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In Vitro Anti-inflammatory Activity of Extract and Fraction Seed Coat Kebiul (*Caesalpinia bonduc* L.)

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Abstract: *Inflammation is the protective reaction of body and supporting element of tissues to injurious stimuli, such as physical injury, tissue and cell damage by pathogens, and protein denaturation. This study aims to compare the antiinflammatory activity of ethanol extract, ethanol fraction, and n-hexane fraction from kebiul (Caesalpinia bonduc* L.*) seed coat. Antiinflammatory activity was tested by measuring the % inhibition of the denatured protein using a UV-Vis spectrophotometer. From the result of the study obtained IC_{50} value 2,10 µg/mL, 7,89 µg/mL, 4,04 µg/mL & 11,03 µg/mL.*

Key word: Antiinflammatory, Caesalpinia bonduc L.*, Protein denaturation.*

INTRODUCTION

Inflammation is a protective reaction normal to tissue damage caused by physical injury, hazardous chemicals and protein denaturation (Chandra et al., 2012). Protein denaturation is a process in which the protein loses its tertiary structure and their secondary structure by external compounds, such as strong acids, strong bases, organic salts, solvents organic, and heating. Protein denaturation is a one of the causes of inflammation. According to (Aditya et al., 2015) the compounds that can inhibit protein denaturation can be used as an anti-inflammatory drug, compounds such as polyphenols include anthocyanins, tannins, xanthones, and phenolic acid compounds.

The Non Steroidal Antiinflammatory Drugs (NSAIDs) have these side effects can cause irritation if use in the long time (Chandra et al., 2012). The natural ingredients which has many bioactive compounds and has potential as an inflammatory drug is Kebiul (*Caesalpinia bonduc* L.). Kebiul seeds are often used by the people of South Bengkulu for treatment such as malaria, diabetes, and kidney stones (Singh & Raghav, 2012) (Rio Putra, 2013). But until now, research using of kebiul seed coat is still not done by many people, especially in Indonesia. Kebiul seed coat is usually only thrown away by traditional people and is not used properly.

The effectiveness of the extract in inhibiting denaturation protein can be known by calculating the IC_{50} value. IC_{50} value is the concentration when the percentage inhibition of protein denaturation reaches a value of 50%, the smaller the IC_{50} value, the greater the activity anti-inflammatory (Farida et al., 2018). Based on this, it is necessary to test anti-inflammatory activity of ethanol extract, ethanol fraction and n-hexane seed coat of kebiul (*Caesalpinia bonduc* L.) in vitro against the inhibitory ability protein denaturation.

RESEARCH METHODS

Procedures

Sample Preparation

Kebiul fruit (*Caesalpinia bonduc* L.) is taken from Padang Guci Subdistrict, Kaur Regency, Bengkulu, Indonesia, then peeled and take the seeds. The seeds are washed with running water then dried under the sun. Kebiul seed coat is then mashed use a mixer to powder kebiul seed coat (Yani & Dirmansyah, 2021).

Sample Extraction

About 230,93 grams of leather powder kebiul (*Caesalpinia bonduc* L.) seed coat macerated with ethanol 96 %. Filtrat was evaporated using rotary vacuum evaporator at 40℃. Five grams extract was fractionation with ethanol : n-hexane (1:1) (Yani & Dirmansyah, 2021). The percentage of extract yield was calculated by using the equation 1 formula (Sani et al., 2014):

$$\% \text{ Yield} = \frac{\text{Weight of extract obtained}}{\text{The weight of the extract simplicia}} \times 100\ \% \qquad \text{(Eq. 1)}$$

Phytochemical Test

a. Alkaloid

A few ml of the extract solution is taken later put into a test tube. On sample add 1-2 ml of Dragendorff's reagennt. Changes that occur are observed, test results are declared positive when a solutionn with a red precipitate was formed orange (Iqbal et al., 2015).

b. Flavonoid

A few ml of samples is taken and put into a test tube. Add to the sample in the form of Mg powder 2 mg and given 3 drops of HCl p.a. The sample was shaken and observed changes occurs, the formation of a red, yellow or orange color in solution showed the presence of flavonoids (Dirmansyah, 2020).

c. Saponin

Take a few ml of the extract solution then put into a test tube as much as 1-2 ml. Hot water added to the sample, the reaction was positive if foam is formed which is stable for 30 minutes and does not lost on the addition of 1 drop of 2 N HCl (Dirmansyah, 2020).

d. Terpenoid and Steroid

Take a few ml of the extract solution then put into a test tubes as much as 1-2 ml, then dripped with *Lieberman-Burchard*, to form a brown bring, red or violet color, this result indicates a positive test for terpenoids, the formation of a green or blue color showed a positive test result for steroids (Faskalia & Wibowo, 2014).

e. Tannin

Prepare a extract solution of 1 ml, then added 3 drops of a solution of $FeCl_3$ 10%. Changes that observed, the formation of a dark blue or black color greenish, color indicates

the presence of tannin compounds hydrolyzed and when a colored precipitate is formed red indicates the presence of tannins condensed (Sari et al., 2015).

