

## RESEARCH ARTICLE

# Design Formulation Ointment and Wound Healing Effectiveness of Onchidiid Slug (*Onchidium typhae*) Extract Ethanolic Assisted by Design Expert Software

Bambang Wijianto<sup>1\*</sup>, Liza Pratiwi<sup>2</sup>

<sup>1</sup>Departement of Pharmaceutical Chemistry Departement, Pharmacy Department, Medical Faculty, Universitas Tanjungpura, Pontianak, Kalimantan Barat, Indonesia.

<sup>2</sup>Departement of Pharmaceutical Technology Departement, Pharmacy Department, Medical Faculty, Universitas Tanjungpura, Pontianak, Kalimantan Barat, Indonesia.

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## ABSTRACT

Research on onchidiid slugs has been conducted regarding their antibacterial activity and wound healing abilities. Objective: This study aims to design the ointment formula as an effective drug delivery base for wound healing. The formula design was obtained using the mixture design method using Design Expert software version 13.0. The proposed and optimum formulas were then evaluated for their physical properties (organoleptic, homogeneity, spreadability, adhesion, and pH). The incision wound was made with a wound length of 1.5 cm with a depth of 0.2 cm on the mouse's back. The assay group was divided into several groups. Group I is the positive control treated with betadine ointment. Group II is the negative control treated with an ointment base without active extract. Group III is the group given crude extract. Groups IV, V, and VI are the treatment groups' optimum formula of onchidiid slug ethanol extract ointment: 1, 3, and 5%, respectively. The intervention for each group was carried out for ten days with smearing once a day. The design results in optimal formulas with the composition of adeps lanae and vaseline album (9,566:70, 344) with a desirability value of 0.934. Tests for adhesion power, spreadability, and optimum formula pH meet the requirements for a good ointment. An ointment containing 1% onchidiid slug extract showed the best wound-healing effectiveness. This formula is 88.31% effective in wound healing. Onchidiid slug ethanol extract can be designed into an effective ointment for wound healing assisted by the mixture design method using Design Expert software.

**Keywords:** *Onchidium typhae*, Ointment, Wound healing, Mixture design method.

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## INTRODUCTION

Wounds can damage tissues and cells, affecting the skin's function. Sharp or blunt objects, chemicals, explosions, or animal bites in humans and animals alike can cause them. The body's natural process of restoring the skin's structure and function is known as wound healing. There are three stages to the wound-healing process: inflammation, proliferation, and maturation.<sup>1-3</sup> Wounds will fail to heal if there are inhibiting factors. Factors inhibiting wound healing include infection, hematoma, and foreign bodies. Wound treatment aims to reduce risk factors that hinder wound healing, speed up the healing process, and reduce the incidence of infected wounds.<sup>4-7</sup> One alternative for wound healing treatment is using traditional medicine. Traditional treatment of wounds by

the coastal communities of West Kalimantan is using onchidiid slugs, known as "Lintah Babi."

The onchidiid slug is a shellless slug whose habitat is mainly in the swamps of the island of Kalimantan. Onchidiid slugs are rich in macronutrients, especially protein content.<sup>8-10</sup> Research endeavors have continued after identifying various chemical constituents such as polypropionates, depsipeptides, and terpenoids within the *Onchidium* genus.<sup>10,11</sup> They have cytotoxic activity against tumor cells and antiviral and bacteriostatic activity.<sup>11</sup> *Onchidium typhae* can be seen in Figure 1.

The onchidiid slug (*O. thyphae*) exhibits inhibitory activity against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* in its methanol, chloroform, and ethyl

\*Author for Correspondence: bam.wijianto@pharm.untan.ac.id



Figure 1: *Onchidium typhae*

acetate extracts.<sup>8</sup> The healing of wounds involves compounds with anti-infective properties, blood cells, platelets, growth factors, and cytokines impacting the complex process of tissue regeneration and repair. This process involves the extracellular matrix (ECM) and includes phases of exudation, proliferation, and remodeling.<sup>2,12,13</sup>

Only a few reports have been on medicinal preparations from onchidiid slugs in the past 40 years. This study reviews the design of ointment formulations and tests their effectiveness on wound healing in vivo for further study, exploitation, and utilization of this resource.

## MATERIAL AND METHODS

### Materials

The materials used in this research were onchidiid slug ethanol extract, adeps lanae, vaselin album, methylparaben, propylparaben, essence, and Aquadest. We have obtained the following materials from PT. Brataco. Ingredients for the wound healing test included betadine ointment (PT. Mahakam Beta Farma), Lidocaine injection (Phapros), alcohol 70% (PT. Brataco), and male white rats (*Rattus norvegicus*) Wistar.

### Tools

The tools used in the research were Expert Design software version 13 trial, glassware (IWAKI), water bath (Memmert), analytical scales (Bonvoisin Lab Scale 5000 x 0.01 g), tools for the effect of wound healing assay including animal cages, razors (Gillette blue 3), dissecting set (Renz Instruments), and Canon EOS 60D digital camera.

