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Accelerated Stability Test of Snakehead Fish Extract (*Channa striata*) and Kelulut Honey (*Heterotrigona itama*) Ointment Combination with BHT as an Antioxidant

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ABSTRACT

The oil phase of adeps lanae was used as a basis in the ointment formulation with snakehead fish extract and kelulut honey, which influences wound healing. Adeps lanae has the potential to become rancid due to the high water content in it (25-30%). The use of oil-soluble antioxidants such as Butyl Hydroxy Toluene (BHT) can overcome the rancidity in oil. This study aimed to see how adding BHT as an antioxidant affects the ointment's stability. The ointment is prepared in three different BHT concentrations: F1 (0.0075%), F2 (0.05%), F3 (0.1%), and a control for comparison. Organoleptic tests, homogeneity, spreadability, adhesion, protection power, and acid number were used to evaluate the stability of the preparation over 28 days at 40 ± 2°C / RH 75 ± 5%. The data obtained were analyzed statistically using One Way ANOVA. The addition of BHT affected the preparation's spreadability, adhesion, and acid number but did not affect the organoleptic, homogeneity, or protective power. At F3, there is a significant difference in spreadability (5.57 ± 0.21 cm) and adhesion (89.00±2.00 seconds) based on statistical analysis. F3 is the best ointment based on the physical characteristics test results and the minimum increase in acid number (8.83 mg KOH/g).

Keywords: Adeps lanae; BHT; kelulut honey; snakehead fish; ointment

INTRODUCTION

Snakehead fish (*Channa striata*) has another name *Ophiocephalus striatus* and is a freshwater fish from the Channa genus used for a long time as consumption fish (Asikin & Kusumaningrum, 2017a). Snakehead fish have a higher albumin protein content than other fish, which has led to its widespread usage as a food fish—an alternative medication mainly in the medical sector (medicinal freshwater fish) (Alviodinasyari *et al*., 2019). The albumin protein found in snakehead fish meat is beneficial for someone who suffers from hypoalbuminemia. Furthermore, it helps heal wounds post-surgery and burns (Evi & Ika, 2013). Albumin is a globular protein with good solubility in acid, salt, and water solvents (Asikin & Kusumaningrum, 2017b). Therefore, the extract of snakehead fish water used as an alternative to postoperative wound treatment only needs fewer costs than albumin serum (Tungadi *et al*., 2011).

Kelulut honey produced by *Heterotrigona itama* bees is one of the well-known types of honey on the island of Borneo. Honey is an excretory product by insects that contains many antioxidants, enzymes, and antibacterials that can accelerate cell growth (Gebremariam & Brhane, 2014) (Wineri *et al.,* 2014). The antibacterial properties of honey can treat wounds with an infection (Santosa & Riyono, 2018). Microorganisms cannot survive in kelulut honey because of its high water content and pH level, ranging from 3.2-4.5 (Karnia *et al*., 2019). Kelulut honey is known to strengthen the immune system and increase erythrocyte function, related to antibacterial, anticancer, antiseptic, anti-inflammatory, and wound healing properties (Mohd Rafie *et al*., 2018). The combination of the two active substances, in the form of a water phase of snakehead fish extract and kelulut honey, is predicted to generate a synergistic effect, increasing its effectiveness in wound healing.

A combination of the two active ingredients is formulated in an ointment using adeps lanae as the oil phase. Both active ingredients were made in ointment preparations for several purposes. The ointment can stay on the skin longer, thus protecting the injured skin from infection. In addition, the ointment forms an occlusive (water-resistant) layer on the skin's surface, which functions to prevent heat and fluid loss, thus providing a moisturizing effect (Hernani *et al*., 2012). Therefore, ointment is an adequate preparation used in wound healing. Adeps lanae has the potential to become rancid due to the high water content in it (25-30%) (Mueller, 2008). The rancidity in oil can be overcome by using antioxidants such as Butyl Hydroxy Toluene (BHT),

