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# DEVELOPMENT OF A TOPICAL FORMULATION FROM CHITOSAN EXTRACTED FROM PLEUROTUS OSTREATUS MYCELIUM: A SUSTAINABLE ALTERNATIVE TO CRUSTACEAN-DERIVED CHITOSAN

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**ABSTRACT**

This study investigates the extraction of chitosan, a macromolecule composed of many glucosamine units (monomers), with a high molecular weight. This chitin-derived biopolymer has antimicrobial and healing properties from the mycelium of the fungus "*Pleurotus ostreatus*", avoiding the bioethical and availability problems associated with crustaceans, its usual source. Chitin was extracted from Pleurotus ostreatus mycelium with a yield of 10.8%. Chitosan was obtained by diacetylation with a diacetylation degree of 82.6%. It was characterized by techniques such as infrared spectroscopy and thermogravimetric analysis. We incorporate into five ointment formulations with different solvents (cetyl alcohol, vegetable wax, petroleum jelly, coconut oil and beeswax). After one month of thermal stability tests to evaluate their organoleptic and functional properties, the most stable formulation showed a pH of 5.5, a viscosity of 2400 cP and an extensibility of 100-200 mm². Pleurotus ostreatus is an ideal source of chitosan with antimicrobial and healing properties, useful for industrial applications and skin care without resorting to crustaceans.

## Keywords

Chitin, polysaccharide, Chitosan, Pleurotus Ostreatus.

## 1. INTRODUCTION

The growing need to prevent or reduce pollution at source, both in industrial and laboratory environments, has stimulated the search for environmentally friendly raw materials. In the same context, we find that bioethical aspects related to the extraction of raw materials of animal origin have led science to find such raw materials in plant sources, as is the case of chitin, whose traditional source of extraction has been the exoskeleton of crustaceans (Baltodano et al., 2019).

Chitosan (or chitosan) is a complex macromolecule obtained by chemical processes from chitin, a natural polysaccharide found in the cell walls of fungi, including oyster mushrooms (genus *Pleurotus* ), as well as in the exoskeleton of crustaceans such as shrimps and crabs (Espinosa et al., 2020). However, this source has a bad reputation due to the overexploitation of these animals in their natural habitat, which limits its potential industrial acceptance. Therefore, fungi can be a good source for obtaining chitin from them (Guadalupe, 2016). This fungus is a sustainable, easy to cultivate and economical source compared to crustaceans. It includes data on the global availability of the fungus and its cultivability in different environments.

Chitin is a precursor of chitosan, which has numerous industrial applications. Chitosan is of natural origin and is environmentally friendly and biocompatible. It is therefore used in various sectors such as food, agriculture, cosmetics, pharmaceuticals and biomedicine. In addition, further research is ongoing to discover other possible applications (Espinosa et al., 2020, Mármol et al., 2016).

The objective of this study was to develop a pharmaceutical formulation based on chitosan macromolecules isolated by deacetylation from the cell wall of the mycelium of the fungus Pleurotus ostreatus. The compounds obtained were analyzed and incorporated into a pharmaceutical form with potential application in the cosmetic and pharmaceutical fields.

## 2. MATERIALS AND METHODS

In order to obtain and characterize the raw material (chitosan), the formulation and the physicochemical and rheological characterization of the ointment were conducted. The extraction and characterization of chitosan from the mycelium of the mushroom *Pleurotus Ostreatus* was first carried out, for which the following steps were performed, adapted from (Zapata, T. et al., 2014).

*Obtaining chitin*

For the production of chitin, the mycelium of the Pleurotus Ostreatus mushroom, a natural source rich in this polysaccharide, was processed and obtained by conditioning, demineralization, deproteinization, and decolorization. To obtain the mycelium of the Pleurotus Ostreatus mushroom, we proceeded according to the procedure of (Guadalupe, 2016), using yeast extract, peptone and YPG glucose enriched with the following salts 0.5 g $(NH_4)2SO_4$; 0.1 g $K_2HPO_4$; 0.1 g NaCl; 0.5 g $MgSO_4$ $7H_2O$; and 0.1 g $CaCl_2$. These values were expressed for

100 mL of water, with an adjusted pH of 6.3. Equation 1 was used to calculate the percentage yield of fungi on a dry basis.

$$\%\ Dried\ base\ mushroom = \frac{Dried\ mushroom\ mass}{Initial\ mass\ of\ the\ wet\ mushroom} * 100 \qquad \text{(Ecu: 1)}$$

Harnessing Pleurotus ostreatus as a source of chitosan not only reduces pressure on marine ecosystems, but also promotes more responsible agricultural and production practices. This sustainable approach represents a significant step towards a greener and more efficient bio economy.

