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In Vitro Antiinflammatory Activity of Bajakah (*Spatholobus littoralis*) Stem Extract

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Abstract. The plant of Bajakah tampala (*Spatholobus littoralis* Hassk) has been utilized in traditional medication. Previous studies have proven the existence of in vivo anti-inflammatory activities of Bajakah plant (*S. littoralis*) in lowering the degree of carrageenan-induced paw oedema in mice. This study aims to determine the anti-inflammatory mechanism of *S. littoralis* extract *in vitro* through an approach of enzyme inhibition involved in the inflammatory reaction. The concentration of ethanol extract of Bajakah used was 0.1; 0.2; 0.4; 0.8; 1.6 mg/ml. The parameters measured were lipoxygenase enzyme inhibition, protein denaturation inhibition, protease enzyme inhibition, as well as plasma membrane stabilization. The results of the study showed the potential of the ethanol extract of Bajakah stems in inhibiting the inflammatory process viewed from the ability to inhibit inflammation-related enzymes. *S. littoralis* extract concentration of 1.6 mg/ml showed the best inhibition of the protein denaturation process (75.9%), the inhibition of trypsin protease enzyme (26.1%) and the stability of erythrocyte membrane (93.7%). However, the extracts of *S. littoralis* did not provide inhibition for the lipoxygenase enzyme in the range of 0.2-3.8%. This study proves the role of *S. littoralis* extract in the anti-inflammatory mechanism. It has the potential to be developed into standardized herbs.

Keywords: enzyme, inflammation, lipoxygenase, membrane stabilization, protein denaturation

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INTRODUCTION

Inflammation is the body's response to eliminate foreign substances, physical and chemical stimuli or microorganisms which get into the body, which is followed by a process of tissue repair. Leukocytes and inflamed tissues will release various inflammatory mediators which aim to hinder invasion and eliminate foreign pathogens. The excessive inflammatory response will induce the increase of free radical levels such as reactive oxygen species and reactive nitrogen species which can cause oxidative stress, cell mutations, DNA damage and contribute to several diseases such as cancer, cardiovascular disease and metabolic disorders (Pizzino *et al*., 2017). The medication for inflammation generally uses non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids. However, long-term use of NSAIDs can cause complications in the form of gastric irritation, ulcer formation in the digestive tract, liver and kidney damage (Harirforoosh *et al*., 2013). Therefore, one of the alternatives to inflammation treatment is to use plants containing bioactive compounds.

The genus *Spatholobus* has been known as a traditional medicine which consists of various bioactive compounds. The distribution of Spatholobus plants takes place in tropical and subtropical Asia (Ninkaew & Chantaranothai, 2014). Among 29 species belonging to the genus Spatholobus, species *S. littoralis* Hassk. and *S. suberectus* Dunn has been studied as medicinal plants. The results of the phytochemical test conducted by Saputera *et al*. (2019) stated that the ethanolic extract of *S. littoralis* positively contains groups of alkaloid, flavonoid and steroid compounds. Some of the pharmacological potentials of *the S. suberectus* Dunn species of the Bajakah plant are to treat peripheral vascular inflammation and thrombosis (Shao *et al*., 2017), as an anti-mutagenic (Inami *et al*., 2017) and to inhibit estrogen receptors in breast cancer (Sun *et al*., 2016). In addition, Novanty *et al*. (2021) stated that the ethanolic extract of *S. littoralis* was able to reduce the ROS levels, visceral fat as well as body weight in obese Wistar rats.

Research conducted by Rousdy *et al*. (2022) has proven the anti-inflammatory effect of ethanol extract of bajakah stem (*S. littoralis*) in vivo by using carrageenan-induced test animal models. The ethanol extract of *S. littoralis* stem at a dose of 2.5 mg/kg had the best inhibition of inflammation in the rat paw oedema of 19.21%,

close to the inhibitory value of diclofenac sodium positive control at a dose of 30.8 mg/kg (21.53%). According to this background, it is necessary to conduct further in vitro studies on the anti-inflammatory activities of the ethanol extract of *S. littoralis* stem observed from the inhibition of enzymes in the inflammatory response. The study aims to figure out the in vitro anti-inflammatory activities of the ethanol extract of *S. littoralis* stem viewed from its ability on lipoxygenase enzymes inhibition, proteinase inhibition, protein denaturation inhibition and hemolytic inhibition of red blood cells. This research contributes to the development of standardized herbal medicines in Indonesia.

