ANTI-INFLAMMATORY ACTIVITY OF ETHANOL EXTRACT AND ETHYL ACETATE FRACTION OF *KEBIUL (Caesalpinia bonduc L.)* SEED COAT AGAINST INHIBITION OF PROTEIN DENATURATION

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Abstract

Inflammation is a normal protective reaction against tissue damage caused by physical injury, harmful chemicals, and protein denaturation. Protein denaturation is a process in which proteins lose their tertiary structure and secondary structure due to foreign substances, external compounds, such as strong acids, strong bases, organic salts, organic solvents, and heating. The purpose of this study was to determine the anti-inflammatory activity of ethanol extract and ethyl acetate fraction of the seed coat of *Kebiul* (*Caesalpinia bonduc L*.) by calculating the IC_{50} value of protein denaturation in the sample. The results of the phytochemical test of the ethanol extract of *Kebiul* seeds contained flavonoids, alkaloids, terpenoids, steroids, saponins, and tannins, while the ethyl acetate fraction only contained tannins and alkaloids. The results of the anti-inflammatory test showed that the highest percent inhibition value of sodium diclofenac, ethanol extract, and ethyl acetate fraction, respectively, at a concentration of 20 ppm was 84.8%; 84.1%; and 50%. The IC_{50} values of sodium diclofenac, ethanol extract, and ethyl acetate fraction were 5.4 μg/mL; 9.9 μg/mL; and 13.3 μg/mL, respectively. The three samples had percent inhibition values exceeding 20% which indicated that all three can be used as an anti-inflammatory.

Keywords*: anti-inflammatory, protein denaturation, Caesalpinia bonduc L.*

Introduction

Inflammation is a normal protective reaction against tissue damage caused by physical injury, harmful chemicals, and protein denaturation (Chandra *et al.*, 2012). Protein denaturation is a process in which proteins lose their quaternary, tertiary, and secondary structures due to foreign substances, external compounds, such as strong acids, strong bases, organic salts, organic matter, and heating (Anggraini, 2008). According to data from the Ministry of Health of the Republic of Indonesia 2020, the Covid-19 disease, which is a hot topic in the world, will give symptoms of inflammation in patients with it. COVID-19 can trigger a cytokine storm and systemic hyper inflammation that causes hypercoagulation (Willim et al., 2020). Protein denaturation can be a cause of inflammation. The criteria for inflammation are that there are local and systematic symptoms, including migration of leukocytes to inflamed tissues (Wilmana, 2007). According to Aditya et al., (2015), compounds that can inhibit protein denaturation can be used as anti-inflammatory drugs.

The most commonly used anti-inflammatory drugs are Non-Steroidal Anti-Inflammatory Drugs (NSAIDs), people often consume (NSAIDs) in their daily life to treat inflammation, but these drugs have a side effect that can cause gastric irritation if consumed in long term (Neal, 2006). In addition to drugs (NSAIDs) can also be used natural ingredients such as herbal plants. The

advantage of natural ingredients as herbal medicines is that they have fewer side effects than synthetic drugs. One of the natural ingredients that has many bioactive compounds and potential as an anti-inflammatory agent is the *Kebiul* plant (*Caesalpinia bonduc L.)*.

According to Subbiah, et al. (2019), the seed coat of *Kebiul* contains many secondary metabolites such as alkaloids, saponins and flavonoids, these compounds are very likely to have anti-inflammatory activity. The ethanol extract of *Caesalpinia bonduc L.* seed kernel and leaf has strong antioxidant activity and anti-inflammatory activity with IC_{50} values of 311.00 g/ml in leaves and 111.90 g/ml in seed kernels (Farida et al, 2018). The purpose of this study was as an introduction to the activity of the anti-inflammatory extract and the ethyl acetate fraction of the seed coat of *Kebiul (Caesalpinia bonduc L.)* by calculating the IC_{50} value in vitro on the ability to inhibit protein denaturation, so that it is hoped that in the future it can also be a solution in handling and healing Covid-19 in Indonesia.

Research Methods

Tools and materials

Analytical balance (OHAUS PA224), pH meter, vortex, water bath, aluminum foil, filter paper, measuring flask, beaker, measuring cup, separating funnel, erlenmeyer, dropper pipette, test tube, test tube rack, stir bar, spatula, rotary vacuum evaporator (Yamato RE301C-W), incubator, P200 micropipette and UV-Visible spectrophotometer instrument.

The plant sample used was the seed coat of *Kebiul (Caesalpinia bonduc L.)* which was taken from Bengkulu Province. The test medium is Bovine Serum Albumin (BSA). Ethanol 96% solvent, n-hexane (Merck), ethyl acetate (Merck), aquades, NaCl (Merck), Tris base and Tris buffer saline, glacial acetic acid (Merck). The standard chemical drug used as a positive control was sodium diclofenac.

