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Anti-Inflammatory Activities of Ethyl Acetate Fraction From Uncaria Gambir Leaves Through the Inhibition of Edema, COX-2 and iNOS Expression

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ABSTRACT
Inflammatory is a body defense mechanism due to respond of tissue on undermine influences, either local or the one that go into the body. Inflammatory can cause various physical dysfunctions. Uncaria gambir Roxb, a high catechin content in ethyl acetate fraction, is widely used as wound healing in Indonesian culture, therefore its mechanism of anti-inflammatory action is still unknown. The objective of our study is to investigated the activity of anti-inflammatory ethyl acetate fraction of Uncaria gambir leaves inhibition of edema, COX-2 and iNOS expression using induction method with carrageenan in rats. This study is experimental research which use 25 white Wistar strain rats as animals assay that divided into 5 groups: negative group (water), positive group (sodium diclofenac 50 mg/kg bw), dose I (5 mg/kg bw fraction), II (10 mg/kg bw fraction) and III (20 mg/kg bw fraction) groups. Each of them were given orally 30 min before carrageenan 3% induced. The paw volume was measured every day for seven day after injection carrageenan using plethysmometer. Inhibition COX-2 and iNOS expression were determined by enzyme-linked immunosorbent assay. The results showed that ethyl acetate fraction of Uncaria gambir leaves in all doses had anti-inflammatory effect on white rats through the mechanism of inflammatory inhibition by reducing edema volume. All three fraction doses can also reduce COX-2 and iNOS expression. The fraction had the highest anti-inflammatory potency by the dose of 20 mg/kg body weight. The conclusion that ethyl acetate of three doses had anti-inflammatory activities through a mechanism of edema volume reduction, COX-2 and iNOS expression.
***Keywords**: anti-inflammatory, edema, COX-2, iNOS*
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1. INTRODUCTION

Inflammation is considered as complex case that happened due to injured body, caused by chemical material or self-destructive (autoimunne). Inflamation is a protective respond as bodies' effort to return the condition as before injury (preinjury) or self-healing after injury. Inflammation respond is protective reaction and an important body restorative because the human body is trying to maintain the homeostatic under harmful envireonment. Inflamation cause many substances out endogenously, known as inflammation mediator. Arachidonic acid is one of the important inflammation mediator. Arachidonic acid important in prostaglandin biosynthesis through cyclooxygenase [1][2].
Control of pain and edema can increase immune respond in wound healing so the cure run better. One way to control pain and edema is by inhibiting cyclooxygenase enzyme. Anti-inflammation non stereoid (ANS) as antiinflammation works through inhibiting cyclooxygenase and inhibiting the migration of inflammation cells and cels adhesion expression. Anti-inflammation non stereoid had serious adverse effect if used as peptic ulcer, decreasing of infection immunity, osteoporosis, muscle athropy and lipid, increasing intraocular pressure, and diabetics [3][4].
Cyclooxygenase-2 (COX-2) is enzyme that influenced by tissue stimulus. The stimulus can be in the form of cytokine, lipopolysaccharides bacteria, inflammation or other pathologic symptoms. Inflammation can cause leukocytes accumulation, especially neutrophils and monocytes in the injured side to eliminate or confine the wound cause agent. Controlling paint and edema can increase immune respond in wound healing so the cure run better. One way to control pain and edema is by inhibiting cyclooxygenase enzyme. Anti-inflammation non stereoid (ANS) as antiinflammation works through inhibiting cyclooxygenase and inhibiting the migration of

inflammation cells and cels adhesion expression. Anti-inflammation non stereoid can cause gastrointestinal bleeding, make bleeding longer, and ruin the kidney function [5][6][7].

Catechin is flavanoid that had proven can inhibit inflammation. One of plantation that considered have antiinflammation effect is gambier (Uncaria gambir Roxb). The plantation used empirically as the medication for burns, wound, sprue, gingivitis, sore throat, diarrhea, dysentery, cough, and fever. The main substance of gambier is catechin (51%), tanner (20-25%), catechutanic acid, quersetin, red catechu, fluoresein gambier, ash, fatty acid, wax, alcaloids, and tanin. Gambier chemical substances that widely use is catechin and tannin.10 The biggest chemical substances in gambier is catechin that part of flavonoid. Flavonoids fuctions as antibacterial through complex substances formation of extracellular protein that disturb the integrity of bacteria cell membrane.11 Tanin works in gambier as antibacterial and antifungi. Tanin can be used as adstringen that contraction skin pores, harden skin, stop small bleeding, antiseptic, and burns.8 Ethyl acetate fraction of gambier leaves inhibitory activity on COX2 and iNOS enzymes unclarified. The objective of our study is to investigated the activity of anti-inflammatory ethyl acetate fraction of Uncaria gambir leaves inhibition of edema, COX-2 and iNOS expression using induction method with carrageenan in rats