### Methods

#### Research procedure

- *Determination of onchidiid slug*

The sample of the onchidiid slug was determined in the Ecology Laboratory at the Faculty of Science and Mathematics, Department of Biology, Universitas Tanjungpura. Based on the results carried out in the laboratory, the animal species used was the onchidiid slug (*O. typhae*).

- *Preparation of onchidiid slug sample*

This research used samples of onchidiid slugs in several areas of Sambas Regency, West Kalimantan Province. The onchidiid

slug used has a wet weight of 15 kg with an onchidiid slug body diameter of 1 to 3 cm and a length of 1 to 5 cm. Onchidiid slug samples were washed to remove mucus, separated from innards, and rewashed for processing. Onchidiid slug meat is dried for 48 hours at 60 to 70°C, then blended into simplicia powder for extraction.<sup>8,9</sup> To obtain the onchidiid slug powder, we used the maceration technique. We soaked the sample in 500 mL of 96% ethanol for 24 hours and filtered it through a Buchner funnel. The extraction results were evaporated at 70°C.

#### Screening of metabolite compounds from extracts

- *Steroid assay*

To identify the presence of terpenoids in onchidiid slug extract, we carried out an experiment in which 2 mL samples were used. These samples had previously been extracted with ethanol solvent. We then added three drops of concentrated HCl and one drop of concentrated H<sub>2</sub>SO<sub>4</sub> to the extract. When a green oil precipitate appears in an extract, terpenoids are present. This analysis method can give us essential information about the extract's composition and aid in further research.<sup>14</sup>

- *Alkaloid assay*

Use Meyer's and Dragendroff's reagents to perform the flavonoid test. Add 2 mL of onchidiid slug extract to two separate tubes. To the first tube, add five drops of Dragendroff's reagent. If the solution turns orange, the test is positive. Add HCl and Meyer's reagent to tube 2. White precipitate indicated alkaloids present, test positive.<sup>15</sup>

- *Flavonoid assay*

About 2 mL of Onchidiid slug extract was extracted using methanol. After heating for 5 minutes, magnesium metal (0.1g) and concentrated HCl (5 drops) were added. An orange-yellow to red color indicated the presence of flavonoids.<sup>16</sup>

### Ointment Preparation

#### Design of formulation

The ointment was formulated using the mixture design method with the help of Design Expert software version 13.0. The formulation process involved several trials, and formula optimization was done by determining the lower and upper limit values for vaselin album and adeps lanae. The form of a

Table 1: Optimization ointment formula using Design Expert 13.0 Trial

Run	Component factors (%)	
	Vaselin album	Adeps lanae
1	72.5	7.5
2	71.6667	8.3333
3	68.3333	11.6667
4	70	10
5	65	15
6	67.5	12.5
7	65	15
8	70	10

**Table 2:** Ointment formulation

Formulation	Component (%b/v)							
	F1	F2	F3	F4	F5	F6	F7	F8
Extract ethanol of onchidiid slug	1							
Adeps Lanae	7.5	8.33	11.67	10	15	12.5	15	10
Vaselin Album	72.5	71.67	68.33	70	65	67.5	65	70
Methylparaben	0.2							
Propylparaben	0.2							
Essen oil	q.s							
Aquadest	Ad 100							

F1= Formula run 1; F2= run 2; F3= run 3; F4= run 4; F5= run 5; F6= run 6; F7= run 7; F8= run 8.

predicted ointment formula is shown in Table 1. This approach was based on previous research.<sup>17-19</sup> The software provided eight formulas with varying adeps lanae and vaseline album concentrations for further evaluation. Eight ointment formulas were tested for physical properties, including spreadability, adhesion, and pH. Results were analyzed by software for recommendations.<sup>17</sup> A comparison of vaseline album vs adeps lanae to obtain the optimal ointment formula can be seen in Table 2.

#### *Ethanol extract of onchidiid slug ointment formulation*

Mix vaseline album and adeps lanae until smooth, then add onchidiid slug extract, methyl, and propylparaben. Add enough essential oil, package, and evaluate through physical tests. These include organoleptic, homogeneity, spreadability, adhesion, and pH.

#### *Organoleptic test*

When creating an ointment, it is essential to conduct an organoleptic test to assess its appearance, scent, and hue. Accurate input of ointment specifications is essential for achieving a semi-solid consistency. The color must align with the initial specifications, and the fragrance must not be stale. The ointment is tested to guarantee its quality and meet standards.

#### *Homogeneity test*

Apply an ointment on a transparent surface to determine if it is uniformly blended. An adequately mixed ointment will have a uniform texture and color and no clumps. To ensure uniformity throughout the product, test the ointment in the top, middle, and bottom areas.