*Chitosan production*

Chitosan (or chitosan) is a macromolecule because it is composed of a large number of repeating units of glucosamine and, to a lesser extent, N-acetyl glucosamine, linked by β-(1→4) -type glycosidic bonds. These characteristics classify it as a polysaccharide. To obtain chitosan, chitin was thermochemically deacetylated in an alkaline solution (Zapata, T. et al., 2014). For this purpose, the chitin was heated at 120 °C with 50% NaOH under constant stirring for 3 days at 8-h intervals each day. After this, the samples were washed with water until neutral and finally dried at 60 °C for 24 h. To calculate the percentage yield of chitosan on a dry basis from the fungus, equation 2 was used.

$$\%\ Chitosan = \frac{Final\ mass\ of\ chitosan\ obtained}{Initial\ mass\ of\ the\ dried\ mushroom} * 100 \qquad (2)$$

Chitosan was characterized by Fourier transform infrared spectroscopy (FT-IR) using an IRTracer-100 spectrophotometer (Shimadzu) and the attenuated total reflectance (ATR) sampling technique. The spectrum recorded over a range of 4000 cm-¹ to 400 cm-¹, was analyzed using LabSolutions-IR® software. This method is crucial for the characterization of chitosan because it allows the confirmation of its production from chitin (Zapata, T. et al., 2014). In general, spectroscopic methods involve the selection of a suitable characteristic band (intensity of a measured band of N-acetyl or amine content) and a suitable reference band (with invariant intensity with respect to the degree of disacetylation, DA) (Kluczka, J. et al., 2024).

The degree of N-acetylation (DA) of chitosan was determined according to Kluczka, J. et al., 2024, Brugnerotto et al. 2021) determined a linear relationship from the infrared spectra of several chitosan samples with different degrees of acetylation (0 to 100%) using transmission spectra. The DA calculation was based on the ratio of the intensities of the peaks at 1417 cm-¹ and 1313 cm-¹. The equation proposed for this calculation is as follows:

$$\frac{A_{1320}}{A_{1420}} = 0.3822 + 0.03133\ XDA \qquad \text{(Ecu:3)}$$

$$DA = \frac{\left(\frac{A_{1320}}{A_{1420}} - 0.3822\right)}{0.03133} \qquad \text{(Ecu: 4)}$$

Equation 5 was used to determine the degree of diacetylation (DD).

$$\text{Degree of diacetylation (DD)} = 100 - DA \qquad \text{(Ecu:5)}$$

Determine the AM/AR ratio, where AM is the intensity of a characteristic band that is a measure of N-acetyl or amine content, and AR is the intensity of a reference band whose intensity does not change with N-acetyl. The N-acetyl groups of the unknown samples can be estimated by comparing the AM/AR values with similar ratios of some reference samples that have known N-acetyl (Equation 6).

$$DA= (31.92 * (A\ 1320 / A\ 1420)-12.20 \qquad (Ecu:6)$$

The properties of chitosan, such as its degree of deacetylation, purity, thermal stability and ability to form films, make it a multifunctional ingredient for skin care products. Its ability to moisturize, protect, regenerate and fight microorganisms makes it ideal for applications in creams, masks, lotions and medical dressings. Characterization techniques ensure that the chitosan used meets the standards required to maximize its efficacy and safety in these products.

*Preformulation studies*

During this phase, solubility tests were performed using different excipients to identify the most suitable matrix for obtaining an ointment with optimal consistency and stability characteristics. Tests were performed to determine the solubility of chitosan in different solvents, includingcetyl alcohol, vegetable wax, Vaseline, coconut oil,, and beeswax. In each test, 0.25 g of chitosan and 10 g of solvent were taken and heated in a water bath at 50 °C with constant stirring for 30 min.

