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TOTAL FLAVONOID CONTENT AND ANTI-INFLAMMATORY PROPERTIES OF INDONESIAN MISTLETOES (*DENDROPHTHOE PENTANDRA* (L.) MIQ.) ETHANOL EXTRACT

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ABSTRACT

Traditionally mistletoes *Dendrophthoe pentandra* (L.) Miq known in Indonesia is used to cure cough, hypertension, diabetes, cancer, ulcers, smallpox, diuretic, skin infection and after child-birth. The result of its phytochemical screening of the n-hexane fraction showed the presence of flavonoids, monoterpenes, and sesquiterpenes, acid ethyl acetate fraction showed the presence of flavonoids, and Quinones, alkaline ethyl acetate fraction showed the presence of flavonoids, polyphenols, tannins, and Quinones, while the water fraction showed the presence of flavonoids, polyphenols, tannins, and Quinones. Calculated the total flavonoid as quercetin showed that levels of total flavonoids of 0.068 kg w/w. Anti-inflammatory activity of the fraction of n-hexane, ethyl acetate acid, ethyl acetate base, and water from mistletoes *Dendrophthoe pentandra* (L.) Miq were conducted against white male Wistar rats. Tests Carried out using the method inducers of carrageenan in the rat right foot. The test material was administered at a dose orally of 1000 mg/kg BW. For comparison used indomethacin 10 mg/kg BWbw, and used as a control PGA 2%. Anti-inflammatory activity was observed for 5 hours after carrageenan inducers. The test results Showed that the ethyl acetate fraction of acid at a dose of 2000 mg/kg bw have the best activity. At this dose acid ethyl acetate fraction Showed the percentage of 41.46% inhibition of inflammation. Anti-inflammatory was thought to be the caused by the flavonoids contained as quercetin. The results of this study are supporting data of the *Dendrophthoe petandra* that can be used as an adjuvant treatment in anti-inflammation caused by the breast cancer.

KEYWORDS: mistletoes *Dendrophthoe pentandra,* phytochemical screening, total flavonoids, anti-inflammation.

INTRODUCTION

Inflammation is a response to tissue injury and infection. When the inflammatory process takes place, where the vascular reaction liquid, the elements of the blood, white blood cells (leukocytes), and chemical mediators gathered at the site of tissue injury. The process of inflammation is a protective mechanism in which the body attempts to neutralize and eradicate harmful substances at the site of injury and to prepare for the state of tissue repair. As the symptoms of inflammatory reactions can be observed reddening, swelling, increased heat, pain, and dysfunction [Kee and Hayes, 1996; Mutschler, 1999].

Mantovani *et al.* [2008] describes the relationship between inflammation and cancer where the cancer followed by acute inflammation. When growing cancer cells can cause inflammation, then the presence of inflammation in a cell will be a fertile ground for breeding can attack the cancer cells to normal cells. It is said that the treatment of inflammation in cancer treatment is the right direction.

Mistletoes *Dendrophthoe pentandra* (L.) Miq traditionally known in Indonesia for treating cough, hypertension, diabetes, cancer, ulcers, smallpox, diuretic, skin infections and after child-birth [Chevallier, 2000; Ishizu *et al*., 2002; Osadebe *et al*., 2004]. Artanti *et al.* [2012] in their paper stated that mistletoes or *benalu* in Bahasa Indonesia was a semi-parasitic plants which was considered as an unwanted plant to economically important horticultural plant. Katrin in her dissertation in 2005 had been able to isolate quercetin and β-sitosterol which was a metabolite of the mistletoes *Dendrophthoe pentandra (*L.) Miq [Katrin *et al*., 2005, 2011].

Our previous report [Mustarichie *et al*., 2012] showed prediction of quercetin function as inhibitors of estrogen (Erα) so that it could be used as an anti-inflammatory drugs on breast cancer. In vivo quercetin had anti-inflammatory activity at 10 mg / kg bw as reported by [Herowati 2005]. B-sitosterol [Mustarichie *et al*, 2014, Awad et al., 2008] could be predicted as inhibitors of the inflammatory process. It has been reported that beta-sitosterol is the induction of apoptosis in MCF-7 cells [Chai *et al*., 2008]. Effect teratogenic on mus musculus of methanol extract of *Dendrophthoe pentandra* (L.) Miq has been reported by Sundaryono [2011]. This study reports on phytochemical screening, total flavonoid content and anti inflammatory properties of mistletoes *Dendrophthoe pentandra* (L.) Miq ethanol extract.

