

Bittern as an Anti-Inflammatory on the Skin of Swiss Strain Mice

D. Pringgenies^{1*}, A. Ridlo¹, N. Alifia¹, and D. Ariyanto²

¹*Department of Marine Sciences, Faculty of Fisheries and Marine Sciences, Diponegoro University, Semarang, Central Java, 50275 Indonesia*

²*Research Center for Oceanography, National Research and Innovation Agency (BRIN), Jakarta, Indonesia*

The study aimed to determine the quality of bittern lotion. Furthermore, this research aimed to determine the potential of bittern lotions as anti-inflammatory tests on the skin of Swiss Strain Mice using histological analysis. The bittern sample used was obtained from a salt pond in Kedung Mutih, Demak District, Central Java Province, Indonesia. The research was conducted by making lotion bittern in the concentrations of 20 percent, 35 %, and 50 %, followed by testing lotion bittern. The data were then analysed by One-Way ANOVA, LSD test, and Independent Samples T-test. Results showed that the three bittern lotions have potential as an anti-inflammatory, with the best results being 35% bittern lotion with an inflammatory inhibition (PI) level of 29.37%. However, based on histological analysis of the number of inflammatory cells (skin tissue), there was a significant difference ($P < 0.05$). Thus, it can be concluded that bittern lotion has the potential as an anti-inflammatory lotion.

Keywords: Bittern; histological analysis; Inflammatory; salt pond; Lotion

I. INTRODUCTION

Inflammation is a defence response to tissue damage caused by physical trauma, harmful chemicals, or pathogenic microbes (Chen *et al.*, 2018). This might result in the cardinal signs of inflammation, such as pain, heat, swelling, redness, or even loss of function in the affected area (Wutsqo & Budiman, 2018). Inflammation itself aims to protect the body, but an uncontrolled inflammation will cause disease (Oh *et al.*, 2012). Generally, inflammatory reaction is divided into two phases called the acute phase and the chronic phase (Kolawole, 2013). Acute inflammation is the body's first line of defence against damage. Inflammation is local and temporary, which is usually characterised by a buildup of fluid in the affected area (Beg *et al.*, 2011). If the stimulus that triggers the inflammatory response is not removed in time, inflammation will proceed to a chronic phase. Chronic inflammation can lead to tissue damage and fibrosis (Furman *et al.*, 2019).

Skin is the largest organ and protect the internal organs, it also involves in the body temperature regulation and homeostasis. As the largest organ, it is more likely to expose to many external harms such as injury, free radical, infection, etc. Skin is a barrier between the body and the external environment that its' essential purpose is to protect the body from pathogens that cause disease and traumatic injury (Lin *et al.*, 2018), so the development of topical anti-inflammatory agents is needed. Some drugs used for inflammation treatment include steroids, non-steroidal anti-inflammatory drugs (NSAIDs), and immunosuppressants. These drugs have serious side effects when used for a long time (Xu *et al.*, 2017).

The search for ingredients from nature that do not have serious side effects is greatly necessary (Azab *et al.*, 2016). Anti-inflammatory drugs are known to have side effects when used for a long time (Oh *et al.*, 2012), so it is necessary to look for natural ingredients that do not have side effects (Ghasemian *et al.*, 2016).

*Corresponding author's e-mail: delianispringgenies@lecturer.undip.ac.id

Bittern is a residual liquid resulting from the process of making sea salt (Jaya *et al.*, 2016). Bittern contains a wide variety of minerals (NaCl) (Pratama *et al.*, 2016). In general, bittern contains elements needed by the human body such as NaCl, MgSO₄, MgCl₂, KCl, and NaBr and microelements such as iodine, molybdenum, selenium, zinc. Research done by (Kim *et al.*, 2010), revealed that the high mineral content is proven can overcome inflammatory problems on the skin. Magnesium, as the main mineral content in bittern, is also known for its ability to reduce inflammation levels (Nielsen, 2018). Therefore, bittern can be used as a topical anti-inflammatory lotion for the skin. The study aimed to determine the quality of bittern lotion by physical testing, pH test, emulsion type test, dispersion test, adhesion test, and viscosity test and to determine the potential of bittern lotion as an anti-inflammatory by testing the skin of Swiss Strain Mice with histological analysis.

II. MATERIALS AND METHOD

A. Study area

This research was conducted in March-August 2021. The material used in this research was bittern obtained from salt ponds in Kedung Mutih, Demak, Java, Indonesia. Bittern was then used as a lotion for easier application to the skin surface. The lotion was then tested for anti-inflammatory using 2-3 months old male mice (*Mus musculus*) obtained from Java Rat Labs, Semarang (No:41/EC/II/FK-UNDIP/IV/2021).