METHODS

Plant collection and extract preparation

Bajakah stems (*S. littoralis*) were collected from Ambawang District, West Borneo. The stem was washed and powdered, sieved using 25 mesh sieves. Bajakah stems were macerated with ethanol 96% for 3 days. Every 24 hours, the extract is filtered and replaced with a new ethanol solvent. The extract was concentrated using a low-pressure rotary evaporator at a temperature of 60 °C with a 30 rpm rotating speed.

Experimental design

Bajakah ethanol extract was made into serial concentrations of 0.1; 0.2; 0.4; 0.8; 1.6 mg/ml. Each concentration was repeated three times. The stock solution was made at a concentration 3 mg/mL and then diluted according to serial concentrations.

Buffer solution preparation

The composition of the phosphate buffer solution (10 mM, pH 7.4) consisted of NaH_2PO_4 (339 mg) and Na_2HPO_4 (2.021 g). All salt dissolved in one litre of sterile distilled water, pH value was adjusted to 7.4 with the addition of HCl or NaOH. The composition of the borate buffer solution (0.1 M, pH 7.4) consisted of boric acid H_3BO_3 (6.18 g) and NaOH (1 g) dissolved in 800 mL distilled water. The volume of the solution was made up to 1 L pH 8.8. The composition of the phosphate-buffered saline solution (PBS 10 mM, pH 7.4) consisted of NaCl (9 g), Na_2HPO_4 (1.15 g), NaH_2PO_4 (0.26 g). All salts were homogenized in 1 litre volume of distilled water and the pH value was adjusted to 7.4. All buffer solution is stored in a dark airtight bottle.

Erythrocyte suspension preparation

The preparation of erythrocyte suspension is referred to Shinde *et al*. (1999). Human blood samples were taken from healthy subjects not taking NSAID inflammatory drugs. Blood samples that had been added with EDTA anticoagulant were centrifuged at 3000 rpm for 5 minutes. The supernatant was separated, and then the pellet was washed with 0.9% NaCl with the same volume. The blood volume was then made to a concentration of 10% (v/v) using a phosphate buffer solution (10 mM, pH 7.4).

Inhibition of erythrocyte membrane haemolysis

Suspension of red blood cells (0.05 mL) was mixed with 0.05 mL of bajakah ethanol extract and 2.95 mL of phosphate buffer pH of 7.4. The mixture was incubated at 54 °C for 20 minutes. After incubation, the mixture was centrifuged at 2500 rpm for 3 minutes. The supernatant absorbance was measured using a UV-vis spectrophotometer at 540 nm wavelength. Control solution using phosphate buffer. The hemolysis value was calculated using the formula (Okoli *et al*., 2008):

$$Haemolysis\ inhibition(\%) = 1 - \left[\frac{\text{Abs sample}}{\text{Abs control}}\right] x100$$

Protein denaturation inhibition

The protein denaturation test was carried out according to the method of Gambhire *et al*. (2009) and Gunathilake *et al*. (2018). 0.2 mL bovine albumin solution 1% was mixed with 4.78 mL PBS and 0.02 mL of sample extract. The mixture was incubated at 37 °C for 15 minutes and heated at 70 °C for 5 minutes. After cooling, the absorbance was measured at a 660 nm wavelength. PBS buffer was used as a control. Calculation of protein denaturation inhibition using the following formula (Gunathilake *et al*., 2018):

$$Protein\ denaturation\ inhibition\ (\%) = 1 - \left[\frac{\text{Abs sample}}{\text{Abs control}}\right] x100$$

Inhibition of protease trypsin

Trypsin inhibition test using the method of Sakat *et al*. (2010) and Gunathilake *et al*. (2018). The total volume reaction of 2 mL consisted of 0.06 mg trypsin, 1 mL of 20 mM Tris-HCl buffer (pH 7.4) and 1 mL sample. Bajakah extract (0.02 mL) was mixed with methanol (0.980 mL) using as a sample. The mixture was incubated at 37 °C for 5 minutes. After incubation 1 mL casein (0.8%,

w/v) as substrate was added and the mixture was incubated for 20 minutes. Perchloric acid 70% was added to stop the catalytic reaction. The mixture was centrifuged and the supernatant absorbance was measured at 210 nm wavelength. PBS solution was used as a control. Calculation of the percentage trypsin inhibition using the following formula (Gunathilake *et al*., 2018).