In Vitro Assay of Antiinflammatory Activity Test

A total of 50 L of each concentration of ethanol extract solution, ethanol fraction, n-hexane fraction and diclofenac sodium (100, 250, 500, 1000 ppm), was added 0.2% Bovine Serum Albumine solution in Tris Buffer Saline solvent pH 6.2- 6.4, until the volume reaches 5 mL. The solution was incubated at 30℃ in a water bath for 20 minutes and then heated for 10 minutes at 80-85℃. Then let stand for 25 minutes at room temperature. After cooling, the solution was vortexed and absorbance was measured with a UV-Visible spectrophotometer at a wavelength of 660 nm which is the maximum wavelength of protein. The treatment was repeated on the negative control using only the same solvent as the test solution. The percentage of protein denaturation inhibition was measured using the equation 2 formula:

$$\% \text{ inhibition} = \frac{\text{Negative control absorbance} - \text{Absorbance of the test solution}}{\text{Negative control absorbance}} \times 100\% \qquad \text{(Eq. 2)}$$

Compounds that inhibited protein denaturation greater than 20% was considered to have anti-inflammatory and can be used as a reference value for drug development (Novika et al., 2021). IC_{50} value is calculated by making a linear regression equation between concentration (X) and % inhibition (Y) (Novika et al., 2021).

RESULT AND DISCUSSION

Preparation and Extraction of Kebiul Seed Coat

The process of withdrawal of secondary metabolites also carried out with 2 different solvents the polarity is ethanol (polar) and n-hexane (non polar). In this process, secondary metabolites polar ones will be attracted to polar solvents, as well as non-polar solvents. This extraction process is understood as the transfer of solute from one solvent to the solvent others (Irina & Barbulescu, 2011). The yield value of the ethanol extract, n-hexane fraction, and ethanol fraction of kebiul seed coat (*Caesalpinia bonduc* L.) can be seen in Table 1.

Table 1. Percent Yield of Ethanol Extract, Ethanol Fraction, and N-Hexane Fraction

Sample	Weight of dry simplicia	Extract weight obtained	% Yield (Extract / Simplicia)
Ethanol extract	230,93 g	27,17 g	11,765 %
N-hexane fraction	5 g	0,084 g	1,68 %
Ethanol Fraction		3,3695 g	67,39 %

The purpose of calculating the yield value is to find out how much compound content bioactive obtained and the percentage of recovery, so that later it can be a reference for making certain amount of viscous extract. Yield of ethanol extract was obtained (11.76%) with brown blackish and chewy texture, ethanol fraction of (67.39%) which has a dark brown color with a thick texture, and in the n-hexane fraction of (1.68%) has a deep yellow color with slightly lumpy texture.

Phytochemical Test

Phytochemical test was carried out to determine qualitative secondary metabolite compounds. Phytochemical test was carried out on 3 samples, namely the extract ethanol, ethanol fraction, and n-hexane fraction kebiul (*Caesalpinia bonduc* L.) seed coat. Metabolites secondary contained in the 3 samples can be seen in Table 2.

Table 2. Test results of secondary metabolites of *Caesalpinia bonduc* L. ethanol extract, ethanol fraction, and n-hexane fraction through phytochemicals

Compound	Ethanol Extract	N-Hexane Fraction	Ethanol Fraction	Color if the result is positive
Flavonoids	+	+	+	Red, Yellow, and Orange
Tannins	+	-	+	Dark blue or blackish green
Terpenoids	+	-	+	Red Purplish
Steroids	+	+	+	Ring chocolate and green
Saponins	+	-	+	Foam
Alkaloids	+	+	+	Red precipitation

Note: (+) Positive test and (-) Negative test

Based on table 2 that the ethanol extract and the ethanol fraction contains flavonoid compounds, tannins, terpenoids, steroids, saponins, and alkaloids. While the n-hexane fraction only has compounds flavonoids, steroids and alkaloids. All samples were positive for flavonoids, this is because flavonoids have many types with different polarities, so that at when fractionating flavonoid compounds can be attracted to the ethanol and n-hexane solvent. Examples of flavonoid non-polar such as luteolin, apigenin, and trisine, while the polar ones are quercetin, kaempferol, quercitrin, rutin, and others (Mottaghipisheh & Iriti, 2020).