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Table I. Ointment Formula

Material	F0	F1	F2	F3
Snakehead fish extract	30%	30%	30%	30%
Kelulut honey	30%	30%	30%	30%
BHT	-	0.0075%	0.05%	0.1%
CMC-Na	3%	3%	3%	3%
Nipagin	0.18%	0.18%	0.18%	0.18%
Nipasol	0.02%	0.02%	0.02%	0.02%
Propyleneglycol	1.6%	1.6%	1.6%	1.6%
Adeps lanae	ad 100%	ad 100%	ad 100%	ad 100%

Description: F0 = without BHT; F1 = BHT (0.0075%); F2 = BHT (0.05%); F3 = BHT (0.1%)

which is in cosmetic products, food ingredients, and drugs. (Rahmatiyah, 2012). The concentration of BHT usually used for topical preparations ranges from 0.0075-0.1% (Rowe *et al*., 2009).

The criteria for a good stock must meet the physical requirements and be stable during the storage period. One of the methods of stability study that can be used is the accelerated stability test. This study uses storage conditions that exceed normal needs for the preparations made. It aims to increase the speed of physical and chemical degradation, and observing the preparations can take a shorter time (Younis *et al*., 2015). The accelerated stability test lasted 28 days at a temperature of 40 ± 2°C / RH 75 ± 5%. Based on the previous description, this study aimed to observe the impact of adding BHT as an antioxidant on the stability of the water-phase combination ointment of snakehead fish extract and kelulut honey.

METHODOLOGY

Materials

The materials used in this study consisted of kelulut honey, snakehead fish extract, CMC-Na (CV. Clorogreen), BHT, Nipagin (Science Lab), Nipasol (Golden Era), propylene glycol (The Dow Chemical Company USP NF Grade), and adeps lanae (Wujiang Jinyu). Snakehead fish (*Channa striata*) was obtained from fish anglers in the Kubu Raya, West Kalimantan. Kelulut honey (*Heterotrigona itama*) was obtained in Bengkayang Regency, West Kalimantan Province. Precisely in RT/RW 002/001 Pangkalan Darat Hamlet, Sungai Pangkalan 2 Village, Sungai Raya District.

Methods

Snakehead fish extraction

This study's extraction procedure used wet rendering, which was steaming and pressing with water. Only the meat of the snakehead fish was used in the extraction procedure. First, ± 3.0 kg of snakehead fish (*Channa striata*), the head and stomach contents were cleaned off, then steamed in a pan at 65-70°C for 30 minutes. After that, the cooked snakehead fish meat was covered in a cotton cloth and pressed under high pressure in a hydraulic press until a cloudy white liquid was obtained. The liquid was centrifuged to separate the water and oil layers in the snakehead fish extract. The water phase of snakehead fish extract was kept in a container with plastic wrap and aluminium foil (Eka & Rochima, 2016).

Ointment preparation

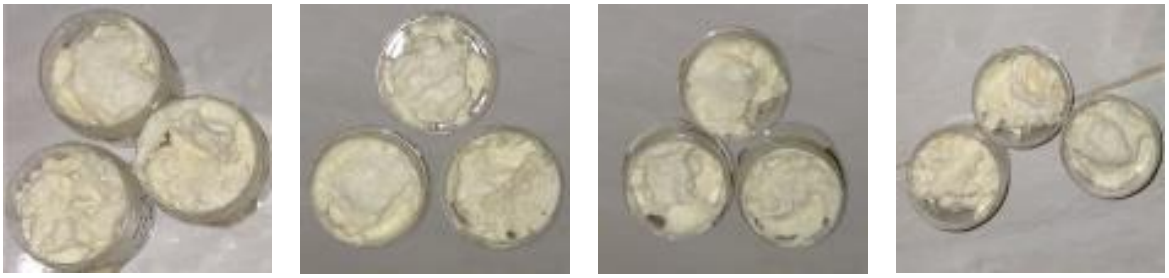
Making the ointment was begun with weighing the necessary ingredients. The active substances used consisted of the water phase of snakehead fish extract and kelulut honey. A magnetic stirrer was used to heat it to 50-60°C for 10 minutes. The mortar was soaked in hot water until the two active ingredients were ready to be crushed. Then, the two active ingredients were put into a preheated mortar to develop CMC-Na and crushed it to form a gel mass (Mixture 1). In a separate mortar, adeps lanae was crushed until it turned yellowish-white. Then BHT, nipagin, and nipasol, which had been dissolved in propylene glycol were added (Mixture 2). Mixture 1 was put into mixture two then mixed until homogeneous.