Extraction process

Kebiul fruit (*Caesalpinia bonduc L.*) was peeled and the seeds were taken and then dried. *Kebiul* seed powder was macerated with 96% ethanol. The filtrate was taken and concentrated with a rotary evaporator until a concentrated extract was obtained and the yield was calculated.

Phytochemical test

1) Tannin test

A 1 ml sample was added with 4 drops of Iron (III) Chloride ($FeCl_3$) solution to form a positive green or blue-black color for tannin compounds (Ruwandha, *et al,* 2021).

2) Alkaloid test

Dragendorff's reagent (Potassium bismuth iodide) is added 1 ml to 1 ml of sample, positive if a brick red precipitate is formed (Yuni, *et al*. 2021).

3) Flavonoid test

The sample was added with 1 ml of HCl, Mg, and ethanol. Positive if the color changes to red or orange (Yuni, et al. 2021).

4) Steroid and triterpenoid test

A sample of 1 ml was added with concentrated H_2SO_4 and 1 ml of anhydrous acetic acid, the color changes to green or blue, then the sample contains steroid compounds, and if the extract changes color to purple or orange, it is positive for triterpenoids (Yani D.F., *et al*, 2021).

5) Saponin test

The saponin test was carried out by means of the foam test. 1 ml of the extract was put into a test tube and aquadest was added and shaken 10 times and if 1–10 cm high foam was formed, it was positive for saponins (Yani DF, *et al*, 2021).

In vitro anti-inflammatory activity

Testing of the anti-inflammatory activity of the ethanol extract and the ethyl acetate fraction from the seed coat of the

Kebiul (*Caesalpinia bonduc L.*) was carried out in vitro through the following steps: (Ashok et al, 2017, and Reynaldi et al, 2021).

Preparation of tris-buffered saline (TBS) Solution

A total of 1.21 grams of tris base and 8.7 grams of NaCl were added with distilled water up to 900 mL. Adjust the pH with glacial acetic acid to pH 6.2–6.5, add aquadest to 1000 ml in a 1000 ml volumetric flask.

Production of 0.2% bovine serum albumin (BSA)

A total of 0.2 grams of BSA was put into a 100 ml volumetric flask. Then added with a solution of TBS to a volume of 100 ml**.**

Preparation of test solution

1 ml of ethanol, ethyl acetate and dichlorofenac extracts were dissolved in ethanol solvent in a 25 ml volumetric flask, then made up with solvent to a volume of 25 ml, so that the concentration as the mother liquor was obtained. The mother liquor was made in series of concentrations so that it became a test solution with a concentration of 2.5 ppm, 5 ppm, 10 ppm, and 20 ppm.

Measurement of anti-inflammatory activity

50 L of solution (test solution and positive control solution), added 0.2% BSA solution to a volume of 5 ml. then incubated at 25 °C for 30 minutes, heated for 5 minutes at 72 °C with a water bath, and allowed to stand for 25 minutes at 23 °C. After cooling, the solution was vortexed and absorbance was measured using UV-Visible spectrophotometry 660 nm (triplo) (Novika, *et al*, 2021).

Determination of IC_{50} value

IC50 value is calculated by making a linear regression equation between concentration (x) and % inhibition (y). So that the IC_{50} value was obtained from the ethanolic extract of the *Kebiul* seed coat (Caesalpinia bonduc L.) and diclofenac sodium. In this inhibition test, if >20% inhibition is produced, it is considered to have anti-inflammatory activity (Reynaldi, *et al*, 2021).

Results and Discussion

Extraction process

The extraction process for secondary metabolites was carried out by the maceration method. This method was chosen because maceration can be used for certain substances that are difficult to dissolve, the process is easy, and simple (Rasul, 2018). During the maceration process, stirring is carried out to enlarge the contact area between the solvent and the sample surface. The solvent softens and damages plant cell walls so that secondary metabolites present in the cells can come out and dissolve in the solvent (Hidayah, 2016).

The process of withdrawing secondary metabolites is also carried out with 2 solvents with different polarity, namely ethanol (polar) and n-hexane (nonpolar) which aims to separate secondary metabolites based on their polarity. In this process, secondary metabolites that are polar will be attracted to a polar solvent, and vice versa. This extraction process is understood as the separation of solutes from one solvent to another (Irina, et al, 2011). Furthermore, the fractionation process is continued with a semi-polar solvent (ethyl acetate) to separate compounds with different polarities.