B. Lotion

The lotion was made with the methods of (Pujiastuti & Kristiani, 2019). The lotion was made with three variations of bittern concentration in the preparation of 20, 35, and 50% (w/w?). Each formula was made in 100 g. The physical quality test of the lotion consisted of a physical test, pH test, viscosity test, spread ability test, adhesion test, and lotion type test. The test method is carried out using the reference of (Megantara *et al.*, 2017). An anti-inflammatory activity test was carried out using male mice of about 2-3 months. Mice were divided into seven groups in each group consisting of 5 mice. Mice were adapted in cages for a week

for the acclimatisation process while maintaining their food and drinking needs.

Mice were divided into 7 groups with each group consisting of 3 mice. Mice were adapted in cages for 1 week for the acclimatisation process while maintaining their food and drinking needs. Group I was a healthy control group, where the test animals did not receive any treatment. Group II is a positive control, where the test animals that have been induced by inflammatory agents are given anti-inflammatory drugs on the market, namely hydrocortisone cream which is known to treat inflammation. Group III is a negative control group, where the test animals were induced by inflammatory agents without being given anti-inflammatory agents. Group IV is a basic control group that received inflammation induction treatment and placebo lotion. Groups V, VI, and VII were treated with inflammation induction and administration of bittern lotion 20%, 35%, and 50%.

The procedure for induction of inflammatory agents is as follows. First, the back of the mice was shaved clean and then 0.1 ml of 2% carrageenan was induced subcutaneously on the back skin of the mice. The application of 0.1 g of lotion was carried out 1 hour after the inflammation appeared which was marked by white patches on the back of the mice. The diameter of inflammation on the backs of mice was then measured every 1 hour for 6 hours using a digital calliper. The mice were sacrificed, and the back tissue of the mice was taken to make Haematoxylin Eosin (HE) staining preparations.

C. Histology

Histology of Mice Skin of Preparations were made on mice that had been treated according to the method of (Alturkistani, Tashkandi & Mohammedsaleh, 2015). Then, histological preparations of the tissue were made for Haematoxylin Eosin (HE) staining. The quality parameters used in this study were the thickness of the epidermis and the number of inflammatory cells (Tewari-Singh *et al.*, 2009; Nguyen & Soulika, 2019). Calculation of the number of inflammatory cells was done by dividing one field of view into three parts. Calculation of the total inflammatory cells was also repeated three times for each treatment.

D. Statistical Analysis

The data from the anti-inflammatory test were then statistically analysed using One-Way ANOVA with a follow-up LSD test using a 95% confidence level. The data from the histology of the skin tissue of mice were analysed using the Independent Samples T-test.

III. RESULT AND DISCUSSION

A. Lotion Quality

The lotion quality test in this study included a physical test, pH test, emulsion type test, spread ability test, adhesion test, and viscosity test. Results of physical tests on lotion bittern 20, 35, and 50%, lotion placebo showed that each form was solid, the colour was milky white, and the smell was the typical smell of a lotion. The pH test, or the acidity value of the bittern lotion with concentrations of 20, 35, and 50%, and the placebo lotion in this research are increasing with the concentration. 6.3, 6.5, 6.6 and 6.5, respectively, ranged from pH 6.33 - 6.63. Lotion with 20% bittern had the smallest pH value of 6.33, and lotion with 50% bittern had the highest pH value of 6.63. Meanwhile, bittern 35% has a pH value of 6.53 and placebo lotion has a pH value of 6.47.

The Emulsion Type Test of the four lotions showed the same type of emulsion, which was the type of oil in water or O/W. The Spread ability Test of the four lotions ranged from 8.4 – 10 cm. The lotion of 50% bittern has the lowest spread ability among the four lotions, namely 8.4 ± 0.27 cm. Meanwhile, the lotion with the highest spread ability was 35% bittern lotion with an average spread of 10 cm. While

20% bittern lotion has a spread of 9 cm and Placebo Lotion has a spread of 9.2 cm.

Results of the adhesion test of the four lotions ranged from 1.27 to 1.66 sec. The lotion with the lowest adhesion was bittern lotion 35% with a time of 1.27 seconds, while the highest adhesion was lotion bittern 20% with a time of 1.66 sec. The lotion of 50% bitter was 1.40 sec, and the Placebo lotion was 1.50 sec. Viscosity test or the thickness of the four lotions obtained high values. Lotion with the lowest viscosity was the lotion of 20% bittern with a viscosity value of 722×10^3 . Meanwhile, the lotion with the highest viscosity was 526×10^3 , and the placebo lotion with a value of 526×10^3 .