Organoleptic test

Organoleptic tests characterized shape or consistency (e.g., solid, viscous, liquid), colour (e.g., yellow, brown), and scent using the senses (e.g., aromatic, odourless) (Daisa *et al*., 2017). The testing was applied on a laboratory scale with three participants. The organoleptic test was on days 1, 3, 7, 14, 21, and 28.

Homogeneity test

This test used 0.1 g of ointment on the slide's surface, covering it with another object glass and it's homogeneity was observed. The absence of lumps due to application, uniform structure, and uniform colour were all homogenous



F0 **F1** **F2** **F3**

Figure 1 shows how the ointment looks

characteristics (Hasrawati *et al*., 2019). The homogeneity test was on days 1, 3, 7, 14, 21, and 28.

Spreadability test

The spreadability test was on days 1, 3, 7, 14, 21, and 28. In this test, 1 g of ointment was placed on a 15 cm diameter round glass. For 1 minute, another glass was put on top, and the diameter of the ointment spread was measured. After that, a load of 50 g, 100 g, and 150 g was added and left for 1 minute to obtain the constant diameter. Topical preparations must have roughly 5-7 cm (Soediono *et al*., 2019).

Adhesion test

The adhesion test was on days 1, 3, 7, 14, 21, and 28. In this test, 0.25 g of ointment was placed on a glass slide. After that, another object-glass was put on top of the ointment and pressed for 5 minutes with a 1 kg load. The object-glass was connected to the test instrument. The 80 g load was released, and the time was recorded until the two beakers were released. The time required for good adhesion is not less than 4 seconds (Sandi & Musfirah, 2018).

Power protection test

The power protection test was on days 1, 3, 7, 14, 21, and 28. This test used 1 g of ointment was applied to filter paper measuring 10 x 10 cm moistened with PP (Phenolphthalein) solution and dried. The smaller filter paper (2.5 x 2.5 cm) was moistened with liquid paraffin on the edges and waited for it to dry. Filter paper smeared with ointment was affixed under the filter paper bordered with liquid paraffin. The area was dripped with KOH solution (0.1 N). Observations were made for 5 minutes (Susilowati, 2013).

Acid number test

This test used 1 gram of ointment into a 200 ml Erlenmeyer, and 25 ml of 96% alcohol was added, which heated for 10 minutes. The mixture was immediately titrated with 0.1 N KOH, and 3-5 drops of 1% PP solution were used as the indicator. The titration procedure continues until the colour changes from colourless to pink, which did not disappear for 15 seconds. The amount of KOH used to neutralized free fatty acids in 1 gram of oil was then noted and quantified (Mardiah *et al*., 2019). The acid numbers test was on days 1 and 28.

Data Analysis

Qualitative data in organoleptic test results, homogeneity, and protective power were presented in tabular and described. Meanwhile, the quantitative data in the spreadability, adhesion, and acid number tests were statistically analyzed using the SPSS version 25 program with a 95% confidence level. The ANOVA method was used to run parametric tests on data that were distributed normally and homogeneous ($p > 0.05$). If the ANOVA test results showed significant differences among groups ($p < 0.05$), the Post Hoc test was used (Tyastirin & Hidayati, 2017).

RESULT AND DISCUSSION

Organoleptic Test

The organoleptic test revealed that after 28 days of storage at a temperature of 40 ± 2°C / RH 75 ± 5%, all formulations for the water phase combination of snakehead fish extract and kelulut honey were lost consistency and showed colour changes. This was evidenced by the thickness of the preparation from stiff to soft on the 3rd day of testing and the change in yellowish-white to yellow on the 7th day. Figure 1 shows how the ointment looks.