#### *Spreadability test*

The ointment sample weighing 0.5 g was applied onto a round glass surface, covered with another glass, and left for 1-minute. The spread diameter of the ointment was then noted. Subsequently, a load of 100 g was added and left for 1-minute, and the diameter was measured. The optimal spread range is between 5 and 7 cm.<sup>20</sup>

#### *Adhesion test*

To test the adhesive properties of an ointment, apply 0.05 g to a piece of glass. Add another glass and a 50 g weight for

30 seconds. Remove the top glass and add a 16 g weight. Record the time taken for detachment. For adequate adhesion, separation time should be at least 4 seconds.<sup>20,21</sup>

#### *Test pH*

To test the pH, use a pH meter to measure a sample that has been diluted with 5 mL of distilled water. The pH value of a good ointment should fall within the range of 4.5 to 6.5, matching the pH of the skin.<sup>22</sup>

#### *Wound-healing assay*

Test animals were anesthetized with ketamine and given a wound on their back. They were divided into three groups and treated for ten days. Group I received betadine ointment, group II received a base ointment, and group III received ointment from onchidiid slug chloroform extract. The wound was made 0.2 cm deep and 1.5 cm long on the male white rat's back using a sterile scalpel.<sup>23</sup>

#### *Analysis data*

Incision wounds on test animals were photographed at a suitable and precise angle constantly with a digital camera. Each photo was quantified using the parameter of the area of the incision wound. The Macbiophotonics Image J computer program was used to assist in quantifying the area of the incision wound. This program can measure research objects' area, number, and intensity. Using this program, we obtained numerical values that can be analyzed further. The research area data obtained will then be analyzed statistically using the SPSS program, which is assisted by the one-way ANOVA test.<sup>24,25</sup> The analysis results were then compared between groups to determine the optimal formula's effectiveness for onchidiid slug ethanol extract ointment in wound healing.

## **RESULT AND DISCUSSION**

The wound-healing process is a complex series of events that unfold in four distinct phases, each following the other. During the hemostatic phase, blood clots form within the incision wound. Typically, this phase lasts for a brief period, ranging from a few minutes to several hours after the injury occurs.<sup>2,26</sup> In the initial stage, the body's blood vessels constrict to reduce blood loss, while platelets in the blood converge at the wound site to form a clot. The inflammatory phase of wound healing

begins a few hours after the injury and usually persists for about one week. In this stage, the body's immune system reacts to the injury by discharging different inflammatory mediators, like cytokines and chemokines.<sup>27</sup> When an injury occurs, mediators are released, causing white blood cells like neutrophils and monocytes to move from the bloodstream to the injury site. Once there, these white blood cells transform into macrophages, which are crucial in removing damaged tissue from wounds. As the proliferative phase of wound healing progresses, new tissue is generated to replace the damaged tissue. This phase can last for several weeks. During this time, new blood vessels form, and fibroblasts create a new extracellular matrix to provide structural support for the new tissue.<sup>28</sup> The remodeling phase, which is the final stage of wound healing, may continue for several months to years as the new tissue matures and reorganizes. The extracellular matrix is further organized and strengthened, and the new blood vessels undergo a process of maturation and stabilization. Ultimately, the wound is replaced by new tissue, and the functional properties of the tissue are restored.<sup>29</sup>

Furthermore, the process of angiogenesis and lymphangiogenesis initiates at this point. Approximately five days post-injury, the third phase extends for the following 10 to 14 days. Throughout this period, both angiogenesis and lymphangiogenesis continue to support the proliferation of fibroblasts, myofibroblasts, and keratinocytes to develop the dermal and epidermal layers. Ultimately, the final (fourth) phase concludes the healing process by promoting collagen deposition and the formation of connective tissue layers consisting of fibroblasts and keratinocytes, leading to the contraction and closure of the wound.<sup>13,29,30</sup>

#### *Preparation of onchidiid slug ethanol extract*

The research involved onchidiid slugs, and the outcomes were validated using such specimens. This study has been approved by ethical review No. 737/UN22.9/PG/2023. Ensuring that the research complies with respecting individuals, promoting benefits, avoiding harm, and upholding fairness is essential. The samples were obtained from different areas in Sambas Regency, including Semparuk district, and the slugs used for the research had a wet weight of 25 to 35 kg. The samples were carefully cleaned and sorted by removing impurities such as mud and mucus and separating fresh meat and offal. The process of making onchidiid slug *simplicia* powder was based on Wijianto (2022), but it was modified to exclude soaking the slugs at a temperature of 70°C.<sup>9,10</sup> The onchidiid slugs were then dried, sorted, and ground to obtain the powder. The onchidiid slug powder was sieved using mesh number 18 to reduce the particle size, ensuring that the contact between the powder and the solvent was comprehensive during extraction. The yield from onchidiid slug *simplicia* powder was 25.96%, while the yield from gross weight to net weight was 14.8%.

The preparation of the onchidiid slug extract involved the use of the maceration method (ethanol 96%), a simple and cost-effective method. The maceration process does not use temperature and requires a small amount of solvent. The

extract from 960.6 g of *simplicia* was 66.5 g yielding 7.1%. Although the yield produced using ethanol solvent was lower than that produced using methanol solvent in other research, it was preferred because it is generally less toxic than methanol. Onchidiid slug extract with ethanol solvent is yellowish-brown with a characteristic odor.

