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Topical anti-inflammatory effect of Ekor Naga (*Rhaphidophora pinnata* (L.f) Schott*)* leaves extract

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ABSTRACT

Ekor Naga (*Rhaphidophora pinnata* (L.f) Schott) leaves are leaves that contain secondary metabolites which can be developed into medicine. Ekor Naga leaves have secondary metabolite compounds of flavonoids, alkaloids, triterpenoid saponins, steroids, tannins, and phenols. This metabolite compound is the basis for testing the anti-inflammatory effect of Ekor Naga leaves extract using topical methods. The purpose of this study was to determine the anti-inflammatory effect of the ethanol extract of Ekor Naga leaves by a topical method. This study used five treatment groups with five mice in each treatment. This research tested the anti-inflammatory activity of Ekor Naga leaves extract by using the combination of 2 methods; namely the method of forming airbags and the formation of artificial edema using the induction of 2% carrageenan solution with the observation parameters being the measurement of exudate volume and differentiation of the number of leukocyte cells observed under a microscope. The results showed that the Ekor Naga leaves extract had an anti-inflammatory effect. The best inflammatory effect is a concentration of 10%, followed by a concentration of 5% and 2.5%.

Keyword: *Ekor Naga leaves extract, anti-inflammatory, leucocyte cells, topical*

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INTRODUCTION

Inflammation is an initial response to the body caused by stimulation from chemical, physical, or foreign microorganism infections that can cause tissue damage. The stimulation of inflammatory mediators such as histamine, serotonin, bradykinin, prostaglandin released in the body can cause vasodilation and an increase in blood vessel permeability (Kumar et al., 2018; Stankov, 2012). The commonly used anti-inflammatory therapies are anti-inflammatory steroids and non-steroidal anti-inflammatory that works by suppressing and reducing inflammation. Anti-inflammatory drugs used in the long term have some side effects that can reduce the function of organs like kidneys, liver, digestive system, and heart. To reduce the side effects of using anti-inflammatory drugs, it is necessary to seek traditional medicine as an alternative to inflammatory therapy that can reduce pain and inflammation with fewer side effects and toxicity (Sukmawati et al., 2015).

Traditional medicine is the non-conventional treatment aimed at improving community health status, including promotive, preventive, curative, and rehabilitative. Ekor Naga leaves (*Rhaphidophora pinnata* (L.f) Schott) is one of the traditional plants that is often used by the public as a cure for cancer, tumors, rheumatism, coughs, and clean dirty blood. Other than that, Ekor Naga leaves contain flavonoids, saponins, tannins, alkaloids, glycosides, and steroids/triterpenoids (Sumaiyah et al., 2018). Ekor Naga leaves also have several supporting research results that can support the anti-inflammatory test. This research was supported by the result of research done by Rahman and Andi (2019), which states that Ekor Naga leave has an effect as a wound healing. It is where one of the phases in wound healing is the inflammatory phase. In the late inflammatory phase of wound repair, T-Lymphocytes appear in the wound bed and may influence the resolution and remodeling of the wound. As inflammation resolves and the number of leucocytes diminishes, the wound undergoes a lengthy period of remodeling and resolution. Although inflammation is not prominent during this resolution phase, many studies suggest that the event of the inflammatory phase has profound effects on the final wound outcome (Koh and DiPietro, 2011). This is because Ekor Naga leaves contain flavonoids that function like *apigenin-8-C-B-D- glucopyranoside (Vitexin)* and several types of phenolic that inhibit cell proliferation and affect cell proliferation repair, and reducing many leukocytes. Meanwhile, the steroid compounds in Ekor Naga leaves can inhibit the phospholipase A2 enzyme in synthesizing arachidonic acid in the formation of inflammatory mediators. Saponin compounds in Ekor Naga leaves can increase the interleukin factor (Borgi et al., 2008; Deli, 2007).