METHODS

Plant material

Materials used in this study was mistletoes *Dendrophthoe pentandra* (L.) Miq taken from the tea plantation in Lembang, West Java.

Animal experiments

White male Wistar rats weighing 170-220 grams and healthy. Mice obtained from the Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran. Prior to use rats adapted to the laboratory environment (quarantine) for one week. If the animal was healthy during the quarantine period body weight increased or remained.

Chemicals

Ethanol 70%, PGA, physiological saline solution (0.9%), α-karagenan, indomethacin, distilled water, n-hexane, ethyl acetate, 1 N HCl, 1 N NaOH, reagents for the phytochemical screening. Unless otherwise stated all chemicals were analytical grade.

Extraction

The extraction method used was maceration with solvent replacement every 24 hours three times. Simplicia powder *Dendrophthoe pentandra* (L.) Miq (706.84 g) put in macerator, then added 70% ethanol until all the powder submerged and allowed to stand for 24 hours while stirring frequently. After 24 hours macerat accommodated and do remaceration back. Macerat obtained was concentrated using a rotary evaporator at low pressure and temperature of 40 ^{0}C until slightly thick. The extract was then poured into a cup vaporizer that had been weighed, then evaporated on a water bath to obtain a viscous extract to be fractionated.

Fractionation

Extract fractionation process of mistletoes *Dendrophthoe pentandra* (L.) Miq proceeded in accordance with the following chart (Figure 1).

