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RESEARCH ARTICLE
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Determination of Flavonoid Levels and Anti-Inflammatory Activity of Lime Root Extract (*Citrus aurantifolia*) by Granuloma Pouch Method

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Abstract:

Non-communicable diseases (NCDs) are still the leading cause of death at the global level. In Indonesia, NCDs contribute 71% of the total mortality rate. One of the causes of non-communicable diseases is the occurrence of oxidative stress in the body which is a condition of imbalance between free radical compounds and antioxidants. One of the sources of free radical formation in the body is when the inflammatory process occurs. Lime is used by the Indonesian people as a seasoning for cooking or empirically as an appetite enhancer, antipyretic and antibacterial. Research states that lime root extract has very strong antioxidant activity with an IC50 value of 24.81 ppm and positively contains alkaloids, flavonoids, saponins and tannins. Flavonoids are thought to act as antioxidants and anti-inflammatory through their ability to donate hydrogen atoms and the ability to bind prostaglandins, COX-1 and COX-2 and lipooxygenase enzymes. The very strong antioxidant activity and the presence of flavonoids contained make lime root have the potential as an anti-inflammatory. Determination of flavonoid levels of lime root extract using UV-Vis spectrophotometry method with quercetin as a comparison and testing of anti-inflammatory activity of lime root extract using the Granuloma Pouch method using mouse test animals. The results showed that the total flavonoid level was 36.8 mg QE (Quersetin Equialen)/g or 3.68%. And itexerts an anti-inflammatory effect through its ability to reduce the volume of exudate, lower the total exudate leukocytes, and lower the number of neutrophils in the exudate, most notably at a concentration of lime root extract of 20%

Keywords: Anti-inflammatory; Flavonoids; Lime; Granuloma

Introduction

Non-communicable diseases (NCDs) are still the leading cause of death at the global level. In Indonesia, NCDs contribute 71% of the total mortality rate. One of the causes of non-communicable diseases is the occurrence of oxidative stress in the body which is a condition of imbalance between free radical compounds and antioxidants. One of the sources of the formation of free radicals in the body is when the inflammatory process occurs[1].

Lime is used by the Indonesian people as a seasoning for cooking or empirically as an appetite enhancer, antipyretic and antibacterial. Research stated that lime root extract has very strong antioxidant activity with an IC50 value of 24.81 ppm and positively contains alkaloids, flavonoids, saponins and tannins[2]. Flavonoids are thought to play an antioxidant and anti-inflammatory role through their ability to donate hydrogen atoms and their ability to bind prostaglandins, COX-1 and COX-2, and lipooxygenaseenzyme [3].

The very strong antioxidant activity and the presence of flavonoids contained make lime root have the potential as an anti-inflammatory. Based on the description above, the urgency of the study is that it is necessary to conduct research to determine the level of flavonoids by the UV-Vis spectrophotometry method and the anti-inflammatory effect of lime root extract given topically on the skin of mice's backs using the *Granuloma method.*

Research Method

Sample preparation

The lime root (*Citrus aurantifolia*) that has been taken is then sorted wet, washed thoroughly, then chopped and dried until dried simplicial is obtained[4]. Dried simplicia is then extracted using the maceration method so that a thick extractis obtained [5]

Tools and Materials

The tools used in the study were Rotary evaporators, analytical scales, becker glasses, maceration bottles, erlenmayers, funnels, petri cups, micropipettes, Uv-Vis spectrophotometry, cuvettes, shavers, syringes, wood shavings. The materials used in this study were Akar jeruk nipis, etanol 96%, aquadest, kertas saring, kapas, aluminium foil, label, mencit, Serbuk Mg, HCl pekat, kuersetin, karagenan

Determination of flavonoid levels

Preparation of quercetin parent raw solution 100 ppm 10 mg of quercetin is dissolved with 96% ethanol, put in a 100 ml measuring flask. Determination of the maximum wavelength of quercetin stock 100 ppm is taken 1 ml, add 1 ml of 10% AlCl3 and 8 mL of 5% acetic acid. Determine the maximum wavelength at a wavelength of 370-450 nm.

Stock 100 ppm is taken 1 ml, add 1 ml of 10% AlCl3 and 8 ml of 5% acetic acid. Absorbance is determined by the wavelength obtained. With a time interval of 2 minutes until stable absorbance is obtained[6]. Manufacture of quercetin standard curves take 100 ppm of 2 ml of stock; 4 ml; 6 ml; 8 ml is diluted with 96% ethanol in a 10 ml flask so that the concentrations obtained are 20 ppm, 40 ppm, 60 ppm, and 80 ppm respectively. Each pipette 1 ml, add 1 ml of 10% AlCl3 and 8 ml of 5% acetic acid to sit during the operating time. Measure the absorption.