Topical treatment provides local treatment by applying it to the skin surface, where the drug must be able to penetrate through the barrier to its place of action. The drug will have an effect depending on the level of the drug that reaches its workplace. The advantages of topical drug administration are that the drug can work locally, avoid the first-pass metabolism, use on irritated and inflamed skin (Cho and Bashaw, 2011).

Based on the data above, the researchers felt the need to make research on the anti-inflammatory activity test of Ekor Naga leaves extract (*Rhaphidophora pinnata* (Lf) Schott) in male white mice smeared on the skin of the mice's back, using a combination method of forming air sacs and artificial edema in rat's back which induces a subcutaneous carrageenan solution. The results obtained can provide additional information about the benefits of using Ekor Naga leaves extract as a natural medicine with anti-inflammatory properties.

MATERIALS AND METHOD

Materials

Fresh Ekor Naga leaves (*Rhaphidophora pinnata* (L.f) Shcott) was taken from Sungai Penuh Kerinci City, Jambi Province in January 2020 Hydrocortisone (PT. Kimia Farma Tbk), carrageenan (CV. Genera Labora), and Vaseline flavum (PT. Brataco), 70% ethanol (PT. Brataco); aquadest; Mayer, Wagner, and Dragendorf reagents.

Method

Simplicia preparation

The Ekor Naga leaves were collected then sorted. The leaves of the petioles were separated then washed with running water three times to remove impurities that were still attached to the leaves. Furthermore the Ekor Naga leaves were chopped into small pieces to 1-2 cm in size to speed up the drying process. The wind method played an important role in drying the Ekor naga leaves. The researcher then weighed the dry sample until it reached the constant weight and ground it to be pollinated.

Preparation of Ekor Naga leaves extract

Extraction of the Ekor Naga leaves was done using the 70% ethanol solvent maceration method. The container was the place to put simplicia powder; next, a 70% ethanol solvent was added until the powder was submerged. Next, it was covered and left for five days, must be protected from the light; it had to be stirred every day. The mixture was filtered by a funnel covered with filter paper. The dregs were macerated by an ethanol 70%, and it was left for two days. The next step was filtering again until the solvent obtained maceration. The macerate was concentrated by a rotary evaporator at a temperature which not exceed 400C until the thick extract was produced.

Anti-inflammatory activity test

The research was conducted using male white mice Swiss Webster with a bodyweight of 20 - 30 grams. All mice were purchased from Galih Farm; the address was in Kota Jambi, Jambi Province. Previously all mice had also been examined by drh. Rospita Pane (from the agriculture and food defense department with a certificate number 524.3/35/DPKP/SKKH/2020). This research had also passed an ethical review with a certificate number 352/UN.16.2/Kep-FK/2020 by the ethics committee team of Andalas University Faculty of Medicine. This research had conducted using five treatments, namely :

Negative Control	=	Mice were given hydrocortisone acetate 2.5%
C1	=	Mice were given extract of Ekor Naga leave 2.5%
C2	=	Mice were given extract of Ekor Naga leave 5%
C3	=	Mice were given extract of Ekor Naga leave 10%

The induction of inflammation on mice refers to the study of Aria et al. (2015), where the back of the mice was shaved with a diameter of three cm and followed by the application of hair removal cream to completely remove the remaining hair and left for twenty-four hours before testing. On the first day of testing, the shaved back was injected with 5 ml of air subcutaneously on the back to form an air sac. On the third day, 3 ml of air was re-injected subcutaneously. Then on the fourth day after air injection, 0.5 ml of 2% carrageenan solution was injected into the airbag to produce an inflammatory response.

The anti-inflammatory test refers to Anilkumar et al. (2017). On the fourth day of testing, after being injected with a 2% carrageenan solution in the treated mice, 0.1 gr of Ekor Naga leaves extract was applied evenly, vaseline flavum in the negative control group, and hydrocortisone acetate 2.5% in the control group positive. The extract was applied on the 4th, 5th, 6th, and 7th days after injection of the 1% carrageenan solution.