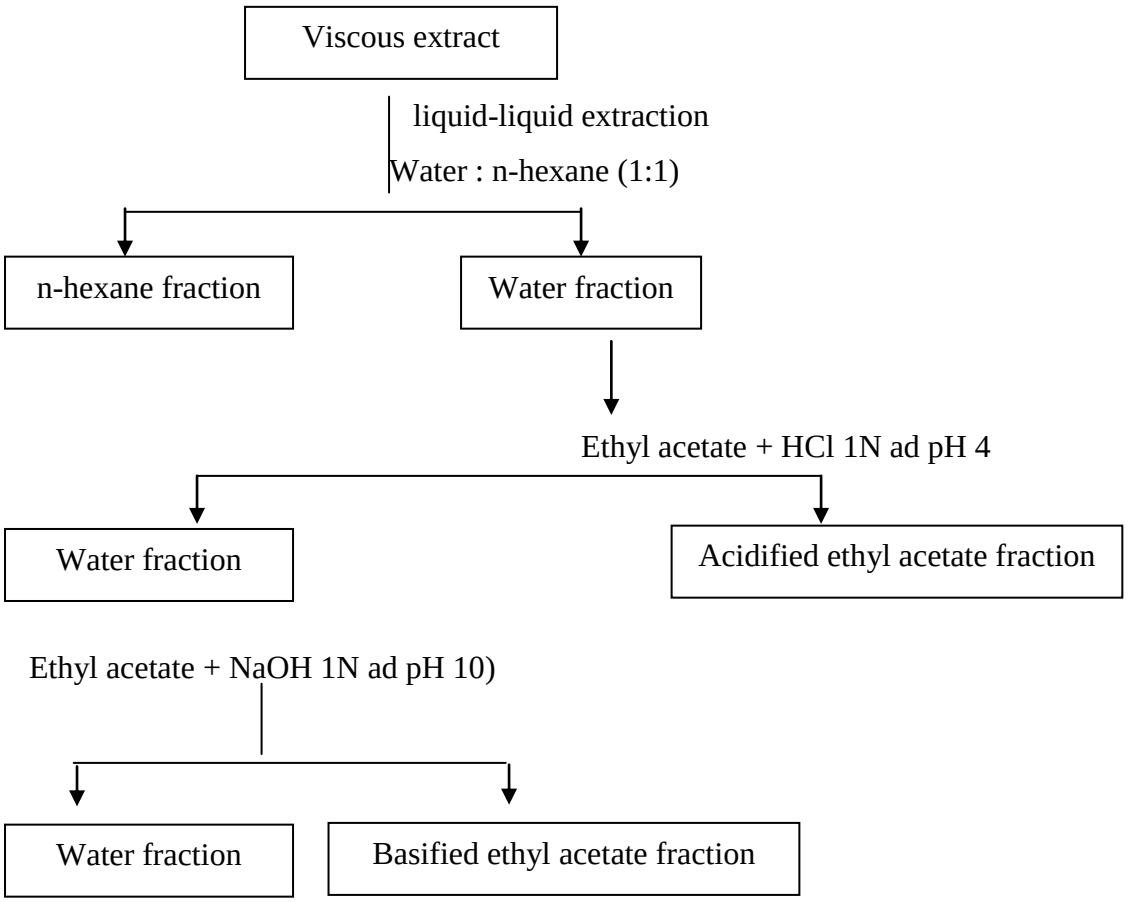


Figure 1: Fractionation chart of mistletoes *Dendrophthoe pentandra* (L.) Miq.

Phytochemical screening

Phytochemical screening performed on crude sample and the fraction of n-hexane, ethyl acetate, and water from mistletoes *Dendrophthoe pentandra* (L.) Miq by the method described by Farnsworth [1966] and Markham [1982].

Analysis of Total Flavonoids

Determination of total flavonoid content in accordance with the studies had been reported previously (Mustarichie *et al*, 2014) based on the method of Chang *et al* (2002). The method used for the analysis of total flavonoids was a standard reagent addition with the help of sliding reagent, $AlCl_3$.

Anti-inflammatory Activity

Anti-inflammatory activity was carried out using a modification of Romay *et al.* (1998) method.

Fraction of n-hexane, ethyl acetate, and water tested antiinflammatory activity by the method of induction of ooedema in the male mice legs. Testing was done by injecting a solution of 1% carrageenan subcutaneously on the soles of the hind legs of mice that can cause ooedema. The amount of ooedema was measured every hour for 5 hours by using plethysmometer.

This study used a randomized design perfectly with 6 kinds of treatments and 3 repetitions. Mice were grouped into 6 groups, each group consisting of 3 mice.

The procedures performed in this study were as follows:

1. Rats were fasted for about 18 hours before testing, water was still given.
2. On the day of testing, each rat was weighed and divided into 6 groups randomly; the positive control group, the negative control group, the group of n-hexane fraction dose of 1000 mg/kg, group acid ethyl acetate fraction dose of 1000 mg/kg BB, ethyl acetate fraction alkaline group dose of 1000 mg/kg, and group water fraction dose of 1000 mg/ kg.
3. At first volume of foot were measured and expressed as the initial volume (Vo) for each rat.

4. Each group was treated orally with doses as follows:

Group 1 was given a 2% suspension PGA as a negative control.
Group 2 was given a suspension of indomethacin 10 mg/kg as a comparison/positive control.
Group 3 was given a fraction of n-hexane mistletoes *Dendrophthoe pentandra* (L.) Miq 1000 mg/kg BW.
Group 4 was given parasite acid ethyl acetate fraction mistletoes *Dendrophthoe pentandra* (L.) Miq 1000 mg/kg BW.
Group 5 was given a fraction of ethyl acetate base mistletoes *Dendrophthoe pentandra* (L.) Miq 1000 mg/kg BW.
Group 6 were given water fraction mistletoes *Dendrophthoe pentandra* (L.) Miq 1000 mg/kg BW.

5. One hour after treated, the right foot of mice were injected with 1% carrageenan suspension 0.05 mL subcutaneously.
6. Further hourly volume right leg all the rats was measured by dipping into plethysmometer tool. Measurements were taken every 5 hours and recorded the volume of the right foot every hour measurement (Vt).

7. Percentage of inflammation every hour for each rat was calculated.
8. Next to each group calculated the average percentage of inflammation. Data in which the percentage of maximal inflammation statistically analyzed ANOVA and Newman Keuls range test.

   Percentage inflammation calculated by the following formula:

$$\% \text{ inflammation} = \frac{V_t - V_o}{V_o} \times 100\%$$

   Where Vo = volume foot carrageenan-induced mice before carrageenan induced.

   Vt = volume of foot mice after carrageenan induced.

