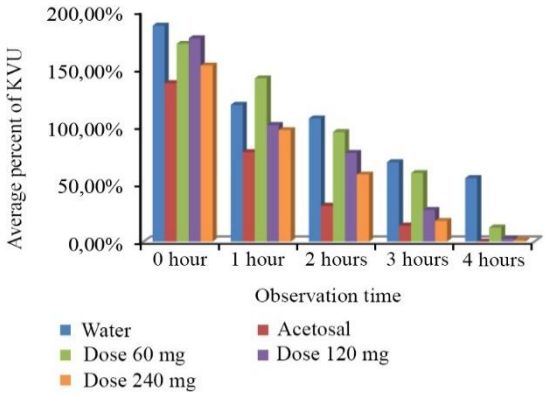

Figure 2. Graph of the correlation between observation time and average percent of IEV.

The average percentage in the volume of edema from each treatment were investigated (Figure 6). It could be seen that the control solvent (water) had the highest percent increase in edema volume. This was very relevant because water did not have an anti-inflammatory effect, and when the soles of the test animals' paws had carrageenan-induced edema, the reduction in edema lasts longer. Acetosal had the highest anti-inflammatory effect with the lowest average percentage of IEV which was 52.12%, while for the extract group, a dose of 240 mg had a % KVU that was close to the positive control (acetosal) which was 62.51% compared to doses of 60 mg and 120 mg.

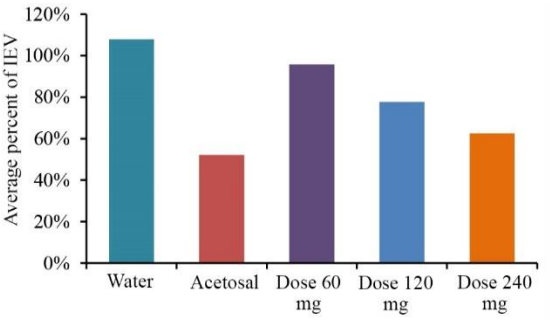

Figure 3. Average percent IEV diagram chart. The average percentage increase in the volume of edema from each treatment.

Ethylacetate Extract Anti-Inflammatory Activity

As shown in Fig. 4, the lowest and highest reduction in edema were occurred in positive control (acetosal) and negative control (oil), respectively. The extract obtained with ethyl acetate at a dose of 240 mg showed the closest value to the positive control. The oil used in this experiment was cooking oil which was neutral so it did not cause side effects and did not damage the active substances contained in *M. calabura* leaves. *M. calabura* leaves was chosen because as a traditional medicine it was believed to have anti-inflammatory properties.

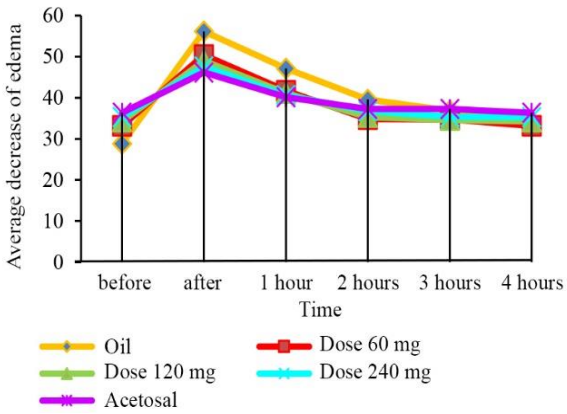

Figure 4. Graph the correlation between observed time with edema decreasing to the mice's feet.