Exudate volume measurement

Exudate volume measurement refers to the research done by Dillasamola et al. (2016). On day eight, the mice were sacrificed by cervical dislocation. Furthermore, the air sac tissue on the skin of the mice's back was dissected and split open to suck up the exudate volume using a syringe and measured using a measuring cup. The inflammation inhibition calculation refers to the average inflammation inhibition calculated by the formula:

$$\% \text{ Inflammation inhition} = \frac{a-b}{a} x\ 100\%$$

a = Negative control group average inflammation volume (ml)
b = An average inflammation volume of the test group or control drug treatment group (mL)

Leucocyte counting

On the eighth day, the blood of the mice's tail was taken, dripped into an object-glass, and made smear preparations. After drying, the smears were fixed using methanol for 3-5 minutes. Next, after drying, color with Giemsa dye for 10 minutes and rinse with aquadest. Then the preparations were given immersion oil. Leucocyte was examined using a microscope with a magnification of 100 times counting each leukocyte. The cells were counted at least 100 cells and calculated the percentage of leukocyte types. If the calculation had reached 100 cells before reaching zone IV, then the calculation was continued so that the number of cells exceeded 100. The relative value of each leukocyte was expressed in percentage. The relative value was obtained by dividing the number of leukocytes in one type of leukocyte by 100, then multiplied by 100% (Hamghalam and Ayatollahi, 2009).

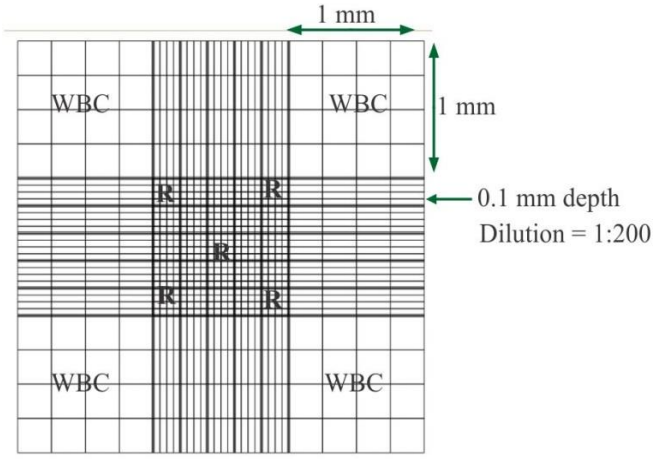

Figure 1. Leucocyte cells counting in the chamber

Data Analysis

The data obtained were analyzed using a one-way ANOVA with a 95% confidence level followed by the Duncan test.

RESULT AND DISCUSSION

The simplicia of Ekor Naga leaves was carried out wet sorting and then dried to air in the open air at room temperature. The purpose of drying fresh leaves is to reduce the water content to prevent rot and the growth of microorganisms while stopping enzymatic reactions and chemical changes. The parameter that shows the leaves have dried completely is that the leaves can be mashed. The result of Ekor Naga leaves simplicia obtained was 25.6%. The purpose of determining the simplicia yield, according to Nuralifah et al. (2018), is to determine the approximate amount of simplicia needed in making thick extracts. Meanwhile, the thick yield of Ekor Naga leaves extract was 12.71%.

The specific parameter tested on the Ekor Naga leaves was the organoleptic test. According to Syukri et al. (2020), determining the specific parameters of the extract aims to conduct a subjective initial introduction to the extract using the senses. The results obtained from the Ekor Naga leaves extract is a thick greenish brown extract, has a chaste taste and distinctive smell. The results of the phytochemical screening test, the data obtained can be seen in Table 1.