These findings provide valuable insights into making onchidiid slug *simplicia* powder and preparing onchidiid slug extract using the maceration method. These insights could be helpful for further research on the use of onchidiid slug extract in various applications.

#### **Ointment Preparations**

The research focused on developing a wound-healing ointment utilizing the active ingredients in the ethanol extract of the onchidiid slug. The scientists utilized the Design Expert 13.0.0 Trial formula to create the ointment, consisting of eight different runs with varying amounts of *adepts lanae* and *vaseline album*. The dosage of these ingredients is important in determining how long the medicinal substance stays in contact with the skin, thereby influencing skin hydration and the drug's percutaneous absorption. By evaluating the adhesive ability, spreadability, and pH of the ointment base, this study aimed to identify the best formulation to enhance the effectiveness of the active components in the ointment.

Ointment based on *adepts lanae* is the basis of an absorption ointment with great drug release and water absorption capacity. *Adepts lanae* forms an o/o emulsion, which, with this type, can attract the active oil ingredients to penetrate the skin.<sup>31</sup> *Album Vaseline* is used for its emollient effect, as a skin protector, and as a sealant to prevent evaporation. *Vaseline album* is a hydrocarbon ointment base that can only absorb a little water. However, with the addition of *adepts lanae*, the combination of the two bases can absorb up to 50% water. This result is because *adepts lanae*, the absorption basis, comes from sheep's wool fat with a water content of no more than 0.25%.

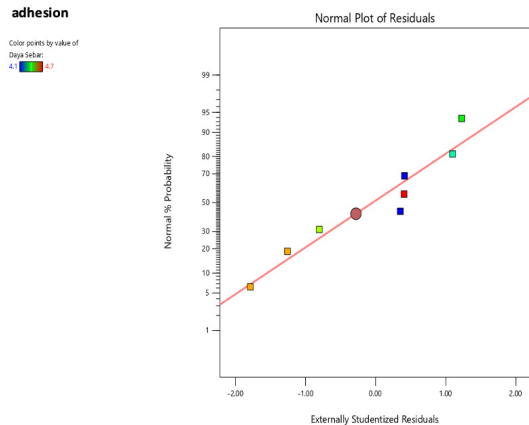
*Cera alba*, commonly known as beeswax, is an ingredient that is frequently used to thicken ointments and increase their viscosity (Rigano and Montoli, 2021).<sup>32</sup> Topical preparations require a viscosity-increasing agent, and *cera alba* is a reliable choice. Stearyl alcohol is another ingredient that can enhance the consistency of ointment preparations, particularly for oil-in-water (O/W) emulsions. Meanwhile, methyl and propylparaben are preservatives to prevent microbial growth in ointment preparations. When combined, these two preservatives can significantly improve the antimicrobial effect, which can help maintain the stability of the ointment for extended periods. To ensure that the ointment is stable, it should remain yellowish-white in color and not have a foul or rancid odor, indicating the absence of bacterial growth. Finally, adding orange essence acts as a pleasant fragrance for the preparation—store ointment in a container to avoid exposure to sunlight and air.

#### **Physic Evaluation of Ointment**

Evaluating an ointment's quality involves measuring its physical properties, including adhesion, spreadability, and

**Table 3:** The results for adhesion, spreadability, and pH test

Run	Adeps Lanae (%)	Vaselin album (%)	Adhesion test (Sec)	Spreadability test (cm <sup>2</sup> )	pH test
F1	7.5	72.5	29	4.1	6.3
F2	8.3333	71.667	28	4.1	5.5
F3	11.6667	68.3333	25	4.3	5.1
F4	10	70	22	4.5	5.4
F5	15	65	18	4.7	5.3
F6	12.5	67.5	24	4.4	5.2
F7	15	65	14	4.6	5.5



**Figure 2:** Normality graph of externally studentized residuals distribution of adhesion data

pH. Each property is quantified using specific units, such as pH degrees on the pH meter, spreadability in cm<sup>2</sup> units, and adhesion expressed in seconds. The Expert Trial 13.0 Design Software analyzes test results to determine the optimal formula.

The criteria for selecting the analysis results are quite specific. One crucial factor is a high R<sup>2</sup> value, which indicates the model's reliability and should be close to 1. An F-value of less than 0.05 (not significant) is also desirable. Adjusted R-square and predicted R-square values should be similar, ideally within 0.2. Models with a high Adeq precision value (over 4) are also preferred. It is good news that all three responses - spreadability, adhesion, and pH - meet these criteria, as Table 3 shows. A lack of fit is also desirable as it suggests that the response data matches the model.

**Adhesion Test**

We measured how long the ointment adhered to a glass object during the adhesion test. The results revealed that runs 1 to 8 had varying adhesion times due to differences in adeps lanae concentration and vaseline album concentration. Our program suggested a linear polynomial model, further supported by ANOVA analysis. This run's suggested model had a significant value where p "prob>F" was smaller than alpha, which is 0.05. The lack of fits value was 0.3176, indicating that the probability is significant and can be ignored. This result shows that the adhesive force response perfectly aligns with the suggested model design.