Steroids occur in nature as a lipid fraction functions to regulate biological activity plants or animals (Ningsih et al., 2016). Generally, steroids and terpenoids can be soluble in non-polar solvents up to semipolar. Tests carried out suspected steroids strong in all samples, this is evidenced by the formation of a dark green color in the sample fraction n-hexane, on ethanol fraction and ethanol extract showing a clear brown ring. Terpenoids test only negative in the n-hexane fraction, it is suspected because the content of steroids is more than terpenoids.

Anti-Inflammatory Activity Test

Ability of a substance to inhibit protein denaturation indicates a real potential for anti-inflammatory activity (Osman et al., 2016). Anti-inflammatory activity of a compound can be determined by calculating percent inhibition of denatured protein. As for the linear regression of protein denaturation inhibition of diclofenac sodium, ethanol extract, ethanol fraction, and the n-hexane fraction is presented in Figure 1.

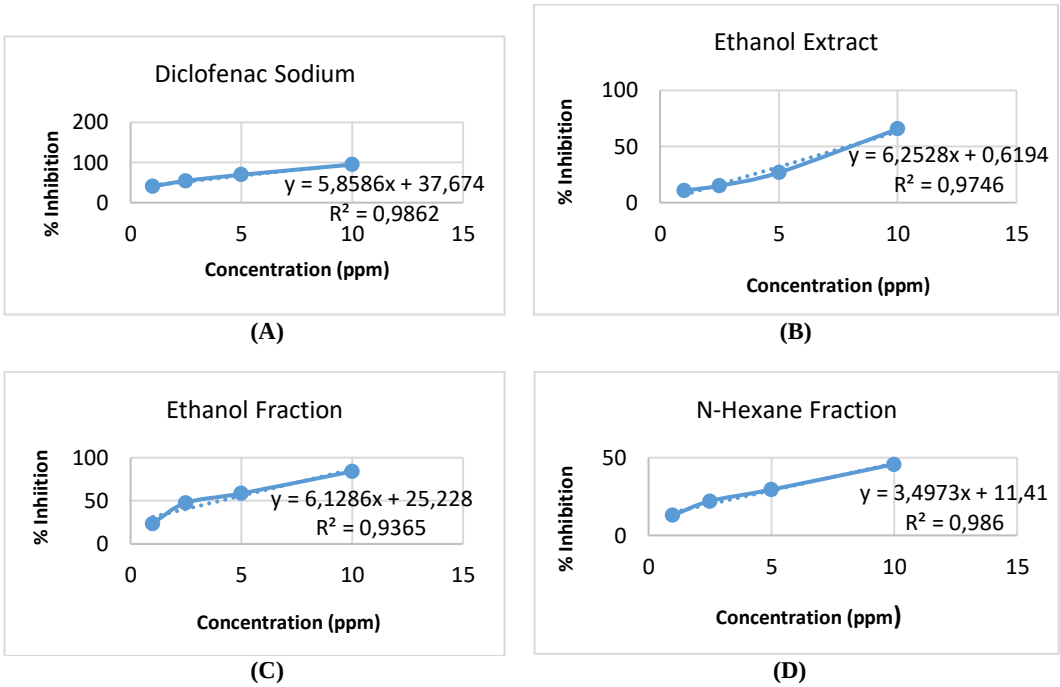

Figure 1. (A) Diclofenac Sodium, (B) Ethanol Extract, (C) Ethanol Fraction, and (D) N-Hexane Fraction

Ethanol extract, ethanol fraction, and n-hexane fraction have secondary metabolites that have potential as anti-inflammatory, namely flavonoids, tannins, saponins, alkaloids, steroids, and terpenoids (Tyagi, 2017) (Souto et al., 2011) (Mohammed et al., 2014). Flavonoids have the potential to inhibit enzymes in arachidonic acid metabolism by decreasing release of inflammatory mediators. Flavonoids can inhibits biosynthesis of prostaglandins, thromboxanes, leukotrienes by inhibiting phospholipase enzyme (Maleki et al., 2019).