Table 1. Phytochemical screening test results

Number	Testing	Information
1.	Alkaloid Test	
	a. Mayer	+
	b. Dragendorff	+
2.	Flavonoid Test	+
3.	Saponin Test	+
4.	Tannin Test	+
5.	Steroid/Triterpenoid Test	+
6.	Fenol Test	+

Notes Information : (+) : contains a group of compounds

The results of the qualitative phytochemical screening test showed that Ekor Naga leaves extract contained alkaloids, flavonoids, saponins, tannins, steroids/triterpenoids, and the phenol test showed positive results. This result is in line with the results of research by Masfria et al. (2014). The experimental results of the average exudate volume obtained can be seen in Table 2.

Table 2. Mean exudate volume ± SD and percentage of inhition

Treatment Group	Exudate Volume (ml)	Percentage of Inhibition (%)
Positive Control	0.006 ± 0.01^{a}	93.47
Negative Control	0.092 ± 0.02^{e}	-
C1	0.071 ± 0.01^{d}	22.82
C2	0.047 ± 0.02^{c}	48.91
C3	0.018 ± 0.01^{b}	80.43

Notes
a. The significance value was determined by one-way ANOVA analysis with a 95% confidence level.
b. Different lowercase superscripts on the same line indicated a significant difference ($P < 0.05$).

Reduction of exudate fluid occurs due to the reduction of damaging membrane lipids, this mechanism occurs through the production of inflammatory mediators such as histamine and prostaglandins so that the formation of exudate fluid can be inhibited and reduced (Abdulkhaleq et al., 2018). In this study, the inducer used was carrageenan. Carrageenan is a mucopolysaccharide obtained from Irish seaweed (Chondrus crispus). Carrageenan is useful in the formation of acute edema (Singh et al., 2008). Where carrageenan functions as a foreign substance (antigen) which when it enters the body will stimulate the release of inflammatory mediators such as histamine so that inflammation occurs due to antibodies that react to these antigens to counter their effects (Necas and Bartosikova, 2013). According to Lee et al. (2015), saponin compounds can inhibit the release of pro-inflammatory substances stimulated by LPS, such as iNOS, IL, and TNF-α, so they can inhibit the formation of fluid exudates and inhibit the permeability of the vascular system. In addition, flavonoids can inhibit the secretion of lysosomes that cause profeliration and exudation so that by reducing lysosome secretion, exudate formation can be inhibited (Abdulkhaleq et al., 2018). The result of the different types of leukocytes measurements can be found in Table 3.

Table 3. The average ± SD number of leukocytes in the Ekor Naga leaves activity test

	Average Number of Leukocyte Cells			
	Stem Neutrophils	**Segmented Neutrophils**	**Monocytes**	**Lymphocytes**
Positive Control	4.40 ± 0.400 [a]	16.40 ± 0.245[a]	1.60 ± 0.245 [a]	85.60 ± 0.400 [e]
Negative Control	35.40 ± 0.245 [e]	39.60 ± 0.400 [d]	3.60 ± 0.245 [c]	25.20 ± 0.374 [a]
C1	34.40 ± 0.245 [d]	40.00 ± 0.316 [d]	2.80 ± 0.200 [b]	33.60 ± 0.244 [b]
C2	29.40± 0. 245 [c]	28.20 ± 0.200 [c]	1.80 ± 0.200 [a]	72.40± 0.244 [c]
C3	12.40 ± 0.245 [b]	14.8 ± 0.375 [b]	1.40 ± 0.245 [a]	79.20 ± 0.374 [d]

Notes Information :

a. The significance value was determined by one-way ANOVA analysis with a 95% confidence level.

b. Different lowercase superscripts on the same line indicated a significant difference (P <0.05).