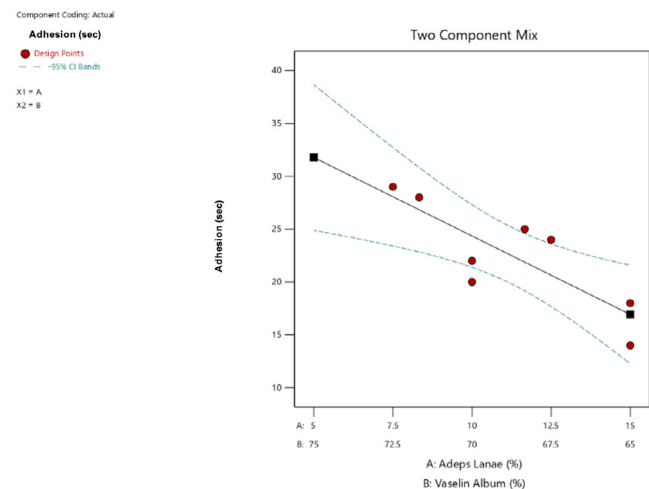
Figure 2 explains that the distribution of distance data between points is spread along the standard line so that it can be stated that the data resulting from the adhesive force response are normally distributed. It also shows the standard deviation that separates the actual response value of adhesive force from the small program predicted value. The polynomial equation for the adhesive force response is seen in equation 1.  $Y = 16.93(A) + 31.78 (B)$ ..... 1

Y = Adhesion

A = Adeps lanae component

B = Album vaseline component

The coefficient A has a positive value of 16.93, suggesting that adeps lanae can enhance adhesion. However, coefficient B has a more significant value of 31.78, implying that album vaseline may have a more significant impact on adhesion. Both adeps lanae and vaseline albums have a positive effect on adhesive strength. Adhesive strength is a crucial parameter in the optimal custom design analysis, where the goal is to maximize it. The acceptable range for sticking power is 4 to 25 seconds, while good adhesion criteria range from 2 to 300 seconds. Figure 3 displays the contour plot of the adhesion test results. According to the optimization results, the contour plot of the adhesion curve in Figure 2 is linear. Increasing the concentration of album vaseline and adeps lanae can improve



**Figure 3:** Countourplot graph of adeps lanae and vaseline album on adhesion test

the adhesion of the ointment, which ultimately affects the preparation's adhesion.

**Spreadability Test**

The spreadability test is an effective way to determine the efficacy of ointments when applied to the skin. A higher spreading power means that there is more comprehensive contact between the ointment and the skin, which results in a faster diffusion of the ointment. As shown in Table 3, the spreadability test was conducted by measuring the diameter of the ointment spreading on a scaled glass, and runs 1-8 had different spread diameters due to variations in the concentration of adeps lanae and vaseline album.

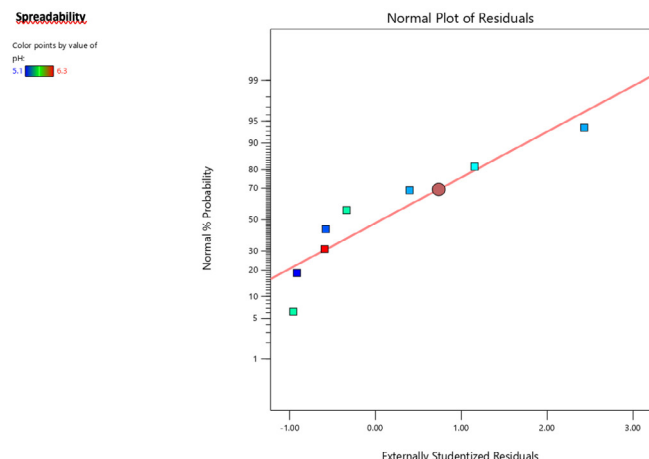
Based on the program's recommendation, a polynomial model was created that fits the data very well. ANOVA analysis supports this model, with a significant "prob>F" value of less than 0.05. The Lack of Fits value is 0.1261, indicating that the "prob" value is insignificant and can be disregarded. Therefore, the results demonstrate that the spread power response follows the recommended model design.

In Figure 4, the graph shows that the distribution of distance data between points is spread along the normal line so that it can be stated that the data resulting from the adhesive force response are distributed normally. This graph also shows that the standard deviation that separates the actual response value of spreadability strength from the program-predicted value is slight. The polynomial equation for the spreadability against response is seen in equation 2.