9. Percent inhibition of inflammatory each test group on the hour in which the percentage of maximal inflammation was calculated.

The percentage inhibition of inflammation was calculated as follows:

$$\% \text{ inhibition} = \frac{\% \text{ inhibition control} - \% \text{ inhibition test}}{\% \text{ inhibition control}} \times 100\%$$

Data Analysis

The data obtained were presented in the form of tables, graphs, and bar charts, statistically analyzed using a random design perfect model of random and Newman Keuls range test [Muth, 2006; Seltman, 2014].

RESULTS AND DISCUSSION

Extraction

Mistletoes *dendrophthoe pentandra* (L.) Miq fresh first dried in the open air with aerated. Drying was done to stop the enzymatic reaction or hydrolysis that occurs in a cell or plant tissue, and to evaporate the water contained in the plant tissue resulting in shrinkage and form pores that could be entered by fluid. Once dried, powdered leaved in a blender to increase the surface in contact

With liquid extraction to obtain more results. In the extraction process used method of maceration with 70% ethanol solvent. Ethanol was chosen as the liquid was able to dissolve almost all of the secondary metabolites contained in the samples. Concenstration of total extract obtained from extraction by maceration with 70% ethanol can be seen in Table 1.

Table 1. Results of Extraction of mistletoes *Dendrophthoe pentandra* (L.) Miq by maceration with 70% Ethanol

Sample weight (g)	706,84
Viscous extract (g)	165,58
Yield	23,42%

Fractionation

The ethanol extract obtained further fractionated using n-hexane, ethyl acetate acid, ethyl acetate base, and water. Extracts were used for fractionation of 60 grams. After fractionated obtained results are shown in Table 2.

Table 2. Fractionation results of n-Hexane, Ethyl Acetate Acid, Ethyl Acetate Bases and Water of Ethanol Extract.

Total extract weight	60 g
n-hexane fraction	1,35 g
Acidified ethyl acetate	5,98 g
Basidied ethyl acetate	0,8 g
Water fraction	50,89 g

Table 2 showed the n-hexane fraction obtained 2.2%, ethyl acetate fraction 9.97% acid, ethyl acetate fraction alkaline 1.3%, and 84.81% water fraction. Percentage highest yield obtained from water fraction. This indicated that the chemical content of *Dendrophthoe pentandra* (L.) Miq more polar so attracted to the fraction of the amount that was more because it had the same polarity with water. While compounds that were semi-polar few. It could be seen from the percentage yield obtained from ethyl acetate fraction of acids and bases. The yield of n-hexane fraction produced the least amount. This suggested that the mistletoes *Dendrophthoe pentandra* (L.) Miq very little to contain compounds that were non-polar.

Phytochemical screening

Screening was done to the fraction of n-hexane, ethyl acetate acid, ethyl acetate base, and water

Dendrophthoe pentandra (L.) Miq and the results of which shown in Table 3 below.

Table 3 Results of phytochemical screening of the fraction of n-Hexane, Ethyl Acetate Acid, Ethyl Acetate and Water Bases *Dendrophthoe pentandra* (L.) Miq.

Compounds	n-hexane fraction	Acidified Ethyl acetate fraction	Based Ethtyl acetate fraction	Water fraction
Alkaloid				
(a) Mayer	-	-	-	-
(b) Dragendorf	-	-	-	-
(c) Bouchardat	-	-	-	-
Flavonoid	+	+	+	+
Tannin	-	-	-	+
Polyphenol	-	-	+	+
Mono and sesquiterpen	+	-	-	-
Triterpenoid	-	-	-	-
Steroid	-	-	-	-
Quinone	-	+	+	+

Notes :

(+): detected

(-): not detected

All fractions shown to contain flavonoids.

Analysis of total flavonoids

Quantitative analysis of total flavonoids done by standard addition method with the help of a sliding reagent $AlCl_3$. The standard addition method chosen to equalize the conditions of matrix sample solution and standard solution, so that the calculation of the levels obtained more accurately. The use of sliding reagent $AlCl_3$ aims to confirm and flavonoids contained in the sample (Markham, 1982). This causes changes in the structure of flavonoids auxochrome group that ultimately affect the wavelength shift produced. Estimates of the total flavonoid levels in this study was calculated as quersetin, because the used raw is quercetin. Quercetin have a common identifier used to analyze flavonoids (Chang *et al*., 2002; Umar, 2008; Gandjar and Rohman, 2007). Absorbance values obtained based on the standard concentration added to the sample can be seen in Table 4.