Table 1. Percentage of ethanol extract yield and ethyl acetate fraction of *Kebiul* seed skin

Sample	Weight of dry simplicia	Weight of extract	Yield
Ethanol extract	230.93 g	27.17 g	11.77%
Ethyl acetate fraction	5.00 g	0.22 g	1.75%

The ethanol extract had a higher yield than the ethyl acetat fraction, because polar secondary metabolites such as flavonoids, alkaloids, steroids, saponins and tannins were more abundant in the kebiul seed sample than semi-polar secondary metabolites such as terpenoids and steroids. The appearance of ethanol extract obtained was blackish brown with a chewy texture, while the ethyl acetate fraction had a brownish yellow color. The difference in texture in each sample is thought to be due to the type of secondary metabolite compound that is attracted and the amount of yield obtained.

Phytochemical test

Table 2. Phytochemical test results of ethanol extract and ethyl acetate fraction

Compound	Color if the result is positive	Ethanol extract	Ethyl acetate Fraction
Flavonoid	Red, Yellow, and Orange	+	−
Tanin	Dark blue or blackish green	+	+
Terpenoid	Red Purplish	+	not
Steroid	Ring chocolate and green	+	not
Saponin	Foam	+	−
Alkaloid	Red precipitation	+	+

Based on Table 2. the ethanol extract contained flavonoid compounds, tannins, terpenoids, steroids, saponins, and alkaloids, while the ethyl acetate fraction only contains tannins and alkaloids while terpenoids and steroids were not tested. Steroids occur in nature as a lipid fraction which functions to regulate biological activity in both plants and animals (Ningsih et al., 2016). The negative flavonoid test on the ethyl acetate fraction showed that there were no semi-polar flavonoids in the skin sample, it is possible that the type of flavonoid in the sample was polar because it was attracted to the ethanol extract (Sari, et al., 2021).

Anti-inflammatory activity test

The choice of anti-inflammatory test method for inhibiting protein denaturation is due to one of the causes of inflammation is protein denaturation in body cell tissues. When the protein from BSA is heated, protein denaturation will occur. This denaturation process can be inhibited by secondary metabolites such as flavonoids. The ethanol extract and ethyl acetate fraction have secondary metabolites that have the potential as anti-inflammatory, namely flavonoids, tannins, saponins, alkaloids, steroids and terpenoids (Tiyagi, et al, 2018; Souto, et al, 2011; Mohammed et al, 2014).

Protein denaturation in body tissues can also be inhibited by secondary metabolites. When the body's cell membranes are damaged due to protein denaturation, phospholipids will be converted into arachidonic acid which is catalyzed by the phospholipase enzyme. This arachidonic acid will then be metabolized by enzymes and cyclooxygenase (COX), in this cyclooxygenase pathway, prostaglandins are synthesized.

Prostaglandins can increase blood flow to areas of inflammation, increase capillary permeability and stimulate pain receptors (Corwin, 2001). The synthesis of these prostaglandins can be inhibited by secondary metabolites (Rusli and Setiani, 2020). The ability of a substance to inhibit protein denaturation signifies a real potential for anti-inflammatory activity (Osman, et al. 2016). The anti-inflammatory activity of a compound can be determined by calculating the percent inhibition of the denatured protein.

Table 3. Percent inhibition of sodium diclofenac, ethanol extract, and ethyl acetate fraction

Concentration (ppm)	Percent Inhibition		
	Sodium diclofenac	Ethanol extract	Ethyl acetate fraction
20	84.8%	84.1%	50%
10	81.8%	65.6%	65.1%
5	69.5%	26.8%	42.0%
2.5	54.4%	15.1%	21.2%

The ethanol extract and ethyl acetate fraction each had a protein denaturation inhibition percentage value greater than 20%. The ethyl acetate fraction had anti-inflammatory activity at a concentration of 10 ppm at 65.1%, and the ethanol extract had anti-inflammatory activity at a concentration of 10 ppm at 65.6%.

However, if we look at the data on the percentage of inhibition in the ethyl acetate fraction at a concentration of 20 ppm, the inhibition decreased again. The % inhibition data on the ethyl acetate fraction is arguably less good than the results of the ethanol extract which overall increases with each increase in concentration. In addition, in his observations, the color of the solution in the ethyl acetate fraction when protein denatured when heated was slightly different from the ethanol extract. The white color that appears in the ethyl acetate fraction is slightly faded so that the change in denaturation is not very clearly visible. In contrast to the ethanol extract, the color changes very clearly to egg white when heated.