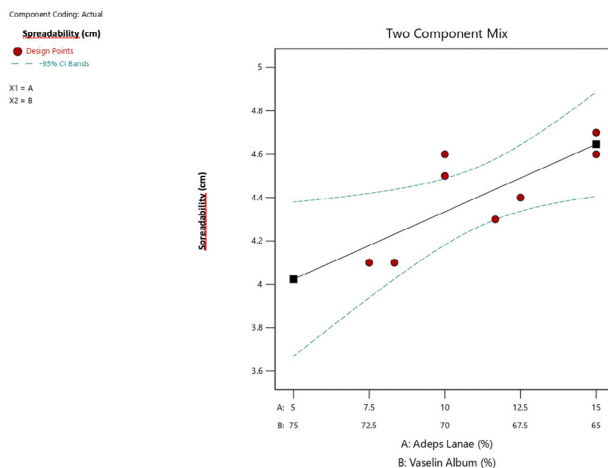
$$Y = 8.69(A) + 3.71(B) \dots\dots\dots 2$$

Y = Spreadability  
 A = adeps lanae component  
 B = vaselin Album component

According to the analysis, the positive coefficient A has a value of 8.69, indicating that adeps lanae can enhance the spreadability of ointments. Comparatively, coefficient B has a value of 3.71, suggesting that adeps lanae is more effective in increasing adhesion than vaseline album. This aligns with earlier research demonstrating that both components positively



**Figure 4:** Normality graph of externally studentized residuals distribution of spreadability data



**Figure 5:** Countourplot graph of adeps lanae and vaseline album on spreadability test

impact adhesion. Adeps lanae can absorb air up to two times, enhancing the spreadability of ointments, while vaseline album reduces the ointment's consistency, making it thinner and more accessible to spread. Together, these components improve the overall spread of the ointment.

The optimal custom design analysis sets adhesive strength as a maximum goal, a crucial parameter in the optimal formula. Spread power is set to 3 to 4.5 cm, while good spreadability in topical preparations is 5 to 7 cm. As shown in Figure 4, the results of the adhesion test counterplot demonstrate that increasing the concentration of Vaseline Album and Adeps Lanae results in increased spreadability of the ointment. The contour plot of the adhesion curve in Figure 5 is linear, indicating the optimization results with optimal custom design.

**pH Test**

Check the pH levels of skincare products before use to avoid skin irritation. Acidic products may irritate, while alkaline ones may dry out the skin. Table 3 shows the results of the pH test. To do the test, we used equipment that's been calibrated with buffers of pH 4, 7, and 10. The pH value displayed on the test equipment is the result of the test. It is worth noting that the pH values of runs 1 to 8 differ due to variations in the concentration of adeps lanae and vaseline album. The quadratic model confirmed significance ( $p < 0.05$ ) with consistent spread power response. A lack of Fit value of 0.2300 means the "prob" value is insignificant.

In Figure 6, the graph shows that the distribution of distance data between points is spread along the normal line so that it can be stated that the data resulting from the adhesive force response are distributed normally. This result also shows that the standard deviation that separates the actual pH response value from the program-predicted value is slight. The polynomial equation for the pH response is seen in equation 3.

$$Y = 5.42(A) + 7.43(B) - 4.38(AB) \dots\dots\dots 3$$

Y = pH  
 A = adeps lanae component  
 B = vaselin Album component

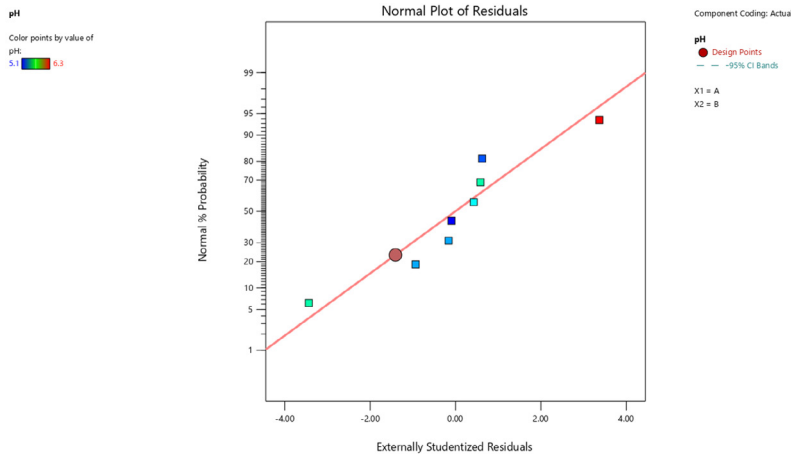


Figure 6: Normality graph of externally studentized residuals distribution of pH data

