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Study on the anti-inflammatory properties of Karamunting (*Rhodomyrtustomentosa* (Aiton) Hassk.) leaf extracts

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Abstract. Karamunting (*Rhodomyrtustomentosa* (Aiton) Hassk.) is one of the most widespread plants in Sumaterawhich areknown to have traditionally used as wound and infection remedy. However, research on anti-inflammatory activity is limited. This study aimedto determine the effect of antiinflammatory of Karamunting (*Rhodomyrtus tomentosa* (Aiton) Hassk.) leaf extracts using acute rat model (carrageenan-induced rat paw oedema). Sodium diclofenac 4,5 $mg.kg^{-1}$BW as standard anti-inflammatory drug, n-hexane, ethyl acetate, and ethanol of Karamunting (*Rhodomyrtus tomentosa* (Aiton) Hassk.) leaf extracts each dose 200 $mg.kg^{-1}$BW were administered orally to 24 Wistar rats 1 h before induction oedema and compared with a negative control given Na CMC 1%. The mean percentage inhibition of paw volume of rats treated with sodium diclofenac was 64,82%, followed by n-hexane extract with 44,75%, ethyl acetat extract with 56,93%, and ethanol extract with 63,56%. All extract have similar antiinflammatory effect with sodium diclofenac ($p>0,05$). This result suggest that all Karamunting (*Rhodomyrtus tomentosa* (Aiton) Hassk.) leaf extracts have antiinflammatory properties comparable withstandard drugs.

1. Introduction

Inflammationis the response to a harmful stimulus. It can caused by mechanical, physical, or biological injury. The inflammatory response of the host is critical for interruption and resolution of the infectious process but also is often responsible for the sign and symptoms of disease.Inflammation may be acute or chronic depending on the disease course. Acute inflammation is characterized by heat, erythema, pain, swelling and loss of function. Chronic inflammation on the other hand results in a progressive shift in inflammatory cells characterized by simultaneous destruction and healing of the injured tissued.

Karamunting (*Rhodomyrtustomentosa* (Aiton) Hassk.) is one of the most widespread plants in Sumatera known to have been traditionally used as wound and infection remedy. Methanolic leaf extract of karamunting with dose 200 mg/KgBW contain 0,132% of total flavonoid have antiinflammatory activity [1]. Flavonoid can ihibit synthesis of $PGE_2$with block mechanism action ofcyclooxigenase and lipooxigenase enzymes [2,3]. Gradient extraction use different organic solvent will affect to type and amount of secondary metabolites.N-Hexane will extract non polar compounds such as lipid, sterol, coumarins and terpenoids. Ethyl acetate will extract semipolar compounds such as flavonoid and tannins.Ethanol will extract polar compounds such as glycosides and phenolics [4].

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Multistage extraction used for investigate the affect of polarity of solvent with antiinflammations activity.

2. Experimental Section

2.1. Materials and Chemicals
The material used in this study were karamunting leaves (*Rhodomyrtus tomentosa* (Aiton) Hassk.), 70% ethanol, 96% ethanol, ethyl acetate, n-hexane, aquadest, Na-CMC, diclofenac Na, carrageenan, magnesium metal (Mg), concentrated HCl, 10 NaOH %, 1% $FeCl_3$, chloroform, sand, ammonia in chloroform, concentrated sulfuric acid, 2 N sulfuric acid, anhydrous acetic acid, wagner reagent, mayer reagent, dragendorf reagent, and $AlCl_3$.

2.2. Test Animals Preparation
The test animals used were male Wistar (*Rattus novergicus*) white rats weighing between 200-250 g and age between 2-3 months. The animals were allowed 1 week to acclimatize before the commencement of the experiment. The animals were kept in room temperature and given enough food and drink.

2.3. Production of Karamunting Leaf Simplicia
Leaves of karamunting weighed 10 kg were obtained from the Indralaya campus of Sriwijaya University, South Sumatra. The samples are weighed, sorted and washed to separate the dirt attached. The leaves drying process is done by air drying under the sun and covered with a black cloth to avoid direct sun exposure. After dryed, the sorting back process is done to obtain simplicia with good quality. After that, the dried leaves are pulverized using a blender and weighed as a net weight.