Flavonoids have the potential to inhibit enzymes in arachidonic acid metabolism by decreasing the release of inflammatory mediators. Flavonoids can inhibit the biosynthesis of prostaglandins, thromboxanes, and leukotrienes by inhibiting the phospholipase enzyme. (Arifin and Ibrahim, 2018). Tannins have anti-inflammatory effects including radical scavenging and inhibition of inflammatory mediators, such as several cytokines and COX-2. Saponins have a mechanism in inhibiting glucocorticoid degradation, inhibition of enzymatic formation and the release of inflammatory mediators (Mohammed, et al., 2014).

Steroids inhibit phospholipase enzymes thereby inhibiting the formation of inflammatory mediators leukotrienes and prostaglandins (Amir, et al, 2016). Terpenoids have mono- and sesquiterpene hydrocarbons and their oxygen-containing derivatives as the main components of plant-derived essential oils, which have strong anti-inflammatory effects (Mohammed, et al, 2014). Alkaloids have anti-inflammatory activity by inhibiting lipoxygenase enzymes, COX-1, COX-2 and prostaglandin synthesis.

Results of inhibition concentration 50 (IC_{50}) value

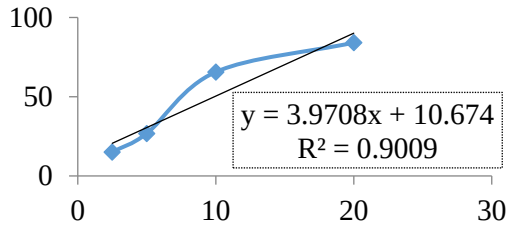
Determination of the half maximal (50%) inhibitory concentration (IC50) is very important to understand the pharmacological and biological characteristics of chemotherapeutic agents (He, et al, 2016).

Table 4. IC_{50} value of sodium diclofenac, ethanol extract, and ethyl acetate fraction

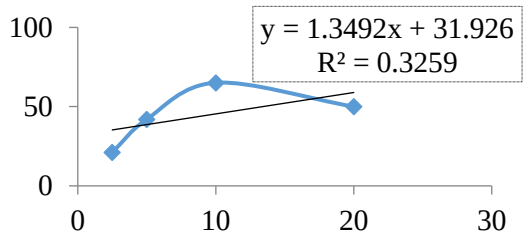
Sample	IC_{50}
Sodium diclofenac	5.4 µg/ml
Ethanol extract	9.9 µg/ml
Ethyl acetate fraction	13.3 µg/ml

The IC_{50} value indicates the ability of anti-inflammatory activity. Based on IC_{50} data, anti-inflammatory activity showed that diclofenac sodium had better anti-inflammatory activity compared to ethanol extract and ethyl acetate fraction. This is because diclofenac sodium is one of the most effective in inhibiting prostaglandin production and has been reported to be stronger than other NSAIDs in its ability to inhibit COX activity (Gan, 2010).

a **Ethanol Extract**



b **Ethyl Acetate Fraction**



c **Sodium Diclofenac**

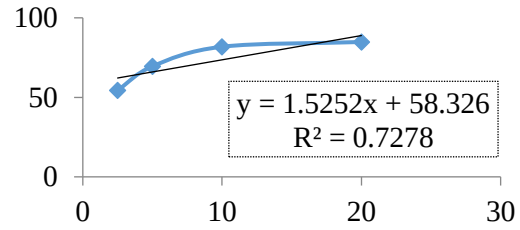


Figure 1. Linear regression curve, a. ethanol extract; b. ethyl acetate fraction; c. sodium diclofenac

From the linear regression curve data, it can be seen that the ethanol extract has a better R-square (R^2) value, which is close to 1 compared to the ethyl acetate and sodium diclofenac fractions. This means that with increasing concentration of ethanol extract, the inhibition value of protein denaturation also increases. This is in accordance with the results of the phytochemical test which states that the compounds in the ethanol extract contain more secondary metabolites than the ethyl acetate fraction. These secondary metabolites are thought to provide anti-inflammatory activity to serum albumin protein when denaturation occurs. However, from all tests, sodium diclofenac was still better than the extract and fraction of kebiul seed coat.

Conclusion

Anti-inflammatory activity at a concentration of 20 ppm from kebiul seed rind (*Caesalpinia bonduc L.*) ethanol extract (84.1%), ethyl-acetate fraction (50.0%), and diclofenac sodium (84.8%). Each sample had a percentage of inhibition greater than 20% but the percentage of inhibition of the ethanol extract and ethyl acetate fraction was not better than the positive control of sodium dichlorophenate. The IC_{50} values of diclofenac sodium, ethanol extract, and ethyl acetate fraction of each sample were 5.4 g/mL, 9.9 g/mL, and 13.3 g/mL, respectively.

Suggestion

It is necessary to isolate and characterize secondary metabolites in the ethanol extract and ethyl acetate fraction of kebiul seed coat, so the compounds that play a role in anti-inflammatory properties can be identified.

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