Anti-Inflammatory Test: The inflammation formed on the back skin of mice was in the form of white patches (Figure 1). Then, the diameter changes were recorded. Results of the diameter measurement of the inflammatory area can be seen in Table 1. Results of the diameter data were then used to calculate the inflammation area, which can be seen in Table 1.



Figure 1. Inflammation of the back skin of mice

Table 1. Diameter and Large of inflammation area at zero hour (mm²)

Treatment	Control	Control (-)	Control (+)	20% Bittern	35% Bittern	50% Bittern
D ₀	0	0	0	0	0	0
D ₁	0	0	0	0	0	0
D ₂	0	0	0	0	0	0
D ₃	2.87	1.83	2.20	3.24	2.76	3.52
D ₄	2.86	1.59	2.02	2.76	2.52	2.94
D ₅	2.64	1.56	1.90	2.44	2.33	2.37
D ₆	2.51	1.48	1.79	2.31	2.14	2.28
A ₀	0	0	0	0	0	0
A ₁	0	0	0	0	0	0

A₂	0	0	0	0	0	0
A₃	9.00	12.24	7.15	12.46	8.35	10.57
A₄	8.43	9.46	4.93	9.26	7.07	7.23
A₅	7.12	8.73	4.34	7.38	6.10	5.58
A₆	6.47	7.36	3.85	6.47	5.15	5.18

Note: D₀ : diameter measurement at zero hour onwards, A₀ : inflammation area at zero hour onwards.

Data of the inflammation area was then used in the AUC (Area Under Curve) calculation. The total AUC value for each treatment group can be seen in Table 2. The negative control group has the highest average total AUC value, with an average AUC of 34.11 mm² h. Meanwhile, the group with the lowest average total AUC value was the positive control group, with an average AUC of 18.35 mm² h. Then the total AUC data was used in calculating the inflammation inhibition percentage (PI), which can be seen in Fig 2. The highest PI value was in the positive control group, namely 46.21%. The lowest PI value was 20% bittern, with a PI value of 5.19%.

Table 2. AUC Value of Each Group

Treatment	AUC (mm² h)
Base control	27.79 ± 5.00
Negative control	34.11 ± 2.26
Positive control	18.35 ± 4.72
20% Bittern	32.34 ± 8.08
35% Bittern	24.09 ± 3.89
50% Bittern	25.97 ± 4.38

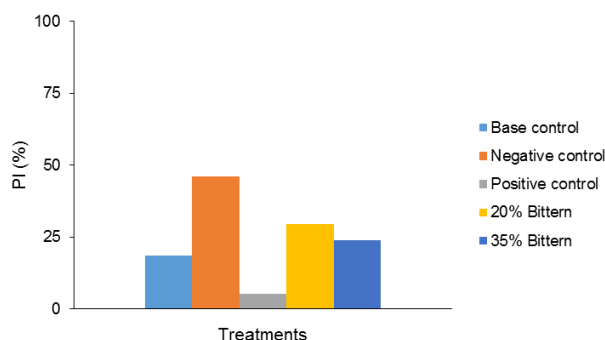


Figure 2. Inflammation Inhibition Percentage (%)

B. Skin Tissue Histology

Results of the skin tissue histology of mice that have previously been tested for anti-inflammatory can be seen in Figure 3. Results of the screening of epidermal thickness and the number of inflammatory cells in the skin tissue of mice for each treatment can be seen in Table 3.

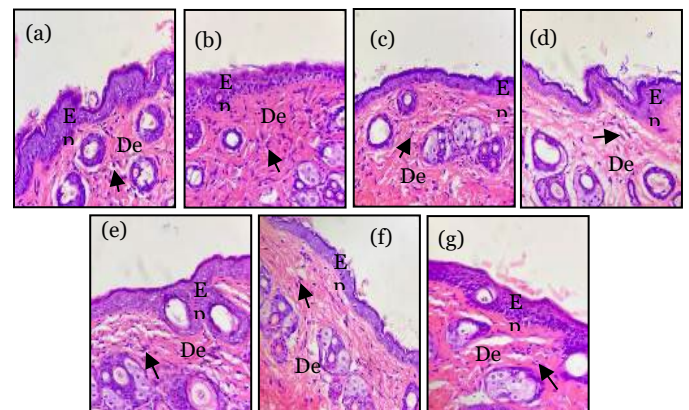


Figure 3. Microphotography of the back skin tissue of the mice with 40x magnification Ep: Epidermis. Der: Dermis. Arrow: inflammatory cells. (Note: (a) healthy control, (b) negative control, (c) base control, (d) positive control, (e) 20% bittern, (f) 35% bittern, (g) 50% bittern)

Results of histological analysis of the skin tissue of mice showed the number of inflammatory cells that filled 1/3 of the sight view were in the group of healthy control, positive control, 20% bittern, 35% bittern, and 50% bittern. The number of inflammatory cells for the base control group filled 2/3 of the sight field, while for the negative control group, the number of inflammatory cells filled the entire sight field. Results of the analysis for the thickness of the epidermis from each treatment group showed no significant difference.