Table 4. Value of absorbance based on the standard addition to the sample

The amount of samples (mL)	The amount of standard (mL)	Standard concentration (ppm)	Absorbance means at 375 nm
5	0	0	0,0010
5	1	2,5	0,2139
5	1,5	3,75	0,3462
5	2	5	0,4215
5	2,5	6,25	0,5400

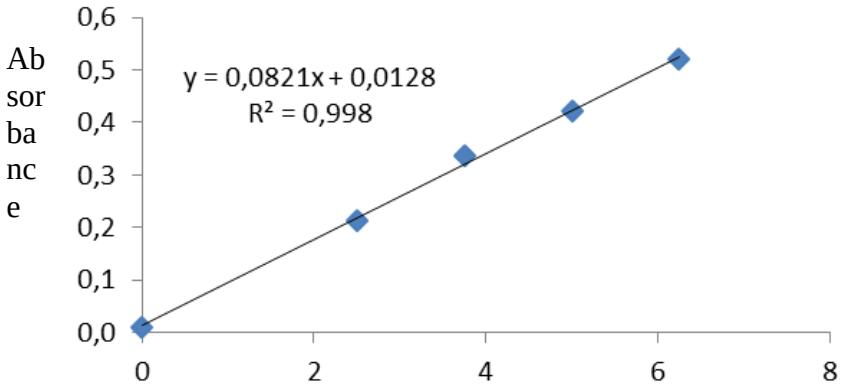


Figure 2. Standard addition Curve

Concentration of total flavonoids calculated as quercetin was calculated by equation from Fig. 2. After obtaining the total flavonoid content, the percentage content of flavonoids in the sample was calculated using the equation:

$$\% \text{ Total flavonoid} = \frac{\text{flavonoid content}}{\text{Sample weight}} \text{ x } 100\%$$

It was found that concentration of total flavonoid were 0.068% w/w.

Anti-inflammatory activity

Fractions of *Dendrophthoe pentandra* (L.) Miq obtained poorly soluble in water, therefore made preparations to use suspending PGA suspension. In testing the anti-inflammatory activity of the

Fraction of n-hexane, ethyl acetate acid, ethyl acetate base, and water *Dendrophthoe pentandra* (L.) Miq used a dose of 1000 mg / kg BW. Suspension PGA 2% was used as a negative control. As a positive control (comparison) used indomethacin dose of 10 mg / KgBW. Indomethacin is a non-steroidal anti-inflammatory drug classes and used as a comparison because it has the most powerful anti-inflammatory power.

In testing the activity of anti-inflammatory drugs are a variety of methods, but in this study used a method of induction of rat foot ooedema. This method was based on the ability of the test drug in reducing ooedema induced in the rat foot. This method was based on one of the signs of inflammation are swelling (oedema). Induction of rat foot ooedema was done by

injecting a suspension of 1% carrageenan subcutaneously. This method was chosen in this study because it was easy to do, and the tools used were available in the laboratory. 1% carrageenan suspension chosen as inducers as a swelling caused by carrageenan was relatively short at around 3-5 hours, to facilitate the observation. Swelling caused by carrageenan would be reduced within 1-2 days without leaving a trace. The volume of the rat foot ooedema was measured using a plethysmometer. Plethysmometer work by Archimedes law, namely the increase in the volume of mercury in the reservoir could be read because of the presence of methylene blue solution in the measuring scale which was proportional to the increase in volume of the rat foot ooedema.

Before the feet of mice injected with carrageenan, measured in advance and called the initial volume (Vo). Once injected volume carrageenan foot were measured and called Vt. The observation of the volume of the initial and final leg of mice can be seen in Table 5.

Table 5. Volume foot mice each treatment group before and after Injected Carrageenan.