*Preparation of ointments*

Based on the results of the solubility tests, five formulations combining an oily and an aqueous phase were developed (Table 1). The five solvents (Cetyl alcohol, vegetable wax, petroleum jelly, coconut oil and beeswax) were selected due to specific criteria of functionality, chemical compatibility and desired characteristics in the final product formulation. The process consisted of preparing a 1% acetic acid solution and gradually adding chitosan with constant stirring until a homogeneous solution was obtained. Simultaneously, an oily mixture was prepared at 70 °C, and an aqueous mixture was gradually added, with constant stirring, until a homogeneous mixture was obtained.

**Table 1.** Composition of the 5 formulations elaborated.

| **Formulations with chitosan** | | |
|---|---|---|
| **Formulations** | **Oily phase** | **Aqueous phase** |
| **1** | 80 percent: Vaseline | 20 percent: 0.25-g of chitosan in 20 mL of acetic acid and 0.2% Sodium benzoate |
| **2** | 85 percent: Vaseline | 15 percent: 0.25-g of chitosan in 15 mL of acetic acid and 0.2% Sodium benzoate |

| | | |
|---|---|---|
| **3** | 85 percent: 5% Beeswax and 95% Vaseline | 15 percent: 0.25-g of chitosan in 15 mL of acetic acid and 0.2% Sodium benzoate |
| **4** | 85 percent: 5% Beeswax, 15% Coconut Oil, and 80% Vaseline | 15 percent: 0.25 g of chitosan in 15 mL of acetic acid and 0.2% Sodium benzoate |
| **5** | 85 percent: 10% Beeswax, 10% Coconut Oil, and 80% Vaseline | 15 percent: 0.25-g of chitosan in 15 mL of acetic acid and 0.2% Sodium benzoate |

*Formulation stability studies and selection of optimal formulations*

The five formulations were subjected to stability studies at room temperature (23-24 °C) and at 40 °C for 30 days, monitoring characteristics such as sandiness, granularity, odor, exudation, consistency, and ease of application (Pongo et al., 2015). These controls were performed every two days to identify the formulation with the best functional properties. In order to achieve better control in the trials, a factorial experimental design was proposed for the optimization of the ointment. A formulation factor was proposed with 5 levels corresponding to the formulations described in Table 1 and a temperature factor with two levels of 23 °C and 40 °C, with the treatments being combinations of the factor levels (Table 2).

**Table 2**. Factors, levels and treatments for statistical analysis of formulations.

| **Factors** | **Formulation** | **Temperature (C°)** |
|---|---|---|
| **Levels** | 1, 2, 3, 4, 5 | 23 y 40 |
| **Treatments** | 10 treatments:<br>1-23, 1-40<br>2-23. 2-40<br>3-23, 3-40<br>4-23, 4-40<br>5-23; 5-40 | |

The response variables are the organoleptic characteristics, which are qualitative; therefore, an experimental design was proposed that allowed it to be carried out under conditions as homogeneous as possible. For the data analysis, it was considered that each treatment was performed 15 times, with 3 observations each, and the treatments were performed randomly.

*Characterization of the optimal ointment formulation*

In order to characterize the optimal formulation, properties such as pH were analyzed and measured using a glass electrode combined with a pH meter to ensure homogeneity of the ointment. Viscosity was also evaluated using a Brookfield dv-ii + pro programmable rotational viscometer operated in a thermostatized environment at 23 °C with a speed gradient of 5–10 rpm and needle #7. In addition, extensibility was measured according to the procedure described in (Pongo et al., 2015) using virion coverslips and masses of 1, 2, 4, 5, 5, 10, and 20

g, with a waiting time of 1 min between each mass. To calculate the area of extensibility (AE), the expression $AE=\pi \times r^2$ was used, where: r = average radius of the 3 measurements (mm).

*Differential scanning calorimetry analysis (DSC) and thermogravimetric analysis (TGA)*

El *DSC,* is based on the absorption of infrared radiation by molecules, which generates specific vibrations (stretching or deformation) in chemical bonds. Each functional group of a molecule has a characteristic absorption spectrum. In the case of chitosan, it is used to identify the functional groups of chitosan, especially those that evidence its deacetylation from chitin. TGA measures the weight loss of a sample as a function of temperature, evaluating its thermal stability and composition. This analysis identifies changes in mass due to decomposition, water evaporation or thermal degradation. In the case of chitosan, it allows evaluating thermal stability and determining the presence of water or impurities. The conditions for the thermal tests are described below: TGA: 0–600 °C, DSC: 0–400 °C with a heating rate of 5 °C/min and nitrogen atmosphere.

