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Potency of Roselle Calyx (*Hibiscus sabdariffa* L.) Extract to Inhibit Protein Denaturation in Vitro

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Artikel Penelitian

Abstract: *Inflammation is a normal response to injury, which is characterized by protein and fluid leakage from blood vessels, causing pain and swelling. This study was conducted to determine the in vitro protein denaturation inhibition activity of roselle calyx extract (Hibiscus sabdariffa L.). Roselle flowers were extracted in stages by maceration with n-hexane, ethyl acetate, and 96% ethanol. The comparator used was diclofenac sodium. Several concentration series of extracts and comparators were prepared, and protein denaturation inhibition experiments on bovine serum albumin (BSA) were conducted. The parameters measured were the inhibition value of protein denaturation at each concentration and the value of 50% inhibition (IC_{50}). The results of the study indicated that n-hexane extract, roselle calyx ethyl acetate, and roselle calyx ethanol (96%) could inhibit protein denaturation. Roselle calyx n-hexane extract exhibited an IC_{50} 46.6748g/mL, ethyl acetate extract exhibited an IC_{50} 225.8391g/mL, and 96% ethanol extract exhibited an IC_{50} 191.5105g/mL, whereas diclofenac sodium exhibited an IC_{50} 8.5437g/mL. The conclusion revealed that roselle calyx extracts in n-hexane, ethyl acetate, and 96% ethanol display potentials as anti-inflammatory potential agents by inhibiting protein denaturation.*

Keywords: *roselle calyx, Hibiscus sabdariffa, anti-inflammatory, protein, denaturation*

Abstrak: Inflamasi merupakan respon normal ketika cedera yang ditandai oleh keluarnya protein dan cairan dari pembuluh darah kapiler sehingga terjadi nyeri dan bengkak. Penelitian ini dilakukan untuk mengetahui aktivitas penghambatan denaturasi protein ekstrak bunga rosela (Hibiscus sabdariffa L.) secara in vitro. Bunga rosela diekstraksi bertingkat dengan maserasi menggunakan pelarut n-heksana, etil asetat, dan etanol 96%. Pembanding yang digunakan adalah natrium diklofenak. Ekstrak dan pembanding dibuat dalam beberapa seri konsentrasi dan pengujian hambatan denaturasi protein dilakukan terhadap bovine serum albumin (BSA). Parameter yang diukur adalah nilai hambat denaturasi protein tiap konsentrasi dan nilai hambat 50% (IC_{50}). Hasil penellitian menunjukkan ekstrak n-heksana, etil asetat bunga rosela, dan etanol 96% bunga rosela dapat menghambat denaturasi protein. Ekstrak n-heksana bunga rosela memiliki nilai IC_{50} 46,6748µg/mL, ekstrak etil asetat memiliki nilai IC_{50} 225,8391µg/mL dan ekstrak etanol 96% memiliki nilai IC_{50} 191,5105µg/mL, sedangkan natrium diklofenak memiliki nilai IC_{50} 8,5437µg/mL. Dapat disimpulkan bahwa ekstrak n-heksana, etil asetat, etanol 96% bunga rosela mampu menghambat denaturasi protein dan berpotensi sebagai agen antiinflamasi.

Kata kunci: bunga rosela, *Hibiscus sabdariffa*, antiinflamasi, denaturasi protein

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Introduction

Inflammation is a normal response to injury. The release of chemical mediators, including bradykinin, histamine, serotonin, and prostaglandins, triggers vasodilation and capillary walls permeability. This stimulation activates pain receptors, leading to the leakage fluid and protein from the capillaries. It also boosts blood flow to the injured site and attracts phagocytic cells to eliminate potentially harmful substances. Excessive redness, swelling (edema), heat, discomfort, and loss of function will result in inflammation (1,2).

Proteins identified in inflammatory or infected regions are having denaturized. When a protein is denatured, the secondary and tertiary structure changes, but the peptide bonds in the primary structure remain intact. This alteration in structure will decrease the protein's activity. Protein denaturation causes a decrease in prealbumin and albumin concentrations (hypoalbumenia) while increasing acute phase protein (c-reactive protein/CRP). As opposed to administering albumin intravenously, the management of hypoalbuminemia needs to concentrate on handling the underlying causes of recurrent inflammation (3,4).