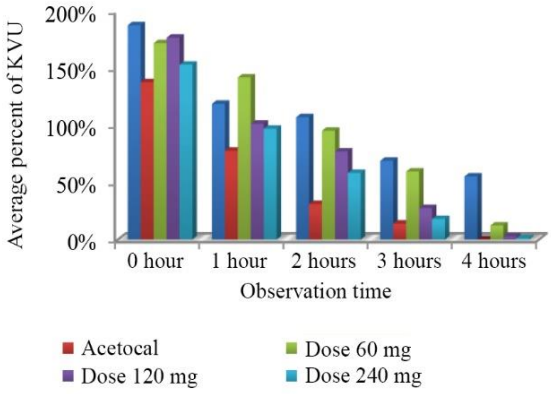

Figure 5. Graph correlation between observed time and average percent of IEV.

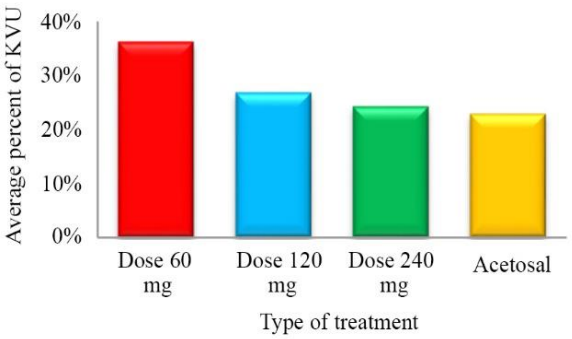

Figure 6. Diagram of the relationship between the type of treatment and the average percent IEV on anti-inflammatory.

Figure 6 showed an average percentage of anti-inflammatory power of positive control (acetosal) was smaller than ethyl acetate extract of *M. calabura* leaves at doses of 60 mg, 120 mg, and 240 mg, because the smaller the percentage of IEV the greater the percentage of the anti-inflammatory effect produced.

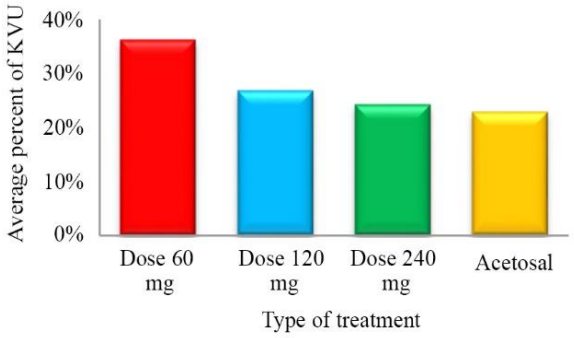

Figure 6. Diagram of the relationship between the type of treatment and the average percent IEV on anti-inflammatory.

Choloroform Extract Anti-inflammatoy Activity

Inflammation is a local protective response caused by injury or damage to the tissue that serves to destroy, reduce, or localize both the injuring agent and the injured tissue. Acute inflammation is made possible by the release of chemical mediators such as vasoactive amines, proteases. plasma, arachidonic acid metabolites, and leukocyte products (Widhihastuti *et al*. 2021).

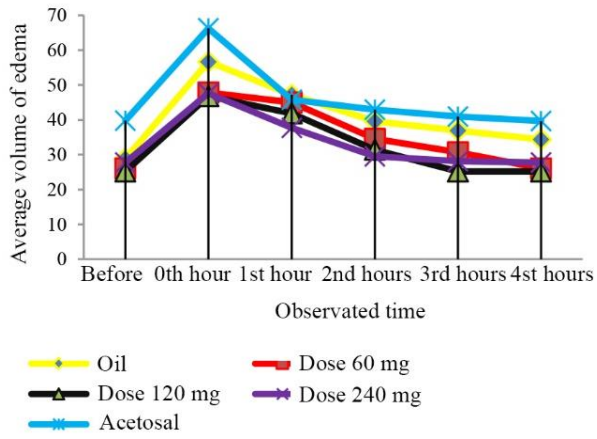

Figure 7. Graph the correlation between edema volume (mm3) with observed time.