*Statistical analysis*

In the stability studies of the formulations and selection of the optimal formulation, we used 5 formulations, which were subjected to stability studies at room temperature (23 – 24 °C) and at 40 °C for 30 days, monitoring characteristics such as grittiness, lumps, odor, exudation, consistency and ease of application (Pongo et al., 2015). These controls were carried out every two days in order to establish the formulation with the best functional characteristics by analyzing the data using the Minitab 2019 software. The design of the experiment had the following characteristics:

Factors, levels and treatments: We considered two factors, formulation with five levels and temperature with two levels, therefore, we have 10 treatments to evaluate.

The experimental unit is the ointment, and as response variables we have the organoleptic characteristics. All these variables are qualitative, therefore, an experimental design was proposed that would allow for an adequate interpretation of the results and to carry it out under homogeneous conditions. For the data analysis, it was taken into account that each treatment was carried out 15 times and these repetitions were carried out randomly.

## 3. RESULTS

*Chitin and chitosan are obtained*

First, Pleurotus Ostreatus was grown in 72.04% ± 0.1701%. In the conditioning, demineralization, deproteinization, and decolorization phases, organic matter, proteins, and minerals were eliminated, and finally, using an alkaline solution, the cellular structures were broken, and the chitosan was released. Average yields of 15% were obtained for chitin and 10.81% of chitosan per 100 grams of mushroom; this value is close to that reported by researchers who used the same extraction source, as well as Lactarius *vellereus* mushroom. In

addition, it is evident that depending on the starting source, different yields of chitosan are obtained (Sequeiros & Zaritzky, 2013, Crestini et al., 1996).

*Characterization of the obtained chitosan*

In the determination of the degree of acetylation (Figure 1), characteristic signals corresponding to chitosan can be observed. The bands between 3400-3500 cm-1 correspond to the elongation vibration of the OH groups; the signals at 2800, 2920, and 1319 cm1 are attributed to the CH2 (pyranose ring) stretching vibration; the signal at 1653 cm1 is also attributed to this vibration.

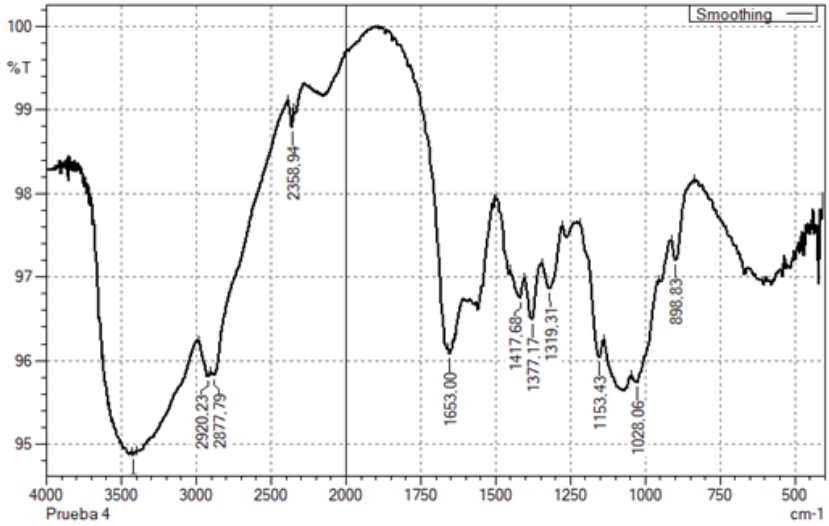


**Figure 1**. Infrared spectrum of chitosan

To calculate the degree of deacetylation (DD) of chitosan, the values of the height of each peak obtained by FTIR of wave number cm-1 1319.21 with an intensity of 96.87 at a height of 1344.38 and 1417.68 with an intensity of 96.75 at a height of 1450.47. This yielded a value of 82.6%, which is within the values reported for commercial and laboratory chitosan samples.

*Thermogravimetric analysis using TGA and differential scanning calorimetry analysis (DSC)*

TGA and DSC analysis (Figure 2) showed three main peaks:
- Moisture loss in a temperature range of 30-120 °C.
- Melting peak at 134.77 °C.
- Thermal decomposition at 291.8 °C.

These results indicate the thermal stability of the obtained chitosan.