Table 3. Epidermal Thickness (ET) and Inflammatory Cell Total

Treatment	ET (μm)	Inflammatory Cell Total
Healthy control	8.00 \pm 1.79	Inflammatory cells fill 1/3 of the sight field
Negative control	7.17 \pm 1.83	Inflammatory cells fill the entire sight field
Positive control	7.67 \pm 2.42	Inflammatory cells fill 1/3 of the sight field
Base control	8.33 \pm 1.97	Inflammatory cells fill 2/3 of the sight field
20% bittern	10.33 \pm 2.50	Inflammatory cells fill 1/3 of the sight field
35% bittern	12.83 \pm 3.76	Inflammatory cells fill 1/3 of the sight field
50% bittern	9.83 \pm 2.04	Inflammatory cells fill 1/3 of the sight field

The active ingredient used in the manufacture of this anti-inflammatory lotion was bittern. Bittern was obtained from the salt-making residual, where seawater can no longer crystallise into salt. This process causes bittern to have a very high salinity with very high minerals (Raesta *et al.*, 2017). Magnesium is the main mineral with the highest concentration in bittern (Marselina *et al.*, 2015). Based on the research of (Kim *et al.*, 2010), high minerals can relieve inflammatory problems on the skin, so the high mineral content in bittern is thought to have anti-inflammatory potential.

The lotion quality test was carried out to ensure that the lotion applied to the skin did not cause irritation and provided a comfortable feeling (Megantara *et al.*, 2017). Based on the physical test, the four lotions have the same shape, colour, and smell, namely in the form of a semi-solid, milky white colour and a distinctive smell of the lotion. This physical test was carried out to determine the level of fairness and comfort of the lotion when applied (Widiputri *et al.*, 2020).

The value of the degree of acidity or pH is one of the essential characteristics because it affects solubility, activity, absorption, and comfort (Widiputri *et al.*, 2000). Measurement of pH is vital to avoid irritation. The pH value should be in the proper range for the skin. The pH value of a good lotion for skin based on SNI 16-4399-1996 is in the

range of 4.5 – 8. If the pH is below 4.5, the lotion is too acidic and has the potential to irritate the skin. Meanwhile, if the lotion pH is above 8, the lotion is too alkaline, which can cause scaly skin (Purwaningsih *et al.*, 2020).

The emulsion type test was carried out to determine the type of emulsion in the lotion (Megantara *et al.*, 2017). The emulsion type can be oil in water (O/W) and water in oil (W/O). The test results showed that the four lotions had an oil-in-water (O/W) emulsion type. The spreadability test was carried out to determine the ability of the lotion to spread on the skin (Dominica & Handayani, 2019). The better the dispersion, the better the medicinal ingredients dispersion because the contact between the lotion and the skin becomes extensive and easily absorbed so that the lotion can work more effectively (Lumentut *et al.*, 2020). Good dispersion ranges from 5-7 cm (SNI 1996). The results obtained from the dispersion test in this study exceeded the dispersion standard.

The viscosity test aims to see the lotion's ability to stay on the skin as indicated by the length of time the lotion is applied. Good lotion adhesion is not less than 4 seconds (Ulaen *et al.*, 2012). The longer the viscosity of the lotion, the better because it allows the active substance to be fully absorbed. Based on the test results, it was discovered that the four lotions have a viscosity of fewer than 4 seconds. The Viscosity Value or thickness determines the tendency of a semi-solid to break up into an oil phase and an aqueous phase. The viscosity also affects the therapeutic effect and comfort at the time of application. The range of viscosity values based on SNI is between 2,000 – 50,000 cP. The viscosity test of the four lotions showed results that exceeded the viscosity range based on SNI. Several factors caused the instability of the viscosity value, including the concentration of the material, temperature, and reaction.