Treatment	No	Foot volume (10^{-3}ml)					
		V_0	V_1	V_2	V_3	V_4	V_5
PGA 2 % (Negative control)	1	18	26	35	36	36	34
	2	18	25	34	35	34	33
	3	20	29	37	38	36	36
Indomethacin 10 mg/Kg BW (Positive control)	1	16	19	20	22	21	19
	2	18	20	23	24	22	21
	3	17	20	21	22	20	19
n-hexane fract. 1000 mg/Kg BW	1	16	20	24	27	23	22
	2	16	21	26	29	25	23
	3	17	22	26	30	26	24
Acidified ethyl acetate fract. 1000 mg/Kg BW	1	21	23	24	27	27	26
	2	22	24	25	29	28	27
	3	22	25	26	32	30	29
Based ethyl acetate fract. 1000 mg/Kg BW	1	18	21	26	27	24	23
	2	17	21	25	28	26	24
	3	16	20	25	27	23	22
Water fract. 1000 mg/Kg BW	1	18	22	30	31	28	26
	2	20	23	29	32	27	26
	3	18	21	29	30	26	25

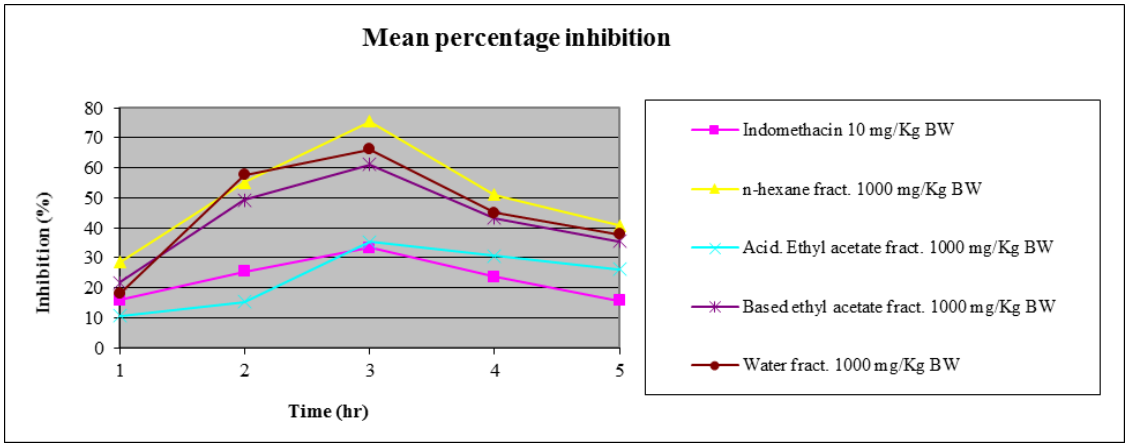


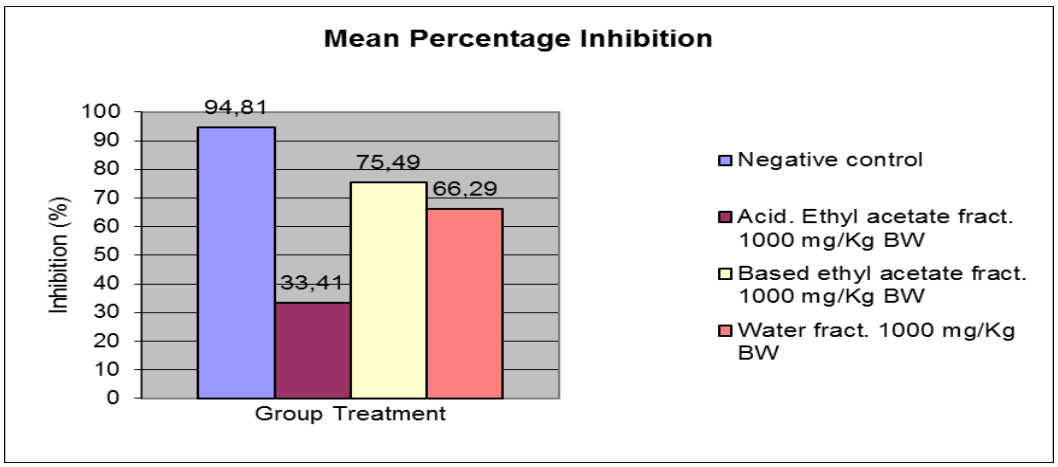
Figure 3. Graph the average percentage of inflamed foot of mice in each treatment during the observation after the injection of carrageenan.