2.4. Preparation of Karamunting Leaf Extract by Multistage Maceration
Simplicia powder (1 kg) was soaked with 4 L n-hexane in a glass container protected from sunlight for 2 x 24 hours. The macerated filtrate is put into container and stored. Remaseration was carried out on the simplicia powder residue using the same solvent as much as 4 liters for 2 x 24 hours. Maseration is repeated up to three times with the same solvent. The filtrate obtained was concentrated by evaporating the solvent using a rotary evaporator at a temperature of $50^{\circ}C$. Solvent replacement is carried out until a clear filtrate is obtained. After that, the simplicia powder pulp was macerated with ethyl acetate and ethanol in the same way. The filtrate obtained from each solvent was evaporated with a rotary evaporator so that a thick extract was obtained. Percent extract yield is calculated as follows:

$$\%Yield = \frac{weight\ of\ extract\ \ obtained}{weight\ of\ extracted\ \ simplicia\ \ powder} \times 100\% \quad (1)$$

2.5. Phytochemical Test of KaramuntingLeaf Simplicia

2.5.1. Alkaloids Identification. One g of sample was crushed in the mortar, a small amount of chloroform and sand were added, then 5 mL of 0,05 N ammonia solutions in chloroform was added. The mixture was shaken for several minutes, then filtered into the test tube. H_2SO_4 2N was added into the filtrate and shaken regularly, two layers are formed. The top solution (water phase) is separated and tested with Mayer, Wagner, and Dragendorff reagents. Sediment formed indicates the presence of alkaloid group compounds [4].

2.5.2. Flavonoids Identification. A total of 0,5 g of sample was put into the test tube, added 5 mL of ethanol, and heated for 5 mins. The extract then filtered and the filtrate was added a few drops of concentrated HCl. Next, added 0,2 mg of magnesium powder approximately. If it appears red, it shows the presence of flavonoid compounds [4,5].

2.5.3. Saponins Identification. A total of 500 mg sample was added into 10 mL of hot water. Then cooled and shake firmly. If there was a stable foam as high as 1 cm or more it showed the presence of

saponin group compounds. Furthermore, addition of 1 drop of HCl 2 N will not make the foam disappear [6].

2.5.4. Tannins Identification. A total of 500 mg of sample was added into 50 mL of distilled water, then boiled for 15 minutes and chilled. 5 mL of filtrate were taken and dripped with $FeCl_3$ 1%. If the color turned into greenish black, it shows the presence of tannin class compounds [7].

2.5.5. Identification of Steroids and Triterpenoids. A total of 2 g samples were crushed in mortar, a small amount of chloroform and sand was added, then added 5 mL of 0,05 N ammonia solutions into chloroform. The mixture was shaken for several minutes, then filtered into the test tube. H_2SO_4 2N was added into the filtrate and shaken regularly, leaving it to form two layers. The bottom solution was separated and dropped onto the drop plate, allowed to dry. After drying, anhydrous acetic acid was added and stirred evenly. Subsequently inserted 3 drops of concentrated sulfuric acid and observed the color that occurred. If the color was blue or green, then this indicates the presence of steroid compounds [4]. If the color was orange or purple, it shows the existence of triterpenoid group compounds [7].

2.6. Determination of Total Flavonoid Content

2.6.1. Determination of Maximum Wavelength. One mL of quercetin solution 20 ppm in methanol p.aadded 1 mL $AlCl_3$ 10% and 1 mL 5% acetic acid. The solution was allowed to stand for 30 minutes, then measure the absorbance using UV-Vis spectrophotometry at wavelengths of 200-600 nm. The maximum wavelength is chosen based on the wavelength that produces the highest absorbance.

2.6.2. Determination of Quercetin Standard Curves. A total of 10 mg quercetin was weighed and then dissolved in 10 mL of methanol p.a. to get 1000 ppm stock solution. The stock solutionwas diluted to 100 ppm and made in concentrations of 10, 20, 30, 40, and 50 ppm. A total of 1 mL of the solution from each concentration series was included, reacted with 1 mL of $AlCl_3$ 10% and 1 mL of 5% acetic acid. The test solution was allowed to stand for 30 minutes then absorbance was measured at λ 434 nm. The standard curve equation is obtained from linear regression between quercetin levels (X) and absorbance (Y).