The addition of CMC-Na can affect the stiff ointment consistency by increasing the viscosity of the preparation and making the system more rigid (Forestryana *et al*., 2020). When temperatures and humidity were high, CMC-Na can absorb moisture from the air into the system, and its water content rises, and the system's consistency decreases

Table II. The average test results of spreadability test (n=3, $\bar{x}$±SD)

Test day	Spreadability $\bar{x}$±SD (cm)			
	F0	F1	F2	F3
1	3.89±0.36	3.92±0.16	3.83±0.07	3.83±0.05
3	3.90±0.13	4.27±0.13	4.43±0.11	4.12±0.40
7	3.46±0.14	4.34±0.13	4.67±0.08	4.27±0.35
14	4.04±0.30*	4.71±0.06	5.09±0.01*	4.36±0.07
21	4.24±0.45	5.23±0.32*	5.24±0.22	5.25±0.07*
28	4.77±0.10*	4.62±0.33*	4.47±0.14*	5.57±0.21*

Description: F0 = without BHT; F1 = BHT (0.0075%); F2 = BHT (0.05%); F3 = BHT (0.1%)

(Sandi & Musfirah, 2018). The soothing ointment's viscosity allows it to spread out more quickly, absorb more rapidly, and leave a softer effect on the skin than the stiff ointment (Dwi Mardani, 2016). The colour of the resulting ointment was yellowish-white. This was because the number of adeps lanae bases used is relatively high and produces a yellowish-white colour after being crushed to dominate the preparation colour. On the 7th day, the ointment preparation had a yellow colour change which the oxidation process could cause. The distinctive odour of kelulut honey and snakehead fish extracts produced by the ointment preparation strongly scent more with the length of time it was stored. Although the ointment preparation experiences instability during the storage period, it was still suitable. It provided comfort when applied to the skin and did not undergo phase separation until the last day of testing.

Homogeneity test

The homogeneity of the ointment formulations can suggest that the materials used were well-mixed, resulting in no aggregates or coarse grains in the final product. The ointment must be uniform not to irritate the skin and widely spread when applied (Naibaho *et al*., 2013). The homogeneity test of the ointment preparation revealed that the results of the control group (F0) and the treatment group (F1, F2, and F3) were comparable. Visual observations were done, and no aggregates or coarse grains were discovered on the preparation when placed on the slide. The homogeneity of the ointment remained stable until the last day of testing.

Spreadability test

It is essential to test the ointment's spreadability to determine its ability to spread on the skin and even result when applied (Dwi Mardani, 2016). The ability to spread was related to how wide the skin's surface was in contact with the preparation when it was applied. The easier the ointment is to be applied to the skin surface, the more comprehensive the contact with the skin surface, and the active substances will be appropriately distributed. The good ranging spreadability for topical preparations is 5 to 7 cm (Soediono *et al*., 2019). Table 2 shows the spreadability testing results of the ointment.

The spreadability generated by each formula increases in proportion to the increase in load. The spreadability test findings in the control and treatment groups on days 1, 3, 7, 14, 21, and 28 showed instability. Most formulas did not meet the spreadability requirements of good topical preparations ranging from 5-7 cm. This was because of the addition of excipient in the form of CMC-Na 3% and the influence of high storage temperatures. When temperatures and humidity were high, CMC-Na can absorb moisture from the air into the system, and its water content rises, and the system's consistency decreases (Sandi & Musfirah, 2018). The addition of BHT will protect the base from damage such as rancidity and oxidation so that no phase separation occurs. Phase separation will have an impact on decreasing the consistency of the ointment and increasing its spreadability. The spreadability data obtained from the test results met the requirements for normal distribution and homogeneity. A parametric analysis was carried out using the ANOVA method followed by the Post Hoc test.