People increasingly prefer traditional medicine, and Indonesia has a vast range of herbs. Several plants, including the Roselle calyx (*Hibiscus Sabdariffa* Linn), have been empirically demonstrated to cure various diseases. The seeds of the Roselle plant can be utilised as a laxative for urination and digestive disorders, whereas the calyx is effective as a medication for vertigo. Roselle calyx contains nutritious compounds such as antioxidants, essential acids, beta carotene, and iron. Roselle calyx, leaves, fruit and seeds are also efficacious as a diuretic, anti-inflammatory and anti-sprue. Aside from therapeutic uses, the fibre in the stems is used to make gunny sacks, cosmetics, and food (5,6).

The acute anti-inflammatory action of roselle leaves demonstrated that a 500 mg/kg BW methanol extract suppressed inflammation in carrageenan-induced test mice (7). In vitro protein denaturation and membrane stabilisation experiments revealed that methanol extracts of calyx roselle flowers and calyx roselle demonstrated inflammatory effects (8,9). No prior research has explored the potential of roselle calyx extract across different polarities. This study seeks to determine the in vitro anti-inflammatory activity of roselle flower extract via protein denaturation.

Material and Methods

Material

Roselle calyx, Bovine Serum Albumin (Sigma Aldrich®), phosphate buffer pH 6.4 (Merck®), Diclofenac Sodium (Kimia Farma).

Methods

Roselle flowers were collected in Cigombong, West Java, and their identification was carried out at the School of Life Sciences and Technology, Institut Teknologi Bandung, West Java, Indonesia. Roselle calyx are sorted, cleaned under running water, then cut. Then, it was desiccated at 50°C for 36 hours. After dry sorting, the Simplicia was powdered and kept in a tight container.

Extraction

Extraction was carried out using maceration, utilizing with solvents with varying polarities, particularly n-hexane, ethyl acetate, and 96% ethanol. 100 g Simplicia were macerated for 24 hours at room temperature 25°C after being soaked in n-hexane, ethyl acetate, and 96% ethanol. Subsequently, the liquid and residue were separated, and the liquid was concentrated into a dense extract using a rotary evaporator at 45°C while under vacuum.

Phytochemical screening

Phytochemical screening consists of identifying alkaloids, flavonoids, tannins, polyphenols, quinones, saponins, monoterpenoids and sesquiterpenoids, steroids, and triterpenoids (10).

Protein denaturation assay

Several concentrations of each extract were tested for their ability to denature proteins. The n-hexane extract was prepared in serial dilutions of 20, 40, 60, 80, and 100 µg/mL. Extracts of ethyl acetate and 96% ethanol were prepared at dilutions of 20, 40, 80, 160, and 320 µg/mL. In comparison, diclofenac sodium dilutions of 2, 4, 8, 10, and 12 µg/mL were used.

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To make a total of 5 mL, mix 0.2 mL of 1% bovine serum albumin (BSA), 4.78 mL of saline phosphate buffer (pH 6.4), and 0.02 mL of extract/diclofenac sodium in one test tube. The mixture was incubated for 15 minutes in a 37°C water bath. Protein denaturation was accomplished by heating the mixture at 70°C for 5 minutes. After cooling, the absorbance was measured at 660 nm using a UV/Vis spectrophotometer. Each test was repeated three times (11). Control in this test is carried out by replacing the extract or diclofenac sodium with the solvent (aquadest). The percentage of protein denaturation inhibition was determined using the following formula:

$$Inhibition(\%) = \frac{Abs\ control - Abs\ test}{Abs\ control} \times 100\%$$

All test data are presented as mean ± standard deviation. From the data obtained, the IC_{50} value was determined using linear regression using GraphPad Prism 8.0 with 95% confidence intervals (12).

Result and Discussion

Roselle specimens were collected in Cigombong Village, West Java. Prior to commencing testing, it was essential to verify the plant authenticity of plant's identity, The verification analysis was conducted at the School of Life Sciences and Technology of Institut Teknologi Bandung (ITB). The results of the analysis confirmed that the plant under study was Roselle scientifically known as *Hibiscus sabdariffa* L., belonging to the Malvaceae family.