2.6.3. Determination of Total Flavonoid. Each 1 mL of 1000 ppm extract solution was added with 1 mL of 10% $AlCl_3$ and 1 mL of 5% acetic acid. The test solution was allowed to stand for 30 minutes then absorbance was measured at λ 434 nm. The measurements were replicated three times. The absorbance measurement was compared to blank. The absorbance value obtained is included in the standard curve equation to determine the total flavonoid concentration in the extract. Calculation of total flavonoid levels in equation 2.

$$TFC = \frac{c \; x \; v \; x \; fp}{g} \quad (2)$$

Description :

TFC = Total Flavonoid content (mg/g)
c = concentration (µg/mL)
v = extract volume (mL)
fp = dilution factor
g = the weight of the sample used (mg)

2.6.4. Analysis of Sample Flavonoid Content. Concentration data series which made from quercetin standards, then made into standard curve equations. Equation of the standard curve y = a + bx with y = absorbance (nm), x = level in ppm (mg / L), a = constant, and b = regression coefficient. The absorbance of karamuntingleaf extract that has been obtained is put into the standard curve equation to get the total flavonoid content of karamunting leaves.

2.7. Test Design in Rats

Test animals, 24 male white rats were selected randomly, and then divided into 6 groups and 12 rats in 3 groups for testing of active extracts. Calculations based on Federer's (1991) formula are calculated by the equation 3[8]:

$$(n - 1)(t - 1) \geq 15 \quad (3)$$

Description: n = number of sample repetitions, t = number of treatment groups.

Based on calculations using the formula in Equation 2, the test animals used in this study were 4 rats per group. The test animals needed in this study were 36 male Wistar white rats, with details in Table 1.

Table 1. Anti-inflammatory Test Treatment Group

Group	Treatment
Normal	Sodium CMC 1% b/v p.o
Negative Control	Sodium CMC 1% b/v p.o + carrageenan 1% b/v s.p
Positive Control	Sodium diclofenac with dose 4,1 mg/KgBW p.o + carrageenan 1% b/v s.p
Group I	n-Hexane extr.with dose 200 mg/kgBW[1] p.o + carrageenan 1% b/v s.p
Group II	Ethyl acetate extr.with dose 200 mg/kgBW[1] p.o + carrageenan 1% b/v s.p
Group III	Ethanol extr.with dose 200 mg/kgBW[1] p.o + carrageenan 1% b/v s.p

2.7.1 Anti-inflammatory Test Procedure

Test animals were weighed and grouped randomly, namely normal groups, negative controls, positive controls, and test groups. Each rat was marked on the measurement boundary in the heel of the right rear leg of the rat. Normal group was given 1% sodium CMC, negative control was given sodium CMC 1% b/v in distilled water and positive control was given sodium diclofenac while the test groups 1 to 3 were given a test solution according to the predetermined concentration. Thirty minutes later, all the right back soles of the mice (except the normal group) were injected subplantarly with 0,2 mL of 1% b/v carrageenan in areas that had been cleaned with 70% ethanol. After thirty minutes of carrageenan injection, the foot volume of the rats was measured by dipping it into a pletismometer. Measurements are carried out every 30 minutes for 3 hours. All data are tabulated and the results of each group are averaged [9]. The percentage of inflammation and percentage of inflammatory inhibitionare calculated according to the following equation:

$$\% \text{ inflammation} = \frac{(Vt - Vo)}{Vo} \times 100\% \quad (4)$$

Description: Vt = Inflammatory volume at time t, Vo = Inflammatory volume before treatment

$$\% \text{ inflamatory inhibition} = \frac{(a - b)}{a} \times 100\% \quad (5)$$

Description: a = Average negative control group, b= Average test treatment group or comparative drug group

2.8. Data analysis

The results obtained from the percentage calculation of total flavonoid content and the treatment group inflammatory inhibition. The realationship between total flavonoid content and anti-inflammatory activity with differences in extraction solvents were analyzed by general linier multivariate model using SPSS® programme.