The spreadability data analyzed first was the data for each formula to the point of the test day. This analysis aimed to determine which formula has the best spreadability stability. The One Way ANOVA test results for each formula showed a significant difference between points on the test day ($p < 0.05$). The Post Hoc test was used to see any significant differences between the groups. The Post Hoc F0 and F2 tests show no

Table III. The average test results of adhesion test (n=3, $\bar{x}$±SD)

Test Day	Adhesion $\bar{x}$±SD (seconds)			
	F0	F1	F2	F3
1	147.33±3.51	160.33±2.51	140.33±2.51	119.00±5.57
3	146.00±6.24	158.67±3.51	138.67±3.51	120.33±1.53
7	147.67±2.52	153.33±4.04	133.33±4.04	122.00±3.00
14	147.33±1.53	70.00±3.00*	48.67±9.01*	120.33±0.57
21	32.00±3.00*	58.00±2.65*	45.67±1.53	82.00±3.60*
28	17.00±2.00*	21.00±2.00*	44.00±3.00*	89.00±2.00*

Description: F0 = without BHT; F1 = BHT (0.0075%); F2 = BHT (0.05%); F3 = BHT (0.1%)

significant difference until the 7th day, whereas for F1 and F3, there was no significant difference until the 14th day.

The spreadability data on day 28 for each formula were then statistically analyzed to determine which formula was the most stable at the endpoint of the test. The choice of the 28th day is because the preparation had undergone many changes. The spreadability data in this condition fulfils the requirements for normal distribution and homogeneity for parametric analysis using the ANOVA method. The One Way ANOVA test yielded a significance value of < 0.05, indicating that the spreadability of each formula differs significantly (F0, F1, F2, and F3). There was a significant difference in F3 based on the Post Hoc test and the average spreadability on the 28th day was 5.57 ± 0.21, which fulfils the spreadability requirements of topical preparations (5-7 cm).

Adhesion test

The adhesiveness of an ointment is its ability to adhere and coat the skin surface during application so that it can maximize its function. The physical stability of an ointment can be seen from the results of measuring its adhesiveness on a regular. (Budiman *et al*., 2013). The adhesion requirement for topical preparations is not less than 4 seconds (Sandi & Musfirah, 2018). The results of testing the adhesion power of the ointment preparation in the water phase of snakehead fish extract and kelulut honey can be seen in Table 3.

The adhesion produced by each formula tends to decrease in proportion to the test time length. This was because of the addition of excipient in the form of CMC-Na 3% and the influence of high storage temperatures. The addition of CMC-Na, which can form a reversible gel, causes this material to melt when heated and form a gel again when left at room temperature (Sayuti, 2015). The addition of BHT will protect the base from damage such as rancidity and oxidation so that no phase separation occurs. Phase separation will have an impact on decreasing the consistency of the ointment and decreasing its adhesiveness. This had an impact on the difference in the adhesion of each formula. The adhesion data of the water phase combination ointment of snakehead fish extract and kelulut honey still meet the requirements for an excellent topical preparation, which is more than 4 seconds until the last day of testing.

The adhesion data obtained from the test results met the requirements for normal distribution and homogeneity. A parametric analysis is carried out using the ANOVA method followed by the Post Hoc test. The adhesion data analyzed first was the data for each formula to the point of the test day. This analysis aimed to determine which formula has the best adhesion stability. Each formula's One Way ANOVA test results showed a significant difference in adhesion between points on the test day ($p < 0.05$). The Post Hoc test is conducted to determine which groups had significant differences. Based on the Post Hoc F1 and F2 test results, it can be seen that there is no significant difference until the 7th day. Whereas for F0 and F3, there is no significant difference until the 14th day.

The adhesion data on day 28 for each formula were then statistically analyzed to determine which formula was the most stable at the endpoint of the test. The choice of the 28th day is because the preparation had undergone many changes. The adhesion power data in this condition fulfils the requirements for normal distribution and homogeneity for parametric analysis using the ANOVA method. The One Way ANOVA test yielded a significance value of < 0.05, indicating that the adhesion of each formula differs significantly (F0, F1, F2, and F3). The Post Hoc test showed a significant difference in F2 and F3, while for F0 and F1, the adhesion power was the same or not significantly different on the last day of testing.

