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 Saputra 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 NaH₂PO₄ (339 mg) and Na₂HPO₄ (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 H₃BO₃ (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), Na₂HPO₄ (1.15 g), NaH₂PO₄ (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 made up to 1 litre volume of distilled water and the pH value was adjusted to 7.4 with the addition of HCl or NaOH. The composition of the phosphate buffer solution (10 mM, pH 7.4).

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).

\[
\text{Trypsin inhibition (\%)} = 1 - \left[ \frac{\text{Abs sample}}{\text{Abs control}} \right] \times 100
\]

### Inhibition of lipoxigenase

Lipoxigenase 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 lipoxigenase (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 lipoxigenase inhibition using the following formula (Gunathilake et al., 2018):

\[
\text{Lipoxigenase inhibition (\%)} = \left( \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \right) \times 100
\]

### 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>
<thead>
<tr>
<th>Extract concentration (mg/ml)</th>
<th>Absorbance of heat incubation</th>
<th>Absorbance of cold incubation</th>
<th>Haemolysis inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.356</td>
<td>0.329</td>
<td>92.38±7.86&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.2</td>
<td>0.358</td>
<td>0.329</td>
<td>91.63±5.52&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.4</td>
<td>0.357</td>
<td>0.316</td>
<td>88.95±9.74&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.8</td>
<td>0.345</td>
<td>0.340</td>
<td>99.92±3.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.6</td>
<td>0.277</td>
<td>0.251</td>
<td>93.73±1.27&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

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 lyed 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 70°C, 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>
<thead>
<tr>
<th>Extract concentration (mg/mL)</th>
<th>Absorbance of heat incubation</th>
<th>Absorbance of cold incubation</th>
<th>Denaturation albumin inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.008</td>
<td>0.009</td>
<td>10.74±0.64&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.2</td>
<td>0.007</td>
<td>0.008</td>
<td>11.11±11.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.4</td>
<td>0.007</td>
<td>0.011</td>
<td>33.23±3.18&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.8</td>
<td>0.009</td>
<td>0.014</td>
<td>37.46±18.58&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.6</td>
<td>0.008</td>
<td>0.032</td>
<td>75.98±2.43&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are expressed as average±standard 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 metabolites include antioxidants that are closely related to the immunomodulatory activity (Fitmawati et al., 2017). Yesmin et al. (2020) stated that several phenolic compounds and alkanoids 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 proteasease 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

<table>
<thead>
<tr>
<th>Extract concentration (mg/mL)</th>
<th>Trypsin (%)</th>
<th>Lipoxygenase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>25.86±0.70&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.812±2.34&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.2</td>
<td>25.98±0.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.261±2.50&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.4</td>
<td>26.03±0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.348±1.19&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.8</td>
<td>25.93±0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.435±0.63&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.6</td>
<td>26.13±0.44&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.217±0.10&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are expressed as average±standard 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 hydroperoxyeicosatetraenoic 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
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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REFERENCES


from the Stems of *Spatholobus suberectus* Using LC-DAD-MSn and Their Inhibitory Effect on Human Neutrophil Elastase Activity. *Molecules*, 18, 7549-7556.