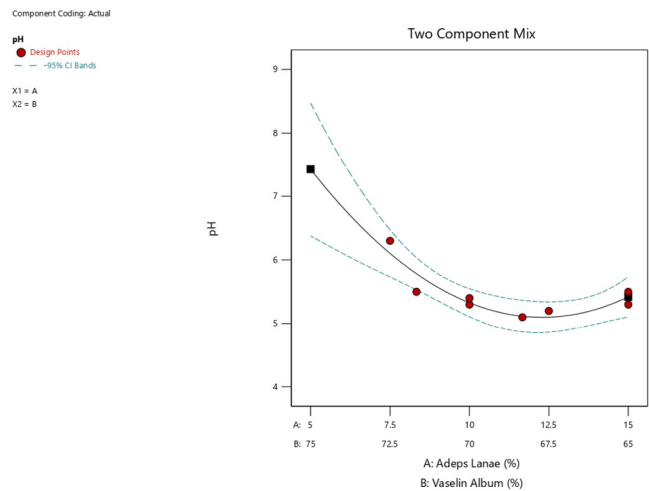


Figure 7: Contourplot graph of adeps lanae and vaseline album on pH test

AB = combination of proportions of adeps lanae and vaseline album

The coefficient A value is positive with a coefficient value of 5.42, which indicates that adeps lanae can increase pH. The value of coefficient A is smaller than that of coefficient B, which is 7.43, which means that the ability of adeps lanae to increase pH is lower than album vaseline. The AB coefficient value, which has a negative sign of 4.38, states that combining adeps lanae and vaseline album can reduce the pH value. Vaseline album is the component that has the most significant influence on the pH response compared to adeps lanae, even though the difference in pH value can be said to be very small (it can be said that variations in base concentration do not affect the pH of the preparation). This result follows Maesaroh's 2020 research, which stated that variations in the concentration of the ointment base did not affect the pH of the preparation. Single-handedly, the amount of vaseline album and adeps lanae positively affected the pH response.<sup>33</sup>

In the optimal custom design analysis for adhesive strength, goals are set in the range of 4.5 to 6. The criteria for a suitable pH in topical preparations are 4.5 to 6.5. The results of the pH test counterplot can be seen in Figure 6. Based on the optimization results with optimal custom design, the contour plot of the adhesion curve in Figure 7 is quadratic, which means that increasing the concentration of vaseline album and adeps lanae reduces the pH results.

**Optimal Custom Design Analysis Results**

We used the highly efficient Design Expert 13.0 Trial program to optimize the formulas. After inputting the physical properties testing data, we processed it using the optimal custom design method, which predicts the ideal formula for each necessary response. As a result, we generated two ideal formulas through numerical optimization with utmost precision. The first formula contained adeps lanae and

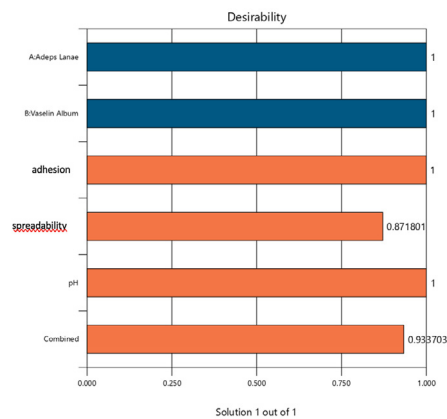


Figure 8: Graph of test response and desirability relationship

album vaseline in a ratio of 9.566:70.344, with a remarkable desirability value of 0.934. Two formulas were generated using the Design Expert 13.0 Trial program's input from physical properties testing data. The second formula had a desirability value of 0.87180 and contained adeps lanae and album vaseline in a 15:65 ratio. The first formula contained adeps lanae and album vaseline in a ratio of 9.566:70.344, with a desirability value of 0.934. The second formula contained adeps lanae and album vaseline in a ratio of 15:65, with a desirability value of 0.87180, as demonstrated in Figure 8.

Determining the optimal formula involves the identification of the desired response criteria (goals) within the feasible range. This process is facilitated by the Design Expert software, which recommends the optimal formula. The recommended formula can be found in Table 4.

According to the data in Table 4, the desirability value achieved is 0.934. The formula with the highest desirability

Table 4: Formula optimum

Adeps lanae	Vaselin album	Adhesion	Spreadability	pH	Desirability	
9.566	70.344	25	4.3	5.423	0.934	Selected

**Table 5:** Result of optimum formula

Replication	Spreadability (cm)	Adhesion (sec)	pH
R1	4.3	25	5.5
R2	4.5	24	5.3
R3	4.3	24	5.4

value is considered the most optimal. The desirability value serves as a measure for optimization, indicating the extent to which the final product meets the specified criteria. As the desirability value approaches 1.00, it signifies a greater level of suitability for the optimal optimization process in achieving the desired response variable.

### Verification of Optimal Ointment Formula

The ointment was prepared using the optimum base ratio determined through optimal custom design analysis. Its spreadability, stickiness, and pH were tested and verified using optimal custom design Software prediction results. The ointment was replicated thrice, as shown in Table 5.

A normality test was carried out using the Shapiro-Wilk test. The results were for adhesive power 1, spreadability 1, and pH 1, with the  $p$ -value  $>$  alpha 0.05, so it met the normality requirements. Next, a homogeneity test was carried out using the Levene test to determine whether the data being analyzed was homogeneous. Homogeneity test results show that the test data has a homogeneous variant with a significant value of 0.115 ( $p > 0.05$ ), so it can be continued with the one-sample T-test. The purpose of carrying out the one-sample T-test is to determine significant differences between the sample test results and the predicted results from optimal custom design. The results for adhesive power 0.517 ( $p > 0.05$ ), spreadability 0.225 ( $p > 0.05$ ), and pH 1 ( $p > 0.05$ ), the results of the ointment samples are not significantly different from the predictions from optimal custom design. Hence, the optimal custom design used is appropriate and can determine the optimum formulation of ointment using a valid and reliable comparison of adeps lanae and vaseline album.