3. Result and Discussion

3.1. Extraction and phytochemical screening result

Karamunting (*Rhodomyrtustomentosa* (Aiton) Hasskleaf extract is carried out by multilevel maceration method. As much as 1 kg of karamuntingsimplicia powder was extracted using solvents of various polarity levels, namely n-hexane, ethyl acetate, then ethanol. The multilevel maceration

process was carried out by soaking the simplicia powder using a suitable solvent for replacement every 2 days and stirring several times was carried out at room temperature until the filtrate was clear. The extract was thickened with a rotary evaporator at 60^0 C. N-hexane, ethyl acetate, and ethanol extract of karamunting leaf (*Rhodomyrtustomentosa* (Aiton) Hassk.) had a yield of 1,66%, 1,98% and 4,46%respectively.The phytochemical screening method was chosen in identifying compounds because the process was simple and easy to do. The phytochemical test results on karamunting leaf extract are listed in Table 2.

Table 2. Phytochemical profiles of Karamunting leaf extracts (*Rhodomyrtustomentosa* (Aiton) Hassk.)

Compound Group	n-Hexane Extract	Ethyl Acetate Extract	Ethanol Extract
Flavonoids	-	+	+
Alkaloids	-	-	-
Triterpenoids	-	-	-
Steroids	+	+	-
Saponins	-	-	+
Tannins	-	-	+

Description: (+) identified, (-) unidentified

Based on Table 2, it can be seen that n-hexane extractof karamunting leaves (*Rhodomytrustomentosa* (Aiton) Hassk) only contains steroid compounds. Ethyl acetate extract of karamunting leaves (*Rhodomytrustomentosa* (Aiton) Hassk) contains flavonoids and steroids. Whereas the ethanol extract of karamunting leaves (*Rhodomytrustomentosa* (Aiton) Hassk) contains flavonoid, phenolic, saponin and tannin compounds. Ethanol extract was not detected by steroid compounds, this was because the compound was non-polar so it was not extracted from ethanol which was polar. Phenolic, tannin, and saponin are polar compounds, so this compound is not extracted in ethyl acetate and n-hexane solvents. Flavonoid compounds were found in ethanol and ethyl acetate extracts. Flavonoid compounds are polar compounds. In this study, flavonoids were not detected in n-hexane extract because according to the principle of like dissolve like, flavonoids were not detected in n-hexane solvents which tended to be non-polar.

3.2. Determination of Total Flavonoid Contents

The total flavonoid content test was carried out using the colorimetric method. The principle of testing flavonoids with the $AlCl_3$ method is based on the formation of complexes between $AlCl_3$ and the keto group on C-4 atoms and also with hydroxy groups on neighboring C-3 or C-4 atoms of flavones and flavonols. The results of quercetin absorbance measurements at various concentrations were used to make the standard curve expressed in the linear regression equation between the absorbance value (y) and quercetin concentration (x). The results obtained were y = 0,0145x – 0,1436 as seen in Figure 1. The results of the average calculation of total flavonoids levels are shown in Table 2.

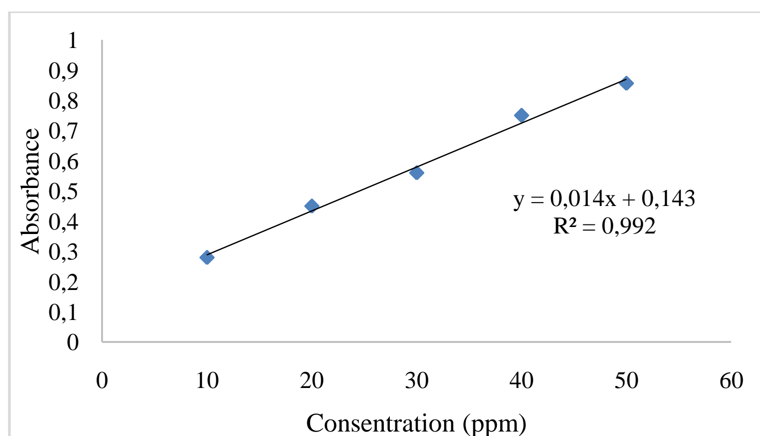

Figure 1. Graph of Quercetin calibration curve

Table 3. Total Flavonoids in various extracts

Extract type	Conncentration (mgQE/g)
n-Hexane	0,048
Ethyl acetat	5,131
Ethanol	15,062