Preparation of lime root extract raw solution: 25 mg of lime root extract dissolved in 96% ethanol, put in a 100 ml measuring flask. A 1 ml pipette is added 1 ml of 10% AlCl3 and 8 ml of 5% acetic acid, let stand during

the operating time. Then measure its absorption at the maximum wavelength obtained

Anti-Inflammatory Activity Test

The grouping of test animals into 5 groups, each group consists of 3 test animals. Groups one and two are negative and positive control groups, groups 2, 3 and 4 are treatment groups that are given 3 test concentrations of 5%, 10% and 20% concentrations, then, 400 mg of caragen was grinded add 20 ml of physiological NaCl little by little while being grinded homogeneously, the result of the 2% caragen concentration obtained was left for 24 hours[7].

Preparation of lime root extract concentration Lime root extract 250 mg; 500 mg and 1,000 mg and added vaseline abu so that concentrations were obtained of 5%, 10% and 20% respectively. The test preparation was given topically as much as 0.2 grams of each treatment given immediately after being injected with a caragen of 0.5 ml subcutaneously.

Test preparations were given for 4 days in each group where: Negative control (K-) is given an album Vaseline, Positive control (K+) given hydrocortisone acetate 2.5%, Treatment group 1 (F1) was given lime root extract 2.5%, Treatment group 2 (F2) was given 5% lime root extract, Treatment group 3 (F3) was given 20% lime root extract.

Induction of Inflammation

The test animals were first trimmed with scissors, then applied Veet® to shed the fur that had not been completely shaved. The skin on the back of the mice that has been shaved is left for 24 hours. The goal is to avoid inflammation caused by shaving and giving Veet®. On the first day of testing, the shaved back was injected with 5 ml of air subcutaneously so that air sacs were formed and at the same time 0.1 ml of carrageen. After 24 hours, the air sac is sucked in with a 5 ml syringe so that the air sac becomes deflated. Then add 0.5 ml of 2% caragen solution to the place where the air bag is. The test preparation is given by applying evenly to the area where the air sac is formed, immediately after the administration of 0.5 ml of carogen. The test preparation was given as much as 0.2 grams for each mouse and the administration of the test preparation was given for 4 days once a day.

Parameter Measurement

Measurement of inflammatory volume is done by taking exudate with a syringe (syringe) and then measuring its volume. Exudate collection is carried out on day 4. The calculation of the percentage of leukocyte cells is carried out by taking one drop of mouse exudate placed on the object glass and flattened with a cover glass so that a smear preparate is obtained, then dried. Once dry, drip with methanol, so that it coats the entire exudate eraser, leave it on for 5 minutes. Then add 1 drop of giemsa's solution and leave for 20 minutes. Wash with distilled water, dry and look under a microscope with 1000x magnification. Count the number of neutrophil cells: the exudate is aspirated with a leukocyte thoma pipette to the 0.5 mark on the pipette, then aspirated with a diluent solution (NaCl 0.9%) to the 11 mark (1:20 diluent) on the thoma pipette. The leukocyte thoma pipette is held in such a way that the two tips of the pipette are located between the thumb and index finger of the right hand and then homogenized for 3 minutes. Before filling the counting chamber, the first 4 drops of exudate are discarded and the tip of the pipette is placed in the counting chamber (*improved neubauer*) right at the limit of the cover *glass*. The exudate is filled into the counting chamber on the 5th drop. The counting chamber

after being filled with exudate is left for 3 minutes and then the total number of leukocytes is calculated on a microscope with a magnification of 40 x 10. The total number of leukocytes is determined by the following formula

Results

The result of the maximum wavelength of quercetin in the range of 370-450 nm was obtained at 428 nm with an absorbance of 0.782. The result *of the operating time* was obtained at the 36th minute. The results of the measurement of the absorbance of the quaercetin standard curve solution are as seen in Table 1.