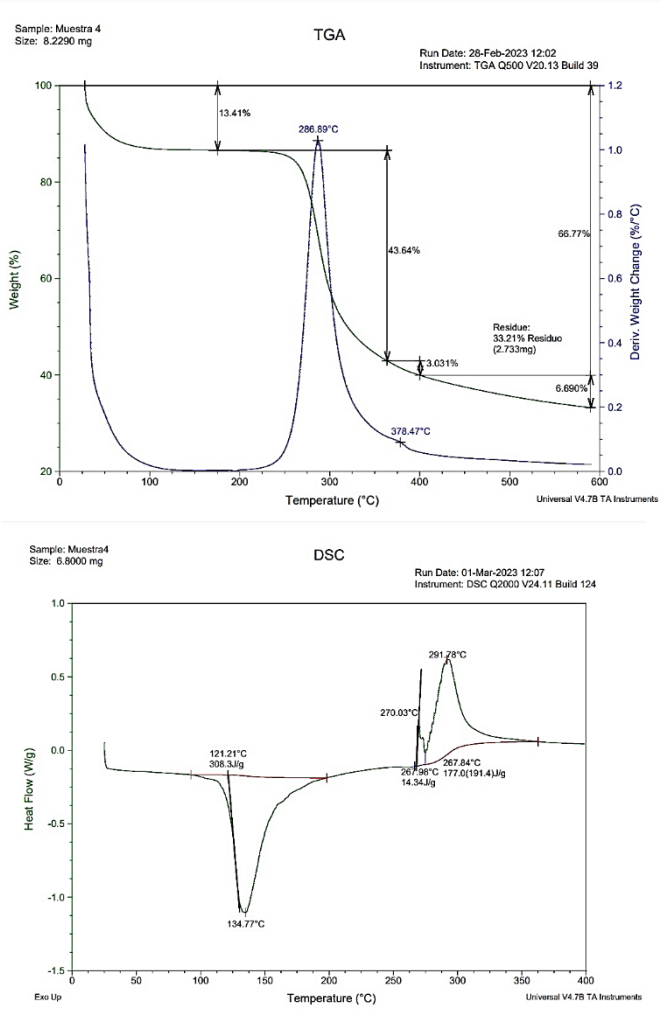


**Figure 2**. Thermal analysis for chitosan.

In the characterization of vaseline (Figure 3), it is expected that in the analysis by differential scanning calorimetry, two endothermic peaks occurred in the temperature range between 40 °C and 80 °C approximately, corresponding to the melting of the solid components present in the mixture. According to (Garcia et al., 2016), the value of 40 and 30 °C obtained can correspond to a change in the mass loss rate of Vaseline. In the DSC curve, the glass transition signal is manifested as a change in the specific heat, suggesting a change in the ability of Vaseline to store thermal energy. According to the work of (Sautina et al., 2014), a glass transition can be observed in the DSC curve of Vaseline near 35 °C, manifesting as a change in specific heat.