From Figure 3 shows that the volume of inflammation reaches a maximum at the third hour for all treatment, and after the third hour volume decreases inflammation. Therefore, made the table

The average percentage of inflammation foot rat at the third hour that can be seen in Table 6. Table 6 furthermore created a bar chart that shows the average percentage of inflamed foot rat at the third hour that can be seen in Figure 4.

Table 6: Percentage inflammation treatment on observations every third hour after injected carrageenan

Treatment	Mice			Amount	Means
	1	2	3		
Negative control	100	94.44	90.00	284.44	94.81
Positive control	37.50	33.33	29.41	100.24	33.41
n-hexane fract.	68.75	81.25	76.47	226.47	75.49
Acid. ethyl acetate fract.	28.57	31.81	45.45	105.83	35.28
Based ethyl acetate fract	50.00	64.71	68.75	183.46	61.15
Water fract.	72.22	60.00	66.67	198.89	66.29



Figur 4. Diagram of the rod average percentage of inflammatory each treatment group at the third hour after the injection of carrageenan.

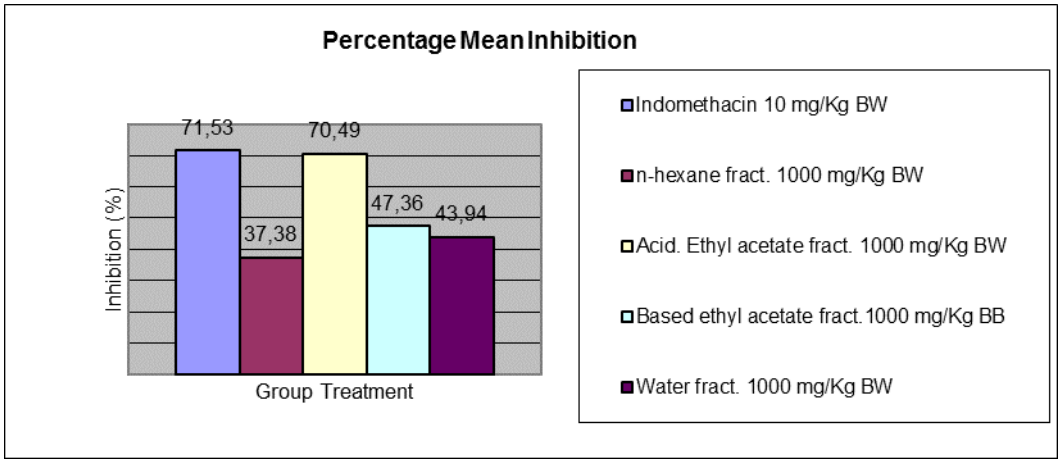


Figure 5. Percentage inhibition of each group

Figure 4 showed that the largest percentage of inflammation which is the group of n-hexane fraction was as large as 75,49% and the smallest was the acid group of the ethyl acetate fraction was equal to 35.28%. While of Figure 5 showed that the group of n-hexane fraction percentage inhibition smallest inflammation was 20.38% and ethyl acetate fraction acid groups had a percentage inhibition of inflammation, most notably 62.79%. The greater percentage of inflammation means the smaller the percentage inhibition of inflammation. This shows that the fraction of n-hexane has anti-inflammatory activity of most small and ethyl acetate fraction acids have anti-inflammatory activity of the greatest.

By using ANOVA table, turned the null hypothesis was rejected, it means that there diffrences in average inflammatory effects for each treatment. Because the null hypothesis was rejected, followed by Newman Keuls range test. Newman Keuls range test was conducted to determine whether there was a similarity between the effects of treatment with each other. Newman Keuls range test results can be seen in Table 7 for $\alpha = 0.05$ and Table 3.10 for $\alpha = 0.01$, respectively.