Table 1. Results of Measurement of Absorbance of Quercetin Raw Curve Solution

Concentration (ppm)	Absorbansi			
	Replicatio 1	Replication 2	Replication 3	Average
0	0	0	0	0
20	0,077	0,079	0,079	0,078
40	0,448	0,450	0,450	0,449
60	0,512	0,512	0,512	0,512
80	0,745	0,743	0,743	0,744
100	1,041	1,041	1,041	1,041

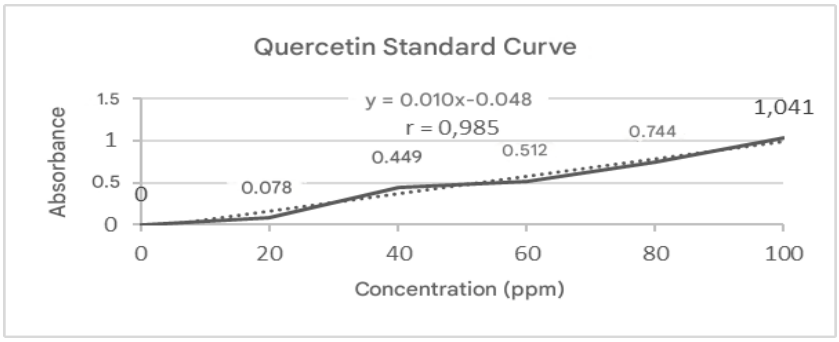

Figure 1. Standard Quartz Standard Curve Graph

Table 2. Determination of Absorbance of Lime Root Extract

Replication	Absorbance	Average
1	0,045	
2	0,048	0,044
3	0,033	

The determination of total Flavonoid levels of lime root extract as seen in Table 3.

Table 3. Determination of Total Flavonoid Levels of Lime Root Extract

and (absorbance)	b	x (mg/L)	a	C (mg/L)	Volume (L)	M (g)	Total Flavonoids Up	
							Mg QE/g	%
0,044	0,010	9,2	-0,048	9,2	0,1	0,025	36,8	3,68

Anti-Inflammatory Activity Test

The average measurement results of exudate volume of mice can be seen ini Table 4.

Table 4. Average Measurement Results of Exudate Volume of Mice

Group	Volume (mL)	Inhibition of Exudate Formation (%)
Towards-	0.72 ± 0.01	
F1	0.68 ± 0.00	5,55
F2	0.51 ± 0.01	29,17
F3	0.32 ± 0.01	55,50
K+	0.29 ± 0.00	59,72

Table 5. Average Total Leukocytes and Percentage of Neutrophils

Group	Total leukosit (/µl)	Neutrophils (%)
K-	17,117 ± 701	8.7 ± 0.58
F1	16,733 ± 709	14.3 ± 1.15
F2	15,267 ± 503	14.3 ± 1.53
F3	14,267 ± 681	13.5 ± 6.36
K+	12.017 ± 711	11 ± Feb 2.65

Based on the data in table 4, it can be seen that in the negative control group (only given ointment without active substances), it can be seen that the average exudate volume is greater than that of the positive control group that is given ointment preparations containing the active substance hydrocortisone. Meanwhile, in the test group, when compared to the negative control group, it was shown that the three variations in the concentration of the test preparation were able to reduce the volume of exudate in mice. The largest decrease in the average volume of exudate was in the concentration of ointment-based extract with a concentration of 20% ointment-based lime root extract, which was 0.32 ml. Based on the percentage of inhibition of exudate formation for the test preparation, the highest result was shown by the ointment preparation with 20% ointment-based lime root extract which was 55.5%. The anti-inflammatory power of a substance can be seen if in test animals induced by 2% carrageen, swelling (% inhibition) is up to 50% or more[7].

Based on the data of table 5 of the results of the observation of total leukocytes in the exudate, there was a difference between the positive control group, the negative control group and the test group. In the negative control group, the average total leukocytes were larger than that of the positive control group. Meanwhile, in the test group, when compared to the positive and negative control groups, it showed that the three variations in the concentration of the test preparation were able to reduce total leukocytes.

Discussion

Quantitative analysis of lime root flavonoid levels (*Citrus aurantifolia)* performed using UV-Vis Spectrophotometry. UV-Vis spectrophotometry is used for the analysis of compounds in the form of colored solutions or compounds in the form of solutions that can be reacted until color is formed. Quercetin is used as a benchmark because it is a type of flavonoid flavonoid that has a keto group in the C-4 atom and also a hydroxyl group in the neighboring C-3 and C-5 atoms[10]. In the determination of flavonoid levels, a reaction occurs between aluminum chloride reagents ($AlCl_3$) added with a keto group on the C-4 atom and a hydroxyl group on the C-3 or C-5 atom on the flavonoid, the reaction that occurs produces a complex compound that is characterized by a yellow color change due to a shift in wavelength towards visible light[11]. The determination of the maximum wave is the wavelength produced by a compound at maximum absorption, aiming to find out the wavelength when it reaches maximum absorption, in addition to having a relatively constant absorption power. The determination of the maximum wavelength of quercetin was

carried out by reading the absorption of the standard raw solution of quercetin of 100 ppm in the wavelength range of 370-450 nm[6]. The result obtained was 428 nm.