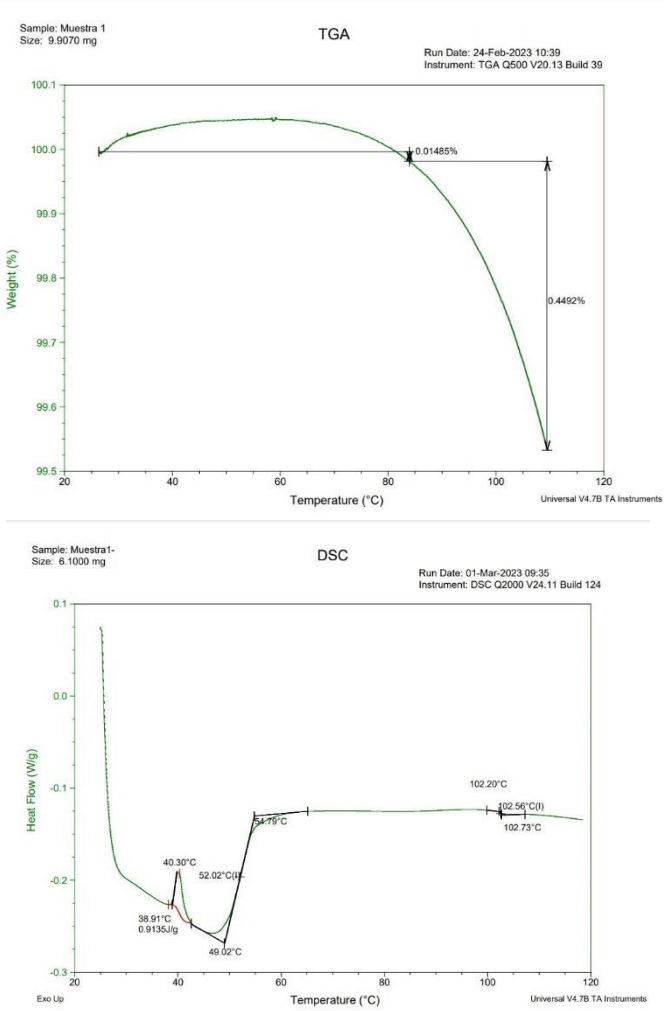


**Figure 3.** Thermal analysis for Vaseline.

In the characterization by thermogravimetric and differential scanning calorimetry analysis of coconut oil (Figure 4), the melting point of coconut oil depends on the variety and quality of the oil. In general, the melting point of coconut oil varies between 23 °C and 26 °C depending on the quality and measurement technique used (Sonwai et al., 2016). These data correspond to the obtained temperatures of 25 °C and 93 °C in the DSC analysis, as shown in Figure 4.

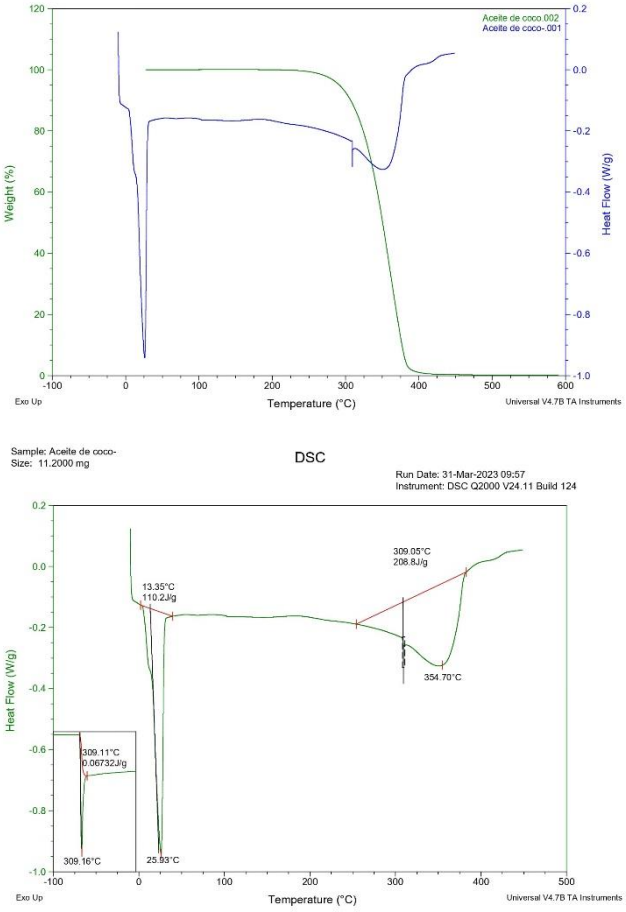


**Figure 4.** Thermal analysis for coconut oil.

In the analysis of beeswax (Figure 5), two main peaks were identified in the TGA and DSC analysis:

- Evaporation peak: in the range of 50-150 °C, related to the evaporation of volatile components, such as short-chain fatty acids.

- Melting peak: in the range of 60-80 °C, related to the melting of solid components, such as Cetyl palmitate. The peak observed at 60.07 °C could be associated with wax melting, while the peak at 46.06 °C could be due to evaporation of volatile compounds. TGA analysis showed that there was no significant mass loss up to 120 °C, suggesting absence of moisture.