$$Trypsin\ inhibition\ (\%) = 1 - \left[\frac{\text{Abs } sample}{\text{Abs control}}\right] x100$$

Inhibition of lipoxygenase

Lipoxygenase inhibition test using the method of Wu *et al*. (1996) and Gunathilake *et al*. (2018). The sodium borate buffer solution (1 mL, 0.1 M, pH 8.8) was mixed with lipoxygenase (10 L, final concentration 8000 U/mL) and 10 mL of bajakah extract. The mixture was incubated in a cuvette at room temperature (30 ℃) for 5 minutes. The reaction of the enzyme activity was started with the addition of a substrate linoleic acid (10 mmol). Then the absorbance was measured at a wavelength of 234 nm. PBS was used as a control. Calculation of the percentage lipoxygenase inhibition using the following formula (Gunathilake *et al*., 2018):

$$Lipoxygenase\ inhibition\ (\%) = \left[\frac{(\text{Abs control} - \text{Abs sample})}{\text{Abs control}}\right] x100$$

Data analysis

The results were analyzed by a One-Way ANOVA test with 95% confidence level. If there is a significant difference between concentrations, the analysis is continued with Duncan's Multiple Range Test.

RESULTS AND DISCUSSION

Inhibition of erythrocyte membrane haemolysis

According to the results of this study, it was found that the ethanol extract of Bajakah stems had the potential to inhibit the process of heat-induced hemolysis of red blood cells. The extract of Bajakah stems with a concentration of 0.8 mg/mL showed the best membrane protection activity of 99.92% and a concentration of 1.6 mg/ml provided a protection activity of 93.73%. The extract with a concentration of 0.4 mg/mL indicated the lowest result of erythrocyte membrane protection at 88.95% (Table 1).

Table 1. Inhibition percentage of erythrocyte membrane haemolysis

Extract concentration (mg/ml)	Absorbance of heat incubation	Absorbance of cold incubation	Haemolysis inhibition (%)
0.1	0.356	0.329	$92.38{\pm}7.86^{ab}$
0.2	0.358	0.329	$91.63{\pm}5.52^{ab}$
0.4	0.357	0.316	$88.95{\pm}9.74^{a}$
0.8	0.345	0.340	$99.92{\pm}3.04^{b}$
1.6	0.277	0.251	$93.73{\pm}1.27^{ab}$

Data are expressed as average±standart deviation. Different superscript letter in the same column showed significant difference (P<0.05)

The erythrocyte hemolysis inhibition method uses the erythrocyte membrane as a model for the lysosomal membrane. Hemolysis is the rupture of erythrocytes in a hypotonic solution or heat induction which destroys the membrane stability. Exposure of erythrocytes to hazardous substances or hot temperatures will cause erythrocytes to lyse. During the inflammatory response, lysosomal organelles in cells experiencing inflammation are lysed or ruptured, to release lysosomal enzymes. The lysosome organelle regulates the release of pro-inflammatory cytokines and anti-inflammatory cytokines depending on stimulus signals (Bonam *et al*., 2019). Lysosomal-related organs are found in neutrophils, eosinophils, basophils, mast cells, T cells and platelets. Lysosomal membrane permeability is affected by various stress factors. Damage of lysosomal membrane permeability leads to the release of intralysosomal components into the cytoplasm, such as cathepsins, inducing an increase in cytosolic pH, unregulated breakdown of cell components and cell death (Wang *et al*., 2018; Sheshacalam *et al*., 2014). Degranulation inhibition of lysosomal enzymes and stabilization of lysosomal membrane is one of the importance mechanism of non-steroidal anti-inflammatory drugs (NSAIDs) indomethacin. Indomethacin 0.2 mg/ml showed 43.98% inhibition of heat-induced haemolysis (Paul *et al*., 2021). The stabilization of

the lysosomal membrane is an important step in the inhibition of inflammation.