The data obtained showed that carrageenan-induced mice experienced acute inflammation as indicated by an increase in the number of stem neutrophils and segment neutrophils, which were an inflammatory response due to the induction of carrageenan chemical compounds Phillipson and Kubes (2019). Inflammation is a physiological response to injury characterized by complex processes which are aimed to restore tissue homeostasis. Its first stage comprises the quick activation and migration of immune cells to the injury site to prevent the invasion of microorganisms and damage by hazardous substances in the absence of tissue integrity. In this context, neutrophils play an important role, arriving at the site of inflammation in a multistep controlled process that encompasses marginalization, slow-rolling, adhesion, and transendothelial or albuminal migration (Dabrowski et al. 2014; Thome et al. 2018). All these processes are dependent on the specific interactions between proteins expressed on endothelial cells and leukocytes, such as integrins and selectins(Schmidt et al., 2013; Thome et al., 2018). The topical administration of Ekor Naga leaves extract in each group showed a significant difference compared to the negative control. It is in line with the research conducted by Pastar et al. (2014) and Behl et al. (2021). The existence of flavonoid compounds found in Ekor Naga leaves according to the research of Benvenutti et al. (2021), can reduce the number of leukocytes when inflammation occurs in inhibiting chemotaxis reactions so that the inflammatory response can be reduced. In addition, saponin compounds contained in Ekor Naga leaves can increase the production of inflammatory mediators, namely growth factors for the formation of vascular endothelium and interleukins, so that they can induce macrophages to areas experiencing inflammation. Other than neutrophils, monocyte cells also have an important role in fighting pathogens that can cause inflammation. The interactions between neutrophils and monocytes/ macrophages enable the host to efficiently defend against and eliminate foreign pathogens that can cause inflammation (Kumar et al., 2018).

Injection of carrageenan also caused a decrease in the number of lymphocytes from its normal value, namely according to Fahrimal et al. (2014) the normal value of lymphocytes in mice was 55-95%. The decrease in the number of lymphocytes induced by carrageenan is caused by the body's compensatory reaction when there is inflammation. The increase in the number of lymphocytes after administration of an Ekor Naga leaves extract was due to the activity of secondary metabolites contained in the Ekor Naga leaves extract, namely flavonoids, alkaloids, saponins, and tannins. These secondary metabolites have mutually supportive effects in increasing the body's response to cell damage or inflammation. Lymphocytes are important cell populations involved in chronic inflammation. Those cells can regulate the immune system, eliminate infected cells and orchestrate immune response by producing and secreting different kinds of cytokines. Cytokines, such as IL-2, TNF-α, and IFN-γ, are important in the progression of an inflammatory response aiming return to the homeostasis (Araújo et al. 2019)

Based on the results of the data above, it can be seen that the secondary metabolite compounds contained in the Ekor Naga leave extract, namely flavonoids, alkaloids, triterpenoids/steroids, tannins, and saponins have anti-inflammatory activity that plays a role in reducing inflammation. According to Abdulkhaleq et al. (2018), flavonoids can inhibit the activity of the cyclooxygenation and lipoxygenase enzymes. The process of inhibiting this enzyme can inhibit the biosynthetic metabolism of the formation of prostaglandins and leukotrienes which are products of the enzymes cyclooxygenase and lipooxygenase. So that the number of leukocytes that have accumulated in the inflamed area can be reduced. In addition, flavonoids can also inhibit lysosome secretion which causes profeliration and exudation. Saponin compounds can inhibit the release of pro-inflammatory substances stimulated by LPS, such as iNOS, IL, and TNF-α, so that they can inhibit the formation of fluid exudates and inhibit the permeability of the vascular system Lee et al., (2015). Then the steroids found in Ekor Naga leaves can also inhibit inflammation through inhibition of the phospholipase enzyme so that the formation of arachidonic acid and prostaglandins can be stopped. Steroids also play a role in inhibiting the migration of leukocyte infiltration.

CONCLUSION

The Ekor Naga leaves extract that applied topically (concentrations of 2.5%, 5%, and 10%) have an anti-inflammatory activity which is characterized by a decrease in the volume of exudate fluid and the number of types of leukocyte cells. The best concentration in overcoming inflammation is a concentration of 10%, with an inhibition percentage of 80.43%.

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