2. METHOD

Materials

Gambier leaves extract from Lima puluh Kota district, catechin standard (Sigma Aldrich, Germany), ethyl acetate, aquadest, methanol HPLC grade (Merck), trifluoroacetic acid (Merck), acetonitrile HPLC grade (Merck), galur wistar mice, COX2 inhibitor screening assay kit, iNOS inhibitor screening assay kit.

Equipment

Fractionation coloumn, rotary evaporator (Buchi R114), oven (Memmert), High pressure liquid chromatography (HPLC) (Waters Alliance 2695), ELISA.

Extraction

Making gambir leaf extract begins with harvesting fresh leaves on a large tree and leaves taken about 5 strands from the shoots. Furthermore, fresh gambier leaves are put into a net and steamed with hot water vapor for 90 minutes. Gambier leaves that have been finished steamed, are removed and compressed using a hydraulic press to obtain gambier sap. Gambir sap drained and collected in a baking sheet, then cooled for 2 x 24 hours at room temperature until hard. Hardened gambier sap is printed with cylindrical molds. To minimize the moisture content, the drying process is continued using an oven at 40-50 ° C for 1 x 24 hours.9

Fractionation

The fractionation process was carried out by using 200 g of gambir leaf extract that crushed until smooth, suspended in n-hexane solvent and homogenized using a sonicator for 10 minutes. The suspension is filtered using filter paper. The residue was dissolved in ethyl acetate solvent, then homogenized with a sonicator for 10 minutes. The solution is partitioned by adding distilled water, then shaken in a separating flask and allowed to stand for 30-60 minutes until there are two layers (ethyl acetate layer at the top and distilled layer at the bottom). The two layers formed are then separated. The ethyl acetate fraction layer was evaporated using a rotary evaporator until thick. The ethyl acetate fraction obtained was evaporated in a fume hood, then continued drying in a vacuum oven at 40-50 ° C until a fixed weight was obtained [9].

Determination of Catechin Levels

Samples of ethyl acetate fraction were analyzed using HPLC Waters, Sun Fire C18 column 4.6 × 150 mm, with a flow rate of 0.45 mL / min, injection volume of 1.0 µL and a detector using UV at a wavelength of 280 nm. The mobile phase is used gradiently with the mobile phase A: 0.1% trifluoroacetic acid in a mixture of acetonitrile: water (5:95) and the mobile phase B 0.1% triflouroacetic acid in acetonitrile. The gradient conditions of the mobile phase are 0-4 minutes (100% A) 4-20 minutes (71.5 A; 28.5 B) and 20-30 minutes (100% B) [10].

Anti-inflammatory Test of Edema Volume Method

Tested animals were completely randomized that divided into 5 treatment groups consisting of negative controls, positive controls, doses I, II and III, namely the groups that were given test samples at doses of 5, 10, 20 mg / kg BW. The treatments for each test group are in table 1.

Table 1. Groups of tested animals

Evaluation Group	Number of rats	Treatment
Negative control	5	Give distilled water orally and 30 minutes later, injected with 3% carrageenan as much as 0.1 mL.
Positive control	5	Diclofenac sodium was given a dose of 50 mg / kg bw orally and 30 minutes later it was injected with 3% carrageenan as much as 0.1 mL.
Dose I	5	The experimental animal group was given gambir extract at a dose of 5 mg / kg bw orally and 30 minutes later it was injected with 3% carrageenan as much as 0.1 mL.
Dose II	5	The experimental animal group was given gambier extract at a dose of 10 mg / kg bw orally and 30 minutes later it was injected with 3% carrageenan as much as 0.1 mL.
Dose III	5	Group of experimental animals which were given gambir extract at a dose of 20 mg / kg bw orally and 30 minutes later at 3% carrageenan injection of 0.1 mL