Determination operating time aims to determine the stable measurement time, which is when the sample reacts perfectly and forms complex compounds. *Operating time* It was carried out using a standard quaercetin 100 ppm raw solution with a time interval of 2 minutes for 60 minutes6. The results were obtained in the 36th minute to the 50th minute. In this time range, the absorption of measured compounds is relatively more stable. This absorbance stability indicates that the formation reaction is optimal. From the measurement *operating time* stable absorbance was obtained in the 36th minute.

The determination of the quercetin standard curve was carried out by measuring the absorbance of the quercetin standard raw solution with various concentrations, namely 20 ppm, 40 ppm, 60 ppm, 80 ppm, 100 ppm which was then left for 36 minutes and measured with a wavelength of 428 nm. The determination of the standard curve of quercetin was carried out with the aim of obtaining a linear regression equation that will be used to determine the level of the sample. The results of absorbance measurement showed that the concentration was directly proportional to the absorbance value obtained, namely the higher the concentration, the higher the absorbance value obtained[6]. Meanwhile, in the linear regression equation, the standard quercetin curve obtained is $y = 0.010x – 0.048$ with a correlation coefficient (r) value of 0.985. The value (r) obtained close to the number 1 indicates that the regression equation is linear, so it can be said that absorbance and concentration have a very strong correlation[6]. The determination of total flavonoid levels in lime root (*Citrus aurantifolia*) was carried out using UV-Vis Spectrophotometry with quercetin raw solution. In order to obtain more accurate data, the test was carried out three replications. In the determination of the flavonoid level of the sample solution, AlCl3 is added, so there is a complex formation between flavonoids and AlCl3, causing a shift in wavelength towards visible light which is characterized by the presence of yellow in the solution. The formation of complex compounds occurs when AlCl3 reacts with a keto group at C-4 and a hydroxyl group at C-3 or C-5 at a flavonoid compound[12]. The addition of acetic acid serves to maintain the wavelength in the visible (visible)[6]

Observations made on the concentration group showed that increasing the concentration of lime root extract could have a total leukocyte reduction effect. The largest decrease in total leukocytes in exudate was in the concentration of preparations with a concentration of lime root extract of 20%, which was 14,267/µl. If inflammation is reduced, then the number of leukocyte cells migrating to the inflamed area will also decrease. This is seen in the number of neutrophils that are less than the negative control.

From the results of the research that has been carried out, it turns out that lime root extract provides an anti-inflammatory effect through its ability to inhibit and reduce exudate volume, reduce total exudate leukocytes, and reduce the number of neutrophils in exudate, so it can be concluded that lime root extract has anti-inflammatory activity. With the increase in the concentration of the extract, the anti-inflammatory activity also increases.

The anti-inflammatory activity is caused by flavonoids contained in lime root extract of 36.8 mg QE *(Quersetin Equialen)*/g or 3.68 %. Where flavonoids

themselves can inhibit the COX-1 and COX-2 pathway prostaglandins, and lipooxygenase enzymes. Flavonoid compounds are able to inhibit the enzymes cyclooxygenase and lipooxygenase, which will cause inhibition of biosynthesis of inflammatory mediators, especially prostaglandins and leukotriene which are the final products of the cyclooxygenase and lyoxygenase pathways, the inhibition of these enzymes can reduce the production of prostaglandin vasodilators so as to decrease vasodilation, then reduce the edema that occurs and the accumulation of inflammatory cells can be reduced.[7]

Neutrophil cells are the first polymorphonulear white blood cells (PMNs) to enter the area of inflammation. Neutrophils have receptors for C3b that act as opsonins (compounds that can attach to the surface of microorganisms[7]. Neutrophils are also the first line of cells that defend in the event of acute inflammation in cell[13].

Based on this, lime root extract is able to provide a restorative effect on inflammatory conditions by lowering neutrophil cells which are the main cells in acute inflammation in a non-specific manner.

Conclusions

Based on data from the study of lime root extract (*Citrus aurantifolia)* obtained the total flavonoid content was 36.8 mg QE (*Quersetin Equialen*)/g or 3.68%. Provides anti-inflammatory effects through its ability to reduce the volume of exudate, lower total exudate leukocytes, and reduce the number of neutrophils in exudate, most notably at a concentration of lime root extract of 20%

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