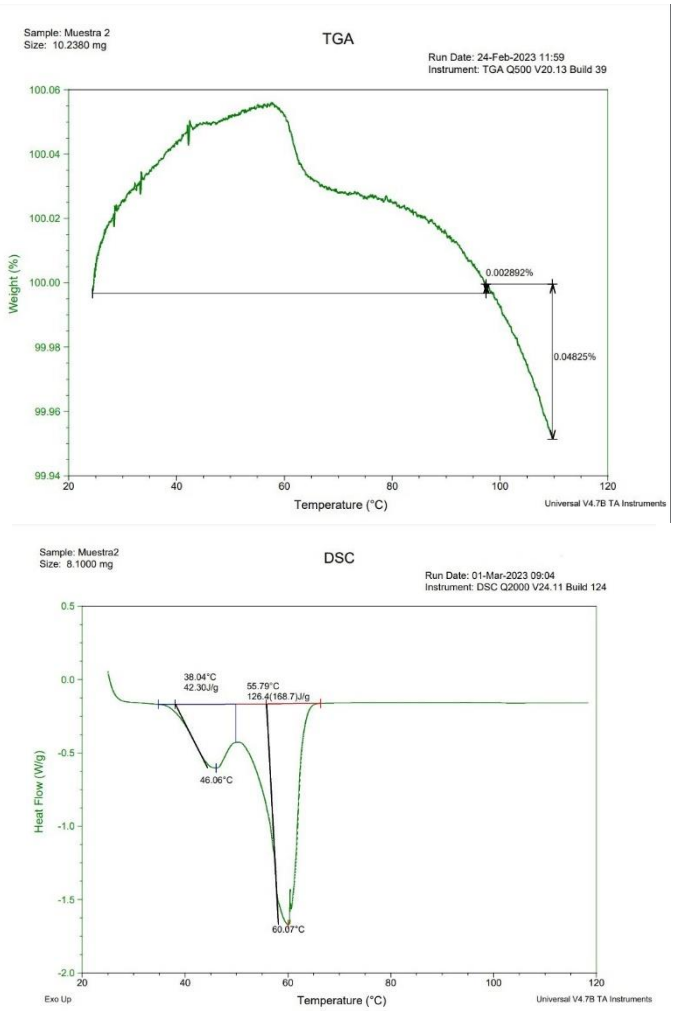


**Figure 5.** Thermal analysis for beeswax.

Characterization by thermogravimetry and differential scanning calorimetry analysis of sodium benzoate (Figure 6), it is established that the most representative peaks are:

The first peak occurs at a temperature of about 120-160 °C and is related to the dehydration of sodium benzoate. The second peak occurs at a temperature of around 450-550 °C and is related to the thermal decomposition of sodium benzoate.

According to (Alaa, 2021), in the results obtained by DSC analysis, we can appreciate melting peaks (146.59 °C) and thermal decomposition (364, 33 °C) of sodium benzoate, as shown in Figure 6. In addition, a gradual mass loss in the range 400–550 °C was observed in the TGA analysis.