Figure 1. The incubation process of the erythrocyte hemolysis test

The other results showed that the ethanol extract of Bajakah stem was able to protect erythrocyte membranes, as a model for lysosomal membranes. Therefore, the ability to maintain the stability of the membranes is one of the mechanisms of anti-inflammatory drugs to prevent the release of hydrolytic enzymes and other inflammatory mediators from lysosomes. The inhibition value of erythrocyte hemolysis from the ethanol extract of the Bajakah stem exposed a greater percentage of inhibition (88 - 99%) than the methanol extract of *Solanum aethiopicum* with the range of the inhibitory value of 46 - 86% (Anosike *et al*., 2012) and *Aloe vera* gel homogenate with inhibitory value 20% (Paul *et al*., 2021). The content of flavonoids and terpenoids in the *S. littoralis* extract is believed to be able to stabilize the lysosomal membrane by binding cations. Tinocrisposide compounds, a terpenoid group from the Brotowali plant (*Tinospora crispa*) showed an increase the stability of erythrocyte membranes (Adnan *et al*., 2019).

Inhibition of protein denaturation

The results of this study showed that the ethanol extract of Bajakah stem had the potential to inhibit the denaturation process of albumin protein. The highest inhibition was given by the extract concentration of 1.6 mg/ml with an inhibitory value of 75.98%. Meanwhile, the lowest inhibition was given by the extract concentration of 0.1 mg/ml of 10.74%. The higher the extract concentration, the higher the inhibition of albumin protein denaturation (Table 2).

In the inhibition test of protein denaturation, the induction of denaturation was carried out by heating at a temperature of 70ºC, causing the albumin to be denatured. Tissue damage during inflammation is often characterized by protein denaturation. When a protein is heated and denatured, it triggers the formation of autoantigens, which are the antigens against the body's cells. Autoantigens are associated with type III hypersensitivity reactions. The manifestations of type III hypersensitivity reactions such as rheumatoid arthritis (Patel & Ziveri, 2014). The mechanism of denaturation likely involves changes in protein conformation structure, hydrogen bonds, hydrophobic bonds and disulfide bonds. Inhibition of albumin protein denaturation at pathological pH (6.2-6.5) is associated with the inflammatory activity of various NSAID drugs (Hasan, 2019). Several NSAID anti-inflammatory drugs such as indomethacin and phenylbutazone, not only do they inhibit the cyclooxygenase enzyme, but also prevent protein denaturation (Elisha *et al*., 2016).

Table 2. Inhibition percentage of albumin denaturation, trypsin and lipoxygenase activity

Extract concentration (mg/mL)	Absorbance of heat incubation	Absorbance of cold incubation	Denaturation albumin inhibition (%)
0.1	0.008	0.009	10.74±0.64[a]
0.2	0.007	0.008	11.11±11.1[a]
0.4	0.007	0.011	33.23±3.18[b]
0.8	0.009	0.014	37.46±18.58[b]
1.6	0.008	0.032	75.98±2.43[c]

Data are expressed as average±standart deviation. Different superscript letter in the same column showed significant difference (P<0.05)

Flavonoid compounds, saponins and tannins are found in the stems of bajakah *S. littoralis* (Saputera *et al*., 2019). Secondary metabolite compounds which are antioxidants are closely related to the immunomodulatory activity (Fitmawati *et al*., 2017). Yesmin *et al*. (2020) stated that several phenolic compounds and alkaloids were responsible for the activity. Molecular analysis also showed that a strong interaction of the flavonoid quercetin caused the formation of hydrophobic, electrostatic and hydrogen bonds of albumin, and therefore increased the thermal stability of albumin (Precupas *et al*., 2016). Phenolic compounds in the aqueous ethanol extract of the *Ribes nigrum* plant also have an anti-inflammatory effect by inhibiting the protein denaturation process and maintaining cell membrane stability (Hasan, 2019).

Inhibition of protease and lipoxygenase

The results of the trypsin protease inhibition test showed that the higher the concentration of *S. littoralis* extract, the greater the ability to inhibit trypsin enzyme activity. The extract concentration of 1.6 mg/ml indicated that the highest inhibition of trypsin protease was 26.13% (Table 3). The trypsin enzyme used in this study was included in the group of protease enzymes. Protease or proteinase is a proteolytic enzyme that plays a significant role in physiological functions such as the processes of digestion, blood clotting, blood pressure control as well as immune response. Excessive protease activity is associated with severe diseases such as cancer, muscle weakness, pulmonary emphysema, and arthritis. Hence, potential anti-inflammatory drugs are believed to work as protease inhibitors.