Table 7: Test Results Treatment Newman Keuls test at $\alpha = 0.05$

Test group	P	RST	Result
II vs IV	1,87	14,992	-
II vs V	27,74	14,992	+
II vs VI	32,88	14,992	+
II vs III	42,08	14,992	+
II vs I	61,40	14,992	+
IV vs V	25,87	18,506	+
IV vs VI	31,01	18,506	+
IV vs III	40,21	18,506	+
IV vs I	59,53	18,506	+

V vs VI	5,14	20,708	-
V vs III	14,34	20,708	-
V vs I	33,66	20,708	+
VI vs III	9,20	22,301	-
VI vs I	28,52	22,301	+
III vs I	19.32	23,519	-

Table 8: Test Results Treatment Newman Keuls test at α = 0.01

Test Group	P	RST	Result
II vs IV	1,87	21,551	-
II vs V	27,74	21,551	+
II vs VI	32,88	21,551	+
II vs III	42,08	21,551	+
II vs I	61,40	21,551	+
IV vs V	25,87	25,439	+
IV vs VI	31,01	25,439	+
IV vs III	40,21	25,439	+
IV vs I	59,53	25,439	+
V vs VI	5,14	27,923	-
V vs III	14,34	27,923	-
V vs I	33,66	27,923	+
VI vs III	9,20	29,749	-
VI vs I	28,52	29,749	-
III vs I	19.32	31,202	-

Notes:

I = the average percentage of inflammation negative control group (PGA 2%).

II = average percentage of inflammation of the positive control group (indomethacin 10 mg / kg BW).

III = average percentage of inflammation group of n-hexane fraction of 1000 mg/kg BW.

IV = average percentage of inflammation group ethyl acetate fraction acid 1000 mg/kg BW.

V = the average percentage of inflammation of ethyl acetate fraction alkaline group 1000 mg/ kg BW.

VI = average percentage of inflammation of the water fraction group 1000 mg/kg BW.

RST = significant range smallest

P = value range

(-) = No significant difference in effect

(+) = There is significant difference in effect

Table 7 appeared that the 95% confidence ethyl acetate fraction acid, ethyl acetate fraction alkaline and water fractions were significantly different effects with the negative control

group, while the fraction of n-hexane did not give effect significantly different from the negative control group. In other words, the 95% confidence ethyl acetate fraction acid, ethyl acetate base, and water of *Dendrophthoe pentandra* (L.) Miq at a dose of 1000 mg/kg BW but had inhibitory effects oedema that occured in the feet of mice, whereas the fraction of n-hexane *Dendrophthoe pentandra* (L.) Miq with a dose of 1000 mg/kg BW no inhibitory effect oedema that occured in the feet of mice. From Table 8 it appeared that the 99% confidence fraction of ethyl acetate and ethyl acetate fraction of acid-base effects are significantly different from the negative control group, while the water fraction and a fraction of n-hexane did not give effect significantly different from the negative control group. In other words that the ethyl acetate fraction of acids and bases of *Dendrophthoe pentandra* (L.) Miq with a dose of 1000 mg/kg BW had inhibitory effects ooedema that occured in the feet of mice, but the fraction of n-hexane and water of *Dendrophthoe pentandra* (L.) Miq at a dose of 1000 mg/kg BW had no inhibitory effect of ooedema. With a confidence of 95% and 99% indicated that the ethyl acetate fraction of acid at a dose of 1000 mg/ kg BW was the fraction that had the best anti-inflammatory activity because it did not have a different effect real difference to the positive control group (indomethacin 10 mg/kg BW).

CONCLUSIONS

The result of phytochemical screening fraction of n-hexane, ethyl acetate, and water of mistletoes *Dendrophthoe pentandra* (L.) Miq showed that the fraction of n-hexane containing flavonoids, monoterpenes and sesquiterpenes, ethyl acetate fraction containing flavonoids and quinones, alkaline ethyl acetate fraction contains polyphenols, quinones and flavonoids, while the water fraction contains flavonoids, polypenols, tannins and quinones. The total flavonoids calculaed as quercetin was found 0.068 % w/w. The test results showed that the anti-inflammatory activity of ethyl acetate fraction acid had anti-inflammatory activity of most good with a percentage of 62.79% inhibition of inflammation. While the percentage of inhibition of inflammation to other fractions were respectively as follows 20.38% for n-hexane fraction, 35.50% for ethyl acetate fraction bases and 30.08% for water fraction. From the resuts of this work, it is suggested to perform further research including effective doses and antimutagenic effect of mistletoes *Dendrophthoe pentandra* (L.) Miq for further clarifications the use of this plant for anti-cancer.

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