Total flavonoid content was found more in ethanol extract compared to ethyl acetate extract. It is possible that there are many polar flavonoids such as flavonoid glycosides and flavonoid aglicon, such as cyanidin-3-galactoside, delphinidin-3-galactoside, pelargonidin-3,5-biglucoside, myricetin-3-O-α-L- furanoarabinoside [10], cyanidin-3-O-glucoside, peonidin-3-O-glucoside, malvinidin-3-O-glucoside, petunidin-3-O-glucoside, delphinidin-3-O-glucoside, pelargonidin-3-glucoside [11], quercetin, and myricetin [12]. Whereas flavonoids extracted in ethyl acetate include flavonoid aglycones that are semipolar like combretol [13].

3.3. Antiinflammatory activity of karamunting extract

The antiinflammatory activity test of karamunting leaf extract (Rhodomyrtus tomentosa (Aiton) Hassk) was carried out on male white wistar rats using carrageenan-induced rat paw edema method. This method was chosen because it is the most commonly used method in anti-inflammatory, inexpensive, and simple processing tests. The anti-inflammatory activity test was carried out by inducing 0,2 mL carrageenan 1% subplantarly on the palm of the right back leg of the rat (leading to the heel). Carrageenan will induce inflammation without leaving a mark, does not cause tissue damage and provides a response that is more sensitive to anti-inflammatory drugs than other irritant compounds [14]. Percent inhibition of rat leg inflammation was calculated based on edema volume that had been measured using a pletismometer.

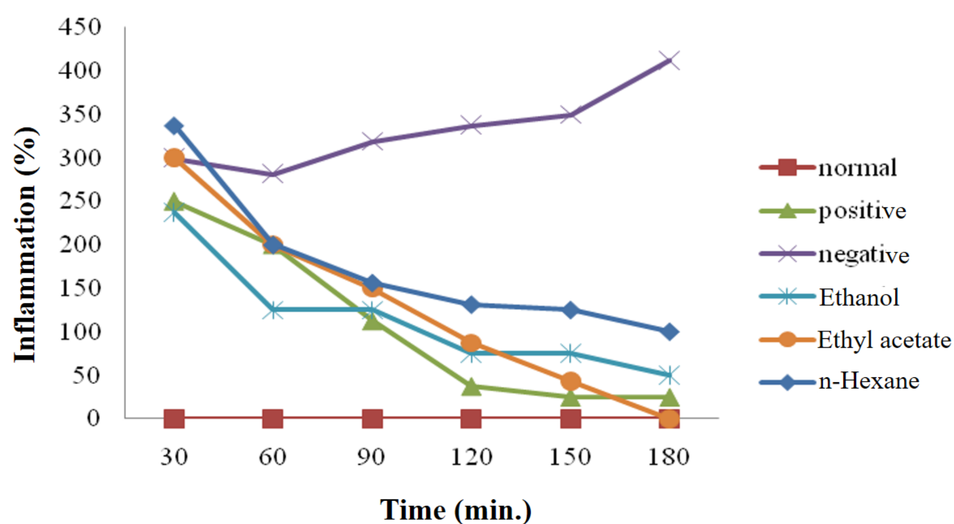

Figure 2. Graph of the relationship of the average of the various treatment groups.

Based on Figure 2, it can be seen that 0,2 mL of 1% carrageenan injected into the back of the right foot of the rat can cause inflammation. Normal groups do not have a percent inflammation value because only 1% sodium CMC is given and carrageenan is not injected. The negative control group has the highest percentage of inflammation because this group is not given drugs to inhibit inflammation. The decrease in the percentage of inflammation in the positive control group and the extract treatment group occurred in the 60th minute. A decrease in the percentage of inflammation occurs with increasing time. This proves that the sodium Diclofenac drug and karamunting leaf extract begin to work in inhibiting inflammation. Onthe 60th minute can be seen that the percentage of

inflammation in the positive control group is greater than the ethanol extract treatment group, but the next minute the percentage of inflammation in the positive control group is smaller than the other extract treatment groups. This is thought to occur because in the 60th minute the positive control group had not worked optimally in inhibiting inflammation. The n-hexane treatment group had a greater percentage of inflammation than the ethanol and ethyl acetate treatment group. The percentage of inflammation in the ethanol and ethyl acetate treatment groups is almost equivalent to the percentage of inflammation in the positive control treatment group.