Figure 7 showed that the most decrease in edema occurred in the positive control (acetosal) followed by *M. calabura* leaf chloroform extract at a dose of 240 mg, while the decrease in edema was the least in the control solvent (oil). This is because the oil has no anti-inflammatory effect whereas acetosal is a chemical drug that has a strong anti-inflammatory effect. The use of cooking oil as a solvent for the chloroform extract of *M. calabura* leaves as well as a negative control because the chloroform extract is non-polar and insoluble in water or other polar compounds, while cooking oil is also non-polar.

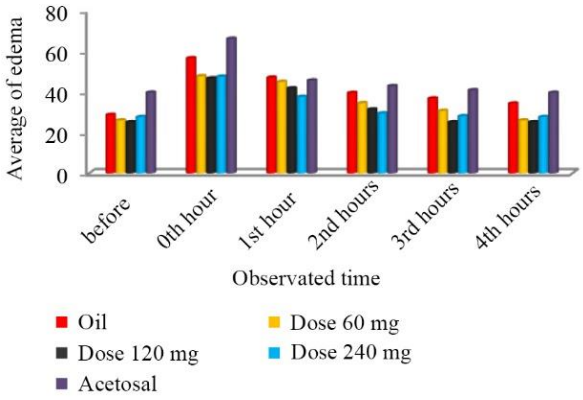

Figure 8. Graph the correlation between observed time with edema average increasing.

The results of increased incidence of edema for 4 h are shown in Figure 8. Experiment on mice with negative control (oil) had a high average number of increases in edema, even at the 4th hour the volume of leg edema in mice had not returned to normal or there was still swelling compared to the experiment in mice with chloroform extract of *M. calabura* leaves at a dose of 60 mg, 120 mg, 240 mg, and also a positive control (acetosal).

In the experimental *M. calabura* leaves chloroform extract at a dose of 60 mg the edema of the mice's feet had partially stopped or had returned to the original size of the mice's feet at the 4th hour, whilst at a dose of 120 and 240 mg the volume of the mice's feet had returned to their original size at the 3rd hour. This was due to the higher the dose given, the faster the reduction of edema in mice. In the graph above, it could be seen that the control solvent (oil) had a very high increase in edema volume.

Figure 9 demonstrated that the volume of edema decreased for the four hours of the experiment. Within four hours there was a decrease in edema in each treatment, but in different volumes. The volume of edema reduction in oil (negative control) showed the least volume of edema reduction among the other control groups. This was because the negative control did not contain active substances that could reduce the volume of edema, while acetosal (positive control) showed the highest volume of edema reduction because acetosal was a

compound that had been tested to have anti-inflammatory activity. In the extract group, a dose of 240 mg had the highest effect on reducing edema among the other doses. It could be seen at the fourth hour that the highest percentage of IEV was oil, which was 19.77%, while acetosal and the extract group at doses of 60, 120, and 240 mg/kg BW were 0%. From these results, it could be seen that the average pattern of edema decreased.

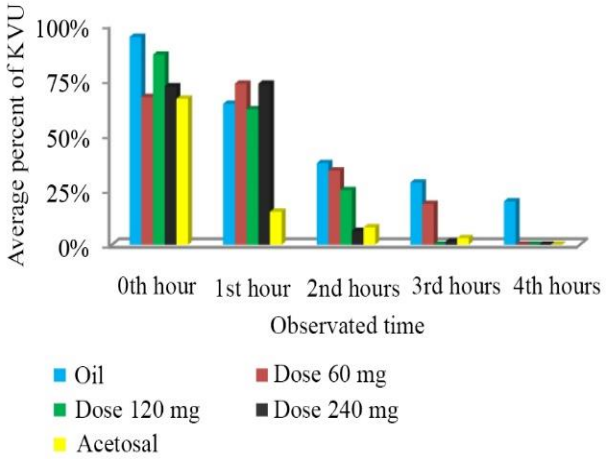

Figure 9. Graph the correlation between observed time with the average decreasing of IEV.