Tannins have anti-inflammatory effects including radical scavenging and mediator inhibition inflammation, such as several cytokines and COX-2 (Mohammed et al., 2014). Saponins have a mechanism of inhibition of glucocorticoid degradation, inhibition of formation enzymatic and inflammatory mediator release (Mohammed et al., 2014).

Steroids occur in nature as a lipid fraction that functions to regulate biological activities in both plants and animals (Ningsih et al., 2016). Steroids inhibit phospholipase enzyme so that inhibit the formation of inflammatory mediators leukotrienes and prostaglandins (Amir et al., 2019). Terpenoids characterized by a pungent smell and formed by aromatic plants as secondary metabolites. Terpenoids have mono and sesquiterpene hydrocarbons and their derivatives containing oxygen as the main component essential oils of plant origin, which has a strong anti-inflammatory effect (Mohammed et al., 2014).

Alkaloids are one of a large class of secondary metabolites. Alkaloids are compounds that contain nitrogen atoms derived from amino acids (Souza et al., 2020). Alkaloids have anti-inflammatory activity by inhibiting enzymes lipoxygenase, COX-1, COX-2 and synthesis prostaglandins (Souto et al., 2011). Other studies have also shown that anti-inflammatory activity is also found in the kernel and leaves of the kebiul plants, This can support the statement that the kebiul seed coat also has an anti-inflammatory effect (Banupriya et al., 2018).

IC_{50} Value Calculation Results

Determination of half inhibition concentration maximum (50%) (IC_{50}) is very important to understand pharmacological and biological characteristics of the agent chemotherapy (He et al., 2016). IC_{50} value is calculated by making a linear regression equation between concentration (X) and % inhibition (Y) as shown Figure 1, and IC_{50} value for each the test solution can be seen in Table 3.

Table 3. IC_{50} value of diclofenac sodium, ethanol extract, ethanol fraction, and n-hexane fraction

Test of Solution	IC_{50} Value
Diclofenac Sodium	2,10 µg/ml
Ethanol Extract	7,89 µg/ml
Ethanol Fraction	4,04 µg/ml
N-Hexane Fraction	>10,00 µg/ml

Table 3 shows that sodium diclofenac has more anti-inflammatory activity better compared to ethanol extract, ethanol fraction, and n-hexane fraction. This is because sodium diclofenac is one of the most effective in inhibition of prostaglandin production and has reported to be more potent than other NSAIDs in its ability to inhibit COX activity (Gan, 2010).

The ethanol fraction has the best anti-inflammatory activity of all the kebiul seed coat test solutions with an IC_{50} of 4.04 µg/ml. The ethanol fraction has the same secondary metabolite content as the ethanol extract, but the ethanol fraction has better anti-inflammatory activity than the ethanol extract which has an IC_{50} value of 7.89 µg/ml. This is presumably because during the fractionation process, the ethanol solvent used attracts more polar secondary metabolites and has more potential for anti-inflammatory activity, such as flavonoids, tannins, saponins and steroids, and also the purity of secondary metabolites in the ethanol fraction was better than that of the ethanol extract. This result is supported by research by (Werdyani et al., 2019) which states that the results of the fraction can increase antioxidant activity compared to the extract, where secondary metabolites can work more strongly when in a single form.

Ethanol extract has an IC_{50} value of 7.89 µg/ml and is still below the ethanol fraction with an IC_{50} of 4.04 µg/ml, the ability of ethanol extract to inhibit inflammation is lower. This is because the ethanol extract contains other impurities such as salt, metal or other substances (Solikhah R. et al., 2019). According to research by (Mehra et al., 2016) which revealed that the metals contained in the kebiul seed extract were Pb, Cd, Zn, and Hg, these metals were thought to inhibit the anti-inflammatory process.

The n-hexane fraction has the lowest IC_{50} value of >10.00 µg/ml, this is because the content of secondary metabolites in the n-hexane fraction is less than the ethanol extract and ethanol fraction, this is evident from the phytochemical results in Table 2 that the n-hexane fraction was only positive for flavonoids, steroids and alkaloids.

CONCLUSIONS

Based on research conducted for determined the antiiflammatory activity of kebiul (*Caesalpinia Bonduc* L.) seed coat against protein denaturation obtained IC_{50} value of

diclofenac sodium, ethanol extract, ethanol fraction, and n-hexane fraction of each sample respectively were 2,10 g/ml, 7,89 g/ml, 4,04 g/ml, and 11,03 g/ml.

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