Table IV. The results of acid number test

Day Test	Formula	KOH (mL)	Acid number (mg KOH/g)
1	F0	1.7	6.82
	F1	1.7	6.82
	F2	1.7	6.82
	F3	1.7	6.82
28	F0	2.7	10.83
	F1	2.6	10.43
	F2	2.4	9.63
	F3	2.2	8.83

Description: F0 = without BHT; F1 = BHT (0.0075%); F2 = BHT (0.05%); F3 = BHT (0.1%)

Protection power test

The protective power test aimed to see if the ointment can protect the skin from external forces. The outward effect can come from dust, pollution, and sunlight. The parameter, in this case, was an alkaline liquid (KOH). This test uses KOH solution as an intervention and phenolphthalein as an indicator. The alkaline KOH solution will react with the PP indicator to produce a pink colour. The PP indicator has a pH route of 8.0-9.6, changing colour to red when in an alkaline environment. The appearance of red stains on the filter paper wet with 0.1 N KOH indicates that a good ointment preparation has protected the skin. Based on the results of the protective power test, it can be shown that all formulas can give reasonable skin protection for 28 days of storage at a temperature of 40 ± 2°C / RH 75 ± 5%, as evidenced by the absence of red stains at each point of the test day.

Acid number test

The acid number test determines how much free fatty acids were present in a given amount of oil or fat (Ketaren, 1986). Furthermore, the acid number can identify the chemical characteristics and stability of the oil phase in concern. The higher this value was, the more free fatty acids were present due to the oxidation process. The oxidation process can cause rancidity, accompanied by ketones and aldehydes, which were acidic. The principle of the acid number test is to calculate the number of mg of KOH required to neutralize free fatty acids in 1 gram of oil. The tests were performed on days 1 and 28. Table 4 shows the results of testing the acid number of the ointment.

The acid number test carried out in this study used the alkalimetric titration method. The principle used in this method is the neutralization reaction between hydrogen ions derived from acidic compounds in the ointment preparation and hydroxide ions derived from KOH, which were used as titrants (Sopianti *et al*., 2017). The alcohol used in the acid number test to dissolve fats or oils in the sample to react with KOH, while the heating process aims to dissolve the oil in alcohol and speed up the reaction (Suroso, 2013). Based on the number of mL KOH used in the titration process, it can be seen that there is an increase in the number of mL KOH on the last day of testing. The amount of KOH is directly proportional to the acid number produced by the ointment preparation. Because of the oxidation process, the higher the acid value, the more free fatty acids were present in the ointment. On the final day of testing, F1, F2, and F3 (10.43 mg KOH/g, 9.63 mg KOH/g, and 8.83 mg KOH/g) did not exceed the control group (10.83 mg KOH/g). The results of the acid number test showed that F3 was the most optimal formula for suppressing the oxidation process, which causes an increase in the acid number in the preparation. The smallest increase in acid number is produced by F3 with the acid number value on the first day of testing of 6.82 mg KOH/g, increasing to 8.83 mg KOH/g on the last day of testing.

Adeps lanae, the oil phase, can form free fatty acids due to heat treatment in the form of high temperatures during storage. The oxidation reaction in the double bond of unsaturated fatty acids can occur faster if there is heating. Free fatty acids formed from the oxidation process can cause the oil phase to experience rancidity, which indicates that the ointment preparation is unstable. BHT has a labile hydrogen atom in the hydroxy group that can be supplied and reduces the free radicals present at the initiation of lipid oxidation. The higher the antioxidant concentration, the greater the activity, but this activity will reach a stable phase (Dewi *et al*., 2007).

CONCLUSION

The results showed that the addition of BHT directly affected the ointment's stability based on the acid number test. The addition of BHT will

protect the base from damage such as rancidity and oxidation so that no phase separation occurs. Phase separation will have an impact on decreasing consistency, decreasing adhesion, and increasing the spreadability of the water phase ointment of snakehead fish extract and kelulut honey. The best formula is F3 (BHT 0.1%), which resulted in the best spreadability (5.57 cm), adhesion (89 seconds), organoleptic test compliance, homogeneity, and protective power, as well as a minor increase in acid number.

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