### Wound Healing Assay

#### Treatment and injuries to test animals

The test animals of the Wistar strain were male white rats (*Rattus norvegicus*). This test animal was chosen because white rats are easy to care for and sensitive to treatment. This type of animal was chosen because rats are relatively

resistant to infection. The rats weighed 150 to 200 g, so they had a large enough area to administer the ointment. An acclimatization process was carried out before injuring the rats. Acclimatization aims for the test rat to get used to their new environment, which can reduce stress and data errors for each experimental animal and have the same uniformity. Changes in the wound were observed in terms of changes in the area of the wound during treatment. Observations were made every day for ten days of observation.

#### Wound healing assay results

A research study analyzed the healing effects of an ethanol extract ointment derived from onchidiid slugs on test animals' wounds. The extent of the wound healing was measured using a specialized computer program called Macbiophotonic Image J. This program accurately quantifies the area, number, and intensity of the research subject, generating numerical values that can be analyzed. The working principle of the Macbiophotonic Image J program is to determine and quantify the area of mouse wounds in mm<sup>2</sup> units. The wound area of the test animal was photographed on days 1, 2, 6, and 10 using a 13-megapixel camera. The wound healing effect was compared to four groups: normal, treatment, negative, and positive, and the data was statistically analyzed to obtain results.

The treatment groups in this study consisted of crude extract (K0), 1% extract (K1), 3% extract (K2), 5% extract (K3), negative control (K-), and positive control (K+) groups. The cream is observed and applied daily, with each 0.1 gram/cm<sup>2</sup> wound administration. Wound healing consists of several phases, including the hemostasis phase, inflammation phase, proliferation phase, and maturation (remodeling) phase. These phases are dynamic and complex.<sup>34</sup> The wound-healing phase in this study began in the hemostasis phase. The hemostasis phase occurs on day 0, marked by the cessation of bleeding after a cut wound. This phase occurs when platelet aggregation occurs, which will stop bleeding in damaged blood vessels.<sup>35</sup> The next phase is the inflammatory phase; swollen and reddish wounds characterize this phase. This phase occurs on day 1. On day 2, scab formation occurred in each group; this entered the wound healing phase, namely the proliferation phase. In the proliferation phase, inflammatory cells, fibroblasts, and collagen fill the wound to form tissue with an uneven surface, which is also called granulation tissue/scab. The wound healing process on days 0, 2, 4, and 10 can be seen in Figure 8. The wound-healing effect is due to the secondary metabolite content in the ethanol extract of onchidiid slugs, especially

**Table 6:** Wound healing ability

Assay group	Observation day (X%; SD; n = 4)				
	0	2	4	8	10
Negative control	0	15.76	40.81	62.03	70.49
Positive control	0	18.92	27.16	59.73	63.79
Crude extract control	0	14.28	25.96	66.22	76.89
Ointment extract 1%	0	19.56	36.68	64.25	88.31
Ointment extract 3%	0	12.02	49.75	81.44	86.85
Ointment extract 5%	0	5.73	19.60	41.11	61.41



alkaloids and steroids. Alkaloids play an antimicrobial role in wound healing and function to re-epithelialize damaged tissue by increasing levels of the enzyme 44 hydroxyproline, which can induce the maturity of collagen tissue in wounds. Alkaloids also play a role in the maturation process of collagen fibrils, namely by preventing cell damage through DNA synthesis. Therefore, new tissue grows more quickly in the wound area. Alkaloids and steroids are anti-inflammatory and act as antioxidants by counteracting free radicals. Onchidiid slugs also have antibacterial and antioxidant activity. The most critical content in onchidiid slugs is protein, namely 67.88%. Protein has a crucial role in the wound healing process by having the function of regenerating damaged cells.

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In a 2005 study by Rodriguez *et al.*, a wound-healing ointment with 1% extract closed an incision wound by 88.85% after ten days of application.<sup>30</sup> The wound healing ability of each test group was statistically analyzed using the Shapiro-Wilk method to check for normal distribution ( $p > 0.05$ ) (Appendix 6). The data was tested using the Levene Test method and found to be consistent ( $p > 0.05$ ) (refer to Appendix 6). The one-way ANOVA test found a significant difference on the 10th day of observation ( $p < 0.05$ ). A post hoc LSD test showed that the onchidiid slug ethanol extract ointment had a significant difference between the negative and positive groups. Based on the table and graph depicting the percentage of wound healing ability, it is evident that the wounds in white rats are decreasing in size daily with an increase in the percentage of wound healing each day. Notably, the ointment group with an extract concentration of 5% had the lowest wound-healing ability.

## CONCLUSION

The results of a study showed that a 1% ethanol extract of onchidiid slug ointment was effective in healing cut wounds. The results are related to its antibacterial ability and the maturation process of collagen fibrils. Histological analysis needs to be carried out around the wound to see IGF-1, FGF-2, and VEGF (parameters of fibroblast, proliferation, angiogenesis, and epimerization), which indicate the proliferation phase.

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