The results of the data obtained from the anti-inflammatory test were then analyzed statistically with the one-way ANOVA test to see a significant difference in the anti-inflammatory effect of the three treatments which must meet the requirements for normality and homogeneity of the data. The data had normality if the significance value of P > 0.05, this meant that the data had the same variance. Normality test was used to measure whether the data had a normal distribution so that it could be used in parametric statistics. Based on the results of the One-Sample Kolmogorov-Smirnov Test, the significance value was 0.677 > 0.05, this meant that the data obtained was normally distributed. The next step, the data was analyzed using the Levene Test, the results obtained a significance value of 0.037 <0.05, the data obtained had an inhomogeneous variant because it had a very large difference.

Then a one-way ANOVA test was carried out, based on the results of the test the probability value listed in the significance column was 0.000 <0.05, then H0 was rejected, which indicated that there was a significant difference between the percent anti-inflammatory power of the *M. calabura* leaves chloroform extract. Furthermore, the Post Hoc Test (LSD) was carried which had a significance value of 0.000 <0.05, then H0 was rejected, there was a significant difference between the administration of acetosal and *M. calabura* leaves chloroform extract.

The dose of acetosal with 240 mg *M. calabura* leaves chloroform extract had a significance value of 0.014 <0.05 then H0 was rejected, there was a significant difference between the dose of acetosal and the dose of 240 mg of *M. calabura* leaves chloroform extract. The dose of 60 mg with acetosal, the dose of 60 mg and 120 mg, the dose of 60 mg and 240 mg had a significant value of P < 0.05 then H0 was rejected, there was a significant difference between the 60 mg and 120 mg doses, the 60 mg dose and 240 mg, a dose of 60 mg and acetosal. The dose of 120 mg with acetosal, the dose of 120 mg with a dose of 60 mg, the dose of 60 mg with 240 mg had a significant value of P < 0.05 then H0 was rejected, there was a significant difference between the dose of 120 mg and acetosal, the dose of 120 mg and the dose of 60 mg, a dose of 120 mg and a dose of 240 mg. The dose of 240 mg with a dose of 60 mg, a dose of 240 mg with acetosal had a significance value of 0.000 <0.05 then H0 was rejected, there was a significant difference between a dose of 240 mg and a dose of 60 mg, a dose of 240 mg and acetosal with a dose of 120 mg having a significance value of 0.014 <0.05 then H0 was rejected, there was a significant difference between a dose of 240 mg and a dose of 120 mg.

From the data above, it could be concluded that the *M. calabura* leaves chloroform extract had anti-inflammatory properties, possibly due to the presence of flavonoids in the preparation. This may also be related to the presence of saponins, flavonoids, and various compounds contained in one. Statistical results on the anti-inflammatory test showed that the *M. calabura* leaves chloroform extract had a significant difference with the positive control (acetosal) of p <0.05. In this study, carrageenan with NaCl solvent was used as a trigger for edema because carrageenan is a compound that can induce cell injury by releasing mediators that initiate the inflammatory process. Edema that occurs due to the release of inflammatory mediators such as histamine, serotonin, brandikinin, and prostaglandins. Edema caused by carrageenan injection is strengthened by inflammatory mediators, especially PEG1 and PEG2 by reducing vascular permeability (Senewe *et al*. 2013)

CONCLUSION

Based on the results of this study, it could be concluded that the *M. calabura* leaves of ethanolic extract showed anti-inflammatory activity. The dose

of 240 mg/kg BW was the most effective dose because the result showed close to positive control (acetosal). The percentage of edema volume decrease as the dose of ethylacetate extract increase is 60 mg by 36.30%, 120 mg by 26.83%, and 240 mg by 24.24% so that it was in contrast to the anti-inflammatory power which was close to Acetosal with a dose of 500 mg/kg BW in mice was 22.89%. Regarding the statistical analysis, the chloroform extract had anti-inflammatory activity similar to the dose of acetosal.

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