The inflammation test was carried out by inducing an inflammatory agent of 2% carrageenan subcutaneously on the back skin of mice. Inflammation was in the form of white patches. These white patches that appear as inflammation appear at 3 hours after induction of inflammatory agents. This was in line with the statement of (Cong, Khaziakhmetova & Zigashina, 2015) that inflammation peaked at 3 hours after carrageenan induction. Area Under Curve (AUC) was used to determine inflammation in a group

of mice. In this study, the AUC value was a representation of the inflammation that appeared.

A low AUC value indicates a decrease in inflammation, meaning an AUC value close to 0 indicates a high anti-inflammatory ability, whereas a high AUC value indicates a low anti-inflammatory power (Santoso *et al.*, 2019). Based on the results of the total AUC, the negative control group has the largest total AUC value, while the positive control group has the lowest total AUC value. The AUC value was then used in calculating the percent inflammation inhibition (PI). The AUC value is inversely proportional to the percent PI, so a low AUC value indicates a high percent inhibition of inflammation (Tristantini & Amalia, 2019).

The AUC values for the group of 20%, 35% and 50% bittern lotion respectively were 32.34; 24.09; and 25.97 mm².hour. The AUC values of 35% and 50% bittern lotion were almost close to the Total AUC of positive control, 18.35 mm².hour. Unlike the lotion bittern 20%, the Total AUC value was almost close to the Total AUC of negative control, 4.11 mm².hour. The same thing was found in the basic control group, with a Total AUC of 27.79 mm².h.

Results of the total AUC calculation were known to be not significantly different ($P > 0.05$). However, the positive control group has the lowest total AUC value and has a big difference from the negative control group. This low Total AUC indicates a high level of inhibition of inflammation. Positive control has the highest level of inhibition of inflammation, which was 46.21%. Based on the calculation results, bittern lotion, which has the best ability to relieve inflammation, was the group of 35% bittern with the PI value of 29.37%, followed by the group of 50% bittern with a PI value of 23.85%. Meanwhile, 20% bittern lotion has low inflammatory inhibition of 5.19%. The base control has an inflammatory inhibition of 18.51%, higher than 20% bittern lotion but lower than the 35% and 50% bittern group. This could be due to the placebo effect in the base control group. The lower decrease in inflammation is a pseudo-effect because the placebo lotion does not contain the active ingredient. This was confirmed by the results of the skin tissue histology of mice, where the readings showed that the number of inflammatory cells in the control group was still high, which filled 2/4 of the sight field. In contrast to the 20%

bittern lotion group, the number of inflammatory cells had decreased and only filled 1/3 of the sight field.

Based on the results of microphotography readings of the skin tissue of mice, there was no significant difference in the thickness of the epidermis for each treatment group ($P > 0.05$). Meanwhile, for the number of inflammatory cells in the healthy control group, the inflammatory cells average only met 1/3 sight field. Likewise, in the positive control group and the bittern group 20%, 35%, and 50%, the inflammatory cells average only met 1/3 of the sight field. This indicates the presence of anti-inflammatory activity because the positive control group and bittern group had the same number of inflammatory cells as the healthy control group. Meanwhile, the base control group showed the number of inflammatory cells that filled 2/3 of the entire sight field. This showed the placebo lotion does not have anti-inflammatory activity. The anti-inflammatory activity of the bittern lotion group came from the addition of bittern. The negative control group was also the same as the base control group, where the number of inflammatory cells filled 2/3 to the entire sight field.

There was a significant difference in the number of inflammatory cells between the positive control group and the negative control group ($P < 0.005$). Significant differences were also shown by the positive control group and the base control group ($P < 0.05$). This shows that carrageenan induction is capable of causing inflammation (Kristanti *et al.*, 2017). Significant differences also occurred between the 20%, 35%, and 50% bittern groups and the negative control group ($P < 0.05$). This shows the anti-inflammatory activity of bittern lotion. And, there was no significant difference in the bittern group of 20%, 35%, and 50% with the positive control group ($P > 0.05$). This shows that the anti-inflammatory activity between the four groups was relatively the same (Kristanti *et al.*, 2017). The base control group was not significantly different from the negative control group ($P > 0.05$).

The placebo lotion does not have anti-inflammatory activity. Therefore, it can be said that bittern contained in bittern lotion has the potential to have anti-inflammatory activity. The bittern lotion met the lotion standards based on the physical test, pH test, and emulsion type test. Bittern lotion has potential as an anti-inflammatory agent and the

best results were obtained in a lotion with a bitter concentration of 35%.

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V. AUTHORS CONTRIBUTION

The authors have an equal contribution in this paper.

- variasi konsentrasi trietanolamin sebagai emulgator serta uji hedonik terhadap lotion', *Jurnal Farmasi Udayana*, vol. 6, no. 1, pp. 1–5.
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