Table 4. Percent inhibition of rat inflammation in various treatment groups

Time(min.)	Percentage of Inflammatory Inhibition of Rat Paw Oedeme(%)					
	Normal	Negative	Positive	Ethanol	Ethyl Acetate	n-heksane
0	0	0	0	0	0	0
30	100	0	16,67	20,83	0	-12,5
60	100	0	28,87	55,55	28	28,88
90	100	0	67,7	60,78	52	50,98
120	100	0	88,88	77,78	74,07	61,11
150	100	0	92,85	78,57	87,50	64,28
180	100	0	93,93	87,87	100	75,75
Average	100	0	64,82	63,56	56,93	44,75

Based on the data in table 4. the negative control treatment group did not have a percentage of inflammatory inhibition because this group was not given drugs to inhibit inflammation. Percentage of inflammatory inhibition from the positive control group and the extract treatment group showed that karamunting leaf extract (Rhodomyrtus tomentosa (Aiton) Hassk.) and diclofenac Na can inhibit inflammation induced by carrageenan. The greater percentage of inhibition indicates the potential for a substance to be anti-inflammatory. The positive control group, and the treatment group of n-hexane, ethyl acetate, and ethanol extract produced a percentage inhibition of inflammation of 64,82%, 44,75%, 56,93% and 63,56%, respectively. Data of total flavonoid content (TFC) and antiinflammation activity were analyzed by SPSS programme with general linier multivariate model that shows at table 5 and table 6.

Table 5. Levene's test of Equality of errorvariances[a]

	F	df1	df2	Sig.
TFC	16,000	2	6	,004
Inflammation_Inhibition	5,567	2	6	,043

[a.]Design : Intercept + Extract

Table 6. Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	TFC	349,801[a]	2	174,901	330625,204	,000
	Inflammation_Inhibition	535,277[b]	2	267,639	2098,030	,000
Intercept	TFC	409,779	1	409,779	774629,582	,000
	Inflammation_Inhibition	26981,348	1	26981,348	211507,820	,000
Extract	TFC	349,801	2	174,901	330625,204	,000
	Inflammation_Inhibition	535,277	2	267,639	2098,030	,000
Error	TFC	,003	6	,001		
	Inflammation_Inhibition	,765	6	,128		
Total	TFC	759,584	9			
	Inflammation_Inhibition		9			
Corrected Total	TFC	349,805	8			
	Inflammation_Inhibition	536,043	8			

[a.] R Squared = 1,000 (Adjusted R Squared = 1,000)
[b.] R Squared = ,999 (Adjusted R Squared = ,998)

The results shows that the difference in solvent extraction significantly affected total levels of flavonoids and their antiinflammatory activity. Based on the percentage of inflammatory inhibition, it can be seen that the ethanol treatment group has a greater percentage inhibition of inflammation when compared to the treatment group of ethyl acetate and n-hexane extracts. This data is directly proportional to the total flavonoid content in each extract. Greater the level of flavonoids in the extract, greater the inhibition value of inflammation of carrageenan-induced rata. Flavonoid compounds have the ability to block the cyclooxygenase and lipooxigenase enzymes of arachidonic acid so that the synthesis of PGE_2 is inhibited [15].

4. Conclusion

Flavonoid levels in every Karamunting (Rhodomyrtus tomentosa (Aiton) Hassk.) leaf extract affect in vivo antiinflammatory activity. Greater level of flavonoid in the extract shows greater antiinflammatory activity.Karamunting (Rhodomyrtus tomentosa (Aiton) Hassk.) ethanolic leaf extract have the greatest total flavonoid content and antiinflammation effect.

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