A phytochemical analysis was conducted on simplisia and its extracts with the aim of qualitatively identifying secondary metabolites present. Simplicia and its extracts are believed to contain secondary metabolites with pharmacological effects. Qualitative analysis of the phytochemical screening involved observing colour reactions and typical precipitations associated with each examined secondary metabolite (**Table 1**). According to these results, roselle flower simplicia contains polyphenolics, tannins, flavonoids, saponins, monosteroids, sesquiterpenoids, and steroids, while the n-hexane extract of roselle flowers contains quinones, sesquiterpenoids, and steroids. Alkaloids, polyphenolics, tannins, flavonoids, quinones, saponins, monosteroids, sesquiterpenoids, and steroids are all present in the ethyl acetate extract. Lastly, the 96% ethanol extract of roselle flowers contains alkaloids, polyphenolics, tannins, flavonoids, and saponins.

This anti-inflammatory assay was conducted using the Bovine Serum Albumin (BSA) protein denaturation method. BSA is the most sensitive indicator compared to other albumin indicators and utilised in the protein denaturation method. The denaturation process in BSA occurs when it is heated. Consequently, substances (including secondary plant metabolites) are capable of stabilizing proteins and preventing denaturation are considered to possess anti-inflammatory activity (13–15).

Table 1. Results of Phytochemical Screening of Dried Simplicia and Roselle Extract

Compound	Dried simplicia	n-hexane extract	Ethyl acetate extract	96% ethanol extract
Alkaloids	–	–	+	+
Polyphenols	+	–	+	+
tannins	+	–	+	+
Flavonoids	+	–	+	+
Quinone	+	+	+	+
Saponins	+	-	+	+
Monosteroids	+	–	+	–
Sesquiterpenoids	+	+	+	–
Steroids	+	+	+	–
Triterpenoids	–	–	–	–

+ = containing the test compound; – = does not contain the test compound

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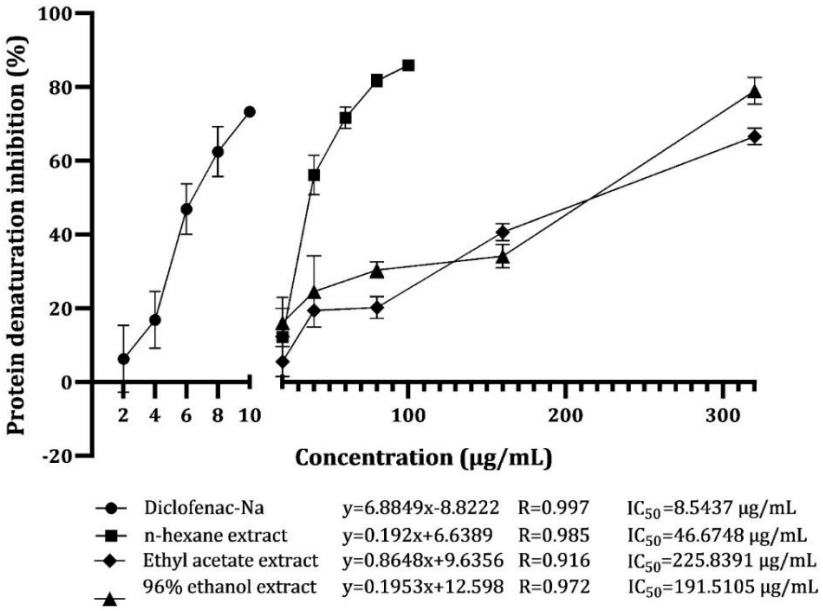

Figure 1. Results of Protein Denaturation Inhibition Test of Roselle Extract and Comparison

In this experiment, a 15-minute incubation was conducted at 37°C, which corresponds to the average temperature of the human body. Subsequently, the mixture was then heated for five minutes at 70°C. This heating is a protein denaturation process that occurs when proteins lose their tertiary and secondary structures due to heat, secondary compounds such as strong acids or strong bases, organic ions, and organic solvents. The rise in temperature causes the protein's constituent molecules to separate. A protein that undergoes denaturation will become less soluble, causing it to precipitate (16).

A variety of concentrations of n-hexane extract, ethyl acetate/ethanol, and the reference diclofenac sodium were used in this study. This difference is based on the rules for computing the IC50 value, which can be done if there are two concentrations with a response less than 50% and two concentrations with a response greater than 50% (17). Therefore, protein denaturation inhibition analysis was carried out at concentrations that provided an inhibitory effect in the range of 5-90%. To be considered effective for anti-inflammatory purposes, an agent needs to reduce protein denaturation by more than 20% (14,18). **Figure 1** shows the % inhibition of protein denaturation test results on concentration.