The test was carried out by weighing the weight of each test animal and marked with an arrow with its left foot using picric acid, then the left leg of the rat was put into a thermometer containing mercury (mercury) which had been prepared until the liquid rose to the upper boundary line, recorded the number on the tool as the initial volume (Vo) that is the volume of the foot before being given medication and induced with carrageenan solution. Each mouse was given a suspension of test material orally according to the group. 30 minutes later each rat's paw was injected intraplantar with 0.1 ml of carrageenan 3% solution and 30 minutes after induction was measured by dipping the rat's left foot into the liquid plestimometer containing mercury liquid until the solution reached the upper border of the left leg mouse and recorded the number obtained. Changes in fluid volume are recorded as rat foot volume (Vt). Measurements were made every 30 minutes for 360 minutes. When measuring, the volume of mercury must always be the same, the boundary markings on the rat's feet must also be clear, and the rat's feet must be immersed to the limit made.[11]
The data obtained in the form of mice foot volume, then used to calculate edema volume. Edema volume is the difference in rat's feet before and after inflammation. The percentage inhibition of inflammation is calculated using the formula [12]:

$$\%\ \text{inhibition of edema} = \frac{a - b}{a}\ x\ 100\%$$

a = average inflammation volume of the negative control group

b = average inflammation volume of the positive test or control sample group

Evaluation for the activity of the cyclooxygenase-2 (COX2) enzyme

The testing of cyclooxygenase inhibition activity was carried out in vitro by the method of inhibiting the activity of COX2 enzymes on the formation of arachidonic acid by using COX2 inhibitor screening assay. The kit used consists of COX Assay Buffer solution, COX Probe (in DMSO), arachidonic acid substrate, COX Cofactor (in DMSO), NaOH, and COX2. The ability to inhibit COX2 is shown by the lower absorbance value of the sample solution compared to the blank solution on measurements using an ELISA microplate reader at a wavelength of 570 nm. Inhibition of enzyme activity is calculated as (%) inhibition [13]:

$$\%\ \text{inhibition} = \frac{\text{control abs} - (\text{sample abs} - \text{blank abs})}{\text{control abs}}\ x\ 100\%$$

Induction nitric oxide synthase (iNOS) inhibitory activity test

The testing of cyclooxygenase inhibition activity was carried out in vitro by the method of inhibiting the activity of the iNOS enzyme against arachidonic acid formation using iNOS inhibitor screening assay. The kit used consisted of iNOS Assay Buffer solution, iNOS Probe (in DMSO), arachidonic acid substrate, iNOS Cofactor (in DMSO), NaOH, and iNOS. The inhibitory ability of iNOS is shown by the lower absorbance value of the sample solution compared to the blank solution on measurements using an ELISA microplate reader at a wavelength of 570 nm. Inhibition of enzyme activity is calculated as (%) inhibition [13]:

$$\%\ \text{inhibition} = \frac{\text{control abs} - (\text{sample abs} - \text{blank abs})}{\text{control abs}}\ x\ 100\%$$

Data Analysis

Data from the experiments were expressed as mean ± SD. The statistical significance of differences between the groups were analyzed with one-way ANOVA, followed by LSD post-hoc test analysis using SPSS software 17.0 version, p values of less than 0.05 were considered to indicate significant difference

3. RESULTS AND DISCUSSION

Results

The protocol of animal study has received the ethical clearance from Health Research Ethics Commitee National Institute Health Research and Development, Ministry of Health number LB.02.01/2/KE.103/2017. Total catechin content in the fraction derived from HPLC analysis was 95,56%. The catechin content which is more than 90% met the requirement of IHP. These result was consistent with Yunarto et al that fractionation process can improve the purity and levels of catechin [14].

Table 2. Inhibition of edema (%)

Groups	Average of % edema inhibition at the -hours					
	1	2	3	4	5	6
Negative control	0.00	0.00	0.00	0.00	0.00	0.00
Positive control	18.04 ± 0.16	26.12 ± 0.36	34.23 ± 0.22	21.72 ± 0.48	12.64 ± 0.31	8.22 ± 0.08
Dose I	17.92 ± 0.12	26.45 ± 0.28	35.14 ± 0.31	22.38 ± 0.25	13.64 ± 0.29	9.04 ± 0.07
Dose II	24.56 ± 0.36	31.18 ± 0.26	39.78 ± 0.24	41.54 ± 0.28	30.64 ± 0.35	18.22 ± 0.16
Dose III	28.12 ± 0.32	35.75 ± 0.42	44.85 ± 0.26	50.22 ± 0.72	38.24 ± 0.42	28.22 ± 0.18

Based on Table 1, at dose III (20 mg / kg body weight) shows the largest percentage of edema inhibition that is 28.12% in the first hour and continues to increase until the fourth hour which can inhibit edema by 50.22%. Dose III had a significant difference ($p < 0.05$) with negative control, positive control, dose I and dose II in the first hour. This difference shows that dose III has a faster onset when compared to other groups. This is because the catechin content at dose III is higher when compared to doses I and II.