Table 3. Inhibition percentage of trypsin and lipoxygenase inhibition

Extract concentration (mg/mL)	Inhibition (%)	
	Trypsin	Lipoxygenase
0.1	25.86±0.70[a]	3.812±2.34[a]
0.2	25.98±0.32[a]	1.261±2.50[ab]
0.4	26.03±0.23[a]	2.348±1.19[ab]
0.8	25.93±0.11[a]	1.435±0.63[ab]
1.6	26.13±0.44[a]	0.217±0.10[b]

Data are expressed as average±standart deviation. Different superscript letter in the same column showed significant difference ($P<0.05$)

Protease enzymes work to degrade collagen fibres and the proteoglycan matrix of connective tissue, bone and cartilage. This destructive effect is worsened by the cytokine IL-1 and TNF alpha which suppress the synthesis of bone matrix, collagen and proteoglycans. Moreover, neutrophil cells are known to contain many proteinases in their lysosomes (Patel & Zaveri, 2014). During the inflammatory response, lysosomes will release hydrolytic enzymes of protease to digest foreign substances which get into the cells. The inhibition of protease activities will play a role in preventing the spread of inflammation to other tissues that are still normal.

The protease inhibition of trypsin is closely related to the content of phenolic compounds, flavonoids and terpenoids in plants (Gunathilake *et al*., 2018). Spatholobus *littoralis* are known to contain secondary metabolites such as flavonoids, saponins and tannins (Saputera *et al*., 2019). The inhibition of the elastase enzymes in neutrophil cells was also found in *S. suberectus* which contains phenolic compounds (Huang *et al*., 2013). The flavonoids such as quercetin, luteolin, kaempferol and apigenin have been investigated to be able to inhibit the trypsin enzymes. The percentage of flavonoid inhibition is determined by the number and position of the flavonoid hydroxyl groups (Li *et al*., 2013).

The results of the lipoxygenase inhibition test revealed that the highest inhibition was found in the extract concentration of 0.1 mg/mL, with an inhibition value of 3.812%. On the other hand, the highest extract concentration of 1.6 mg/mL, provided the lowest inhibition of lipoxygenase enzyme, which was 0.217% (Table 3). Lipoxygenases (LOXs) are a group of oxidative enzymes which catalyze the arachidonic acid substrate into hydroperoxyeicosetraenoic acids (HPETEs). Then, HPETE is catalyzed again by lipoxygenase to leukotrienes and other inflammatory factors leukotrienes or anti-inflammatory mediators (Wisastra *et al*., 2014).

.The inhibition of the lipoxygenase enzyme depends on the dose or the extract concentration. The inhibition value of the lipoxygenase enzyme provided by the ethanol extract of Bajakah *S. littoralis* was not consistent with the increasing

dose and was smaller (0.2 - 3.8%) than the ethanol extract of *Dissotis thollonii* of 66.79 to 95.31%. This thing is assumed to be caused by the different types of secondary metabolites, particularly flavonoids which are contained in the extract. The active site of the lipoxygenase enzyme is composed of non-heme iron atoms. The lipoxygenase inhibitors are thought to work by blocking one or two histidine residue ligands surrounding Fe (Rissyelly *et al*., 2022). The bonding of flavonoids and ligands is strongly affected by the polarity of the compound and the number of hydroxyl groups which form flavonoids. Therefore, it is presumed that the type of flavonoid in the extract of Bajakah *S. littoralis* is less polar so that it cannot block the active site of lipoxygenase. However, it is necessary to conduct further research regarding this matter. Further research on the inhibition of the lipoxygenase enzyme can be carried out towards the expression of the lipoxygenase gene.

CONCLUSION

The ethanol extract of *Spatholobus littoralis* Hassk. stems showed the ability of enzyme inhibition which is associated with the inflammatory process. The extract concentration of 1.6 mg/ml revealed that it is the best protein denaturation inhibition, trypsin protease inhibition and heat-induced hemolysis of erythrocytes inhibition. The extract concentration of 0.1 mg/ml showed the best inhibition of the lipoxygenase enzyme. Based on this research, the ethanol extract of *S. littoralis* stems can be developed as an anti-inflammatory drug. Suggestions for further research are to use *in silico* molecular docking analysis and gene expression in studying the inhibitory activity of enzymes involved in inflammation.

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