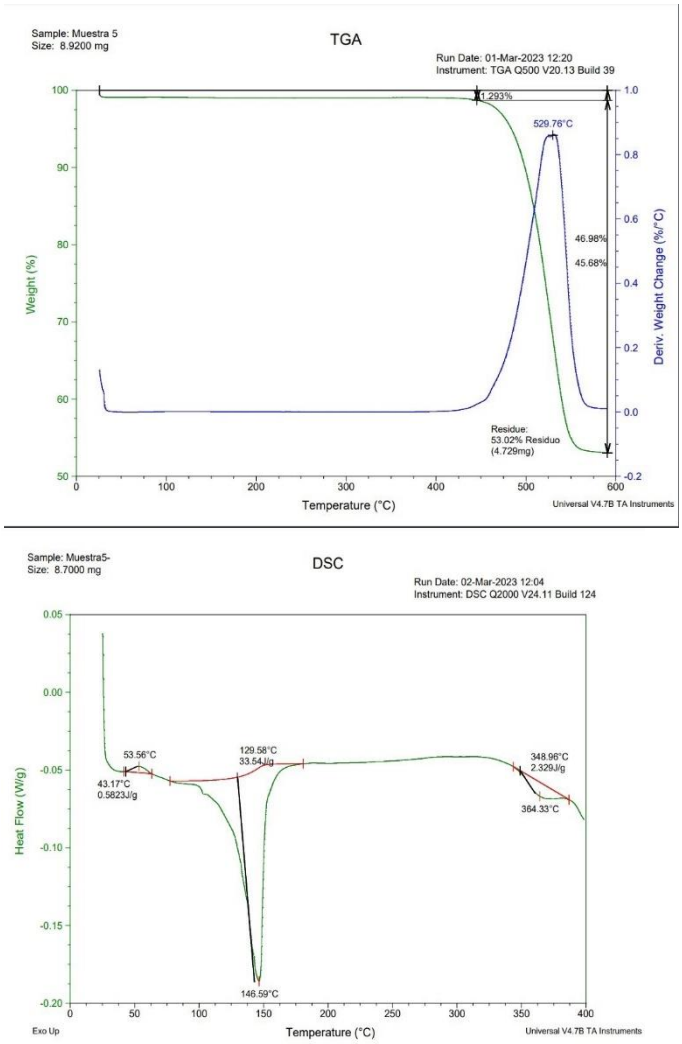


**Figure 6.** Thermal analysis for sodium benzoate.

*Preformulation studies*

The results obtained from the solubility study showed that Vaseline, beeswax, and coconut oil were soluble (+) and the substances Cetyl alcohol and vegetable wax were insoluble (-). These results are consistent with those reported by Reichenbach et al., 2019 and Villarreal, 2004, given that the interaction between chitosan and cetyl alcohol can be limited in oily phases even with the cationic nature of chitosan and the anionic nature of the carboxyl groups present in fatty acids (Cusihuamán Noa et al., 2018).

This is complemented by the fact that Cetyl alcohol is a solid fatty alcohol with a long and linear hydrocarbon chain, which makes the solubilization of chitosan difficult, although solubility can be improved by mixing Cetyl alcohol with polar solvents (Leyva & Hernández, 2013). Furthermore, both chitosan and Cetyl alcohol are insoluble in oils such as sunflower and

mineral oils (Khan et al., 2014. and Zhu et al., 2017). Moreover, both chitosan and Cetyl alcohol are soluble in polar and apolar solvents, and their solubility may depend on factors such as concentration, pH, and temperature. Some studies have shown that these two components are non-soluble to each other (Khan et al., 2014. and Zhu et al., 2017).

Regarding the positive evidence, one possible explanation is that the solubility of chitosan in petroleum jelly is due to its ability to dissolve the fatty acids present in chitin. According to some studies, petroleum jelly can act as a plasticizer, which means that it softens and makes chitosan more flexible, allowing it to dissolve in petroleum jelly (Boroumand, 2021).

Another possible explanation is the solubility of chitosan in Vaseline due to the presence of hydroxyl groups in the chitin structure. It has been shown that Vaseline can interact with these hydroxyl groups, allowing chitosan to dissolve in Vaseline (Chandra et al., 2008).

Finally, chitosan was soluble in coconut oil due to the presence of fatty acids that interact with the amino groups of chitosan, facilitating its dissolution. Solubility of chitosan in beeswax was also observed, which could be attributed to the presence of fatty acid esters that interact with the amino groups of chitosan, allowing its dissolution.

*Preparation of ointments*

With the information from the Preformulation studies and the literature review, the components of the formulations were selected according to their properties. Therefore, a concentration of 0.25% was used in the formulations developed, as this concentration has been determined to be effective and safe in previous studies (Tadić et al., 2021, Kumar et al., 2018, Dharmalingam et al., 2015). Too low a concentration may not be effective in stimulating healing; too high a concentration may cause adverse effects such as irritation and allergic reactions.

This is of great relevance because organoleptic properties such as color and odor are key factors in the acceptability of personal care products by users. In the case of skin-healing ointments, these characteristics can influence the perception of product efficacy, safety, and overall appeal:

- **Color** can influence product efficacy perception. According to a study (Terra, A. et al., 2018), users have higher confidence in a product's ability to improve skin appearance if the product is white or light beige compared to other colors. In addition, color can influence the overall appearance of a product, which can affect its user acceptability.
- **Smell** can affect the perception of product quality and efficacy. Users prefer products that have a pleasant aroma compared to those that are unscented or have an unpleasant aroma. In the case of skin-healing ointments, odor is especially important because the product is applied directly to the skin (Schnettler, B. et al., 2014).
- **The Consistency** of the ointment can influence the ease of application and perceived efficacy of the product. In (Terra, A. et al., 2018) found that users prefer ointments that have a smooth, non-greasy texture, as this facilitates application and absorption of the product into the skin.

- **The Skin sensation** on the skin after the application of the ointment can influence the perception of the efficacy of the product. According to (Bernal, 2016), users prefer ointments that provide a smooth and pleasant sensation after application compared to those that provide a sticky or greasy sensation.

*Formulation stability studies and selection of optimum formulation*

*Lumpiness analysis*

According to the European Pharmacopeia (2019), ointments should