Table 3. Inhibition of COX2 and iNOS activity (%)

Groups	COX2 (%)	iNOS (%)
Negative control	-	-
Positive control	47,60 ± 1.28[a]	18,24 ± 0,20[a]
Dose I	45,23 ± 2,11[a]	17,82 ± 0,13[a]
Dose II	52,23 ± 1,98[b]	28,83 ± 0,19[b]
Dose III	64,49 ± 6,71[c]	37,95 ± 0,12[c]

Percent inhibition of COX2 and iNOS activity shown that compared to positive control, doses II and III of fraction gambir leaves extract were able to inhibit of COX2 and iNOS activity. ($p<0.05$).

Discussion

The mechanism of action of ethyl acetate fraction of gambir leaves inhibiting COX-2 expression causes arachidonic acid did not turn into cyclic prostaglandin endoperoxide. Cyclic endoperoxide prostaglandin is a precursor for all prostaglandins so that prostaglandin biosynthesis is stopped. Prostaglandins function to increase the permeability of blood vessels causing neutrophil edema and chemotaxis. Thus the inhibition of cyclooxygenase enzyme activity by ethanol extract of Moringa leaves will reduce edema volume and COX-2 expression through neutrophils [15].

Poor oxidative status, as well as the production of oxidized lipids from arachidonic acid, has also been linked to chondrocyte apoptosis, activation of latent matrix metalloproteinases, and cartilage matrix degradation due to upregulation of inflammatory gene expression. Catechins are flavonoid of polyphenol groups that function as a free radical scavenger to provide the hydrogen atom. The structure that allows the radical scavenging activity of polyphenols is the presence of 3,4-dihydroxyl (catechol structure) in the B ring, which acts as an electron donor and became the target of radical. The 3-OH structure of ring C is also beneficial to the activity of antioxidant polyphenols. Conjugation bond at C2-C3 with 4-keto group plays a role for the electron delocalization of the B ring, which in turn increases free radical scavenging capacity. Besides that, the 3-OH group and 5-OH, in combination with 4-carbonyl function and C2-C3 double bond, also raise antioxidant activity. In the absence of o-dihydroxy structure in the B ring, the hydroxyl substituents on the catechol ring A can be compensated and the ability antiradikal activity of polyphenols is increased [16][17].

In our study, ethyl acetate fraction of gambir rich catechin reduced both PLA_2 activity and nitrite levels in macrophages. This suggests that catechin may act to limit the conversion of phospholipids from damaged cell membranes to AA upstream of the COX and 5-LOX metabolic pathways. In addition, the reduction in nitrite, the stable breakdown product of NO, may prevent the production of matrix metalloproteinase from macrophages present in the synovium. In support of these findings, flavocoxid was previously shown to reduce iNOS protein production by an antioxidant mechanism. Since it is also known that NO levels are positively affected by increases in cytokine production, catechin cytokine-reducing effect may inhibit the increase in inos expression and NO levels [18].

Stable nitrite is used as a measure of NO produced from iNOS from synovial macrophages in OA. Nitric oxide may be involved in the destruction of proteoglycan in cartilage by inducing the production of matrix metalloproteinase. To assess catechin effect on the production of this highly reactive oxidative molecule, LPS-stimulated rat peritoneal macrophages expressing higher levels of iNOS were used in the presence of increasing levels of the flavonoid preparation [19].

4. CONCLUSION

The ethyl acetate fraction of Uncaria gambir leaves in all doses had anti- inflammatory effect on white rats through the mechanism of inflammatory inhibition by reducing edema volume. All three fraction doses can also reduce COX-2 and iNOS expression. The fraction had the highest anti-inflammatory potency by the dose of 20 mg/kg body weight.

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