The test results indicated that N-hexane extract at 20-100 µg/mL inhibited protein denaturation by 12.22-85.90%, ethyl acetate extract at 20-320 µg/mL inhibited protein denaturation by 5.50-66.59%, ethanol extract at 20-320 µg/mL inhibited protein denaturation by 16.11-78.93%, and diclofenac sodium at 2-12 µg/mL inhibited protein denaturation by 6.22-73.28%. The percentage inhibition of protein denaturation at each concentration, even with variations, was not statistically significant ($p>0.05$). Each concentration shows variability in the percent inhibition of protein denaturation, but this variation is still below 20% so it can still be continued for IC50 calculations (17). BSA denaturation is a kinetic reaction characterized by a hydrodynamic radius of protein aggregation that is strongly influenced by time (19). Therefore, the variability in the percent inhibition of BSA denaturation in this study could be influenced by temperature, time and pH.

IC_{50} was determined using a linear regression equation relating % inhibition of protein denaturation (y) to concentration (x). The IC50

for the n-hexane extract was 46.6748g/mL, the IC50 for the ethyl acetate extract was 225.8391g/mL, the IC50 for the 96% ethanol extract was 191.5105g/mL, and the IC50 for diclofenac sodium was 8.5437g/mL. Although not as powerful as diclofenac sodium, the three roselle extracts show the ability to prevent protein denaturation.

According to the findings of the phytochemical screening, various chemicals present in roselle may give anti-inflammatory benefits. Specifically, flavonoids, phenolics, and steroids/triterpenoids, due to their hydroxyl groups and conjugated double bonds, have the capability to bind the amino acid residues within the BSA structure. This interaction stabilizes the protein structure, preventing denaturation when exposed to heat (20). In inflammation in the gastrointestinal tract, flavonoids have also been shown to reduce PGE 2 release by regulating controlling COX-2 activity. They also play a role in limiting leukocyte migration, inhibiting reactive nitrogen species generation, lowering pro-inflammatory mediators, and suppressing NF-B activity (21).

The pharmacological effects of plants depend on the extraction method and solvent. The crude extract will contain more secondary metabolite compounds. Although crude extracts have the potential to provide a greater synergistic effect, the mixture of these various components can provide contradictory or inhibitory effects. In line with this theory, some isolates have higher effects than extracts (22). In this study, n-hexane extract had a higher ability to inhibit protein denaturation than other extracts, even though it contained fewer compounds. Based on these results, it is suspected that the compounds that play a major role in inhibiting protein denaturation are triterpenoids and steroids.

Steroids in the n-hexane extract are considered to have anti-inflammatory properties. Several other plant steroids (e.g., *Trigonella foenum* graecum L., *Solanum xanthocarpum* L., *Boswellia serrata* Roxb., *Glycyrrhiza glabra* L., *Commiphora mukul*, and *Withania sominifera*) exhibit a glucocorticoid-like structure and be used to treat diseases with inflammatory symptoms (23). Plant steroids can also influence lipocortin and bind to phospholipase A2, thereby preventing the release of prostaglandins, which are chemical mediators that cause inflammation (24).

There were various limitations in this research, including the lack of isolation, identification, and testing of the pharmacological activity of compounds with anti-inflammatory potential. Additional testing is required to ensure the efficacy of roselle extract as an anti-inflammatory. Additional experiments may be performed either in vivo or in vitro. In vitro assessments may comprise membrane stabilization experiments, enzyme activity inhibition tests (lipooxygenase, cyclooxygenase, and hyaluronidase), as well as RAW264.7 cell experiments induced with lipopolysaccharide. The in vivo test was performed on animals induced with carrageenan, formalin, egg albumin, dextran, serotonin, or histamine (25).

Conclusion

The n-hexane extract, ethyl acetate extract, and 96% ethanol derived from flowers demonstrate the ability to inhibit protein denaturation, with n-hexane extract exhibiting the highest potency. While the n-hexane extract's effectiveness in inhibiting protein denaturation is notable, it does not yet match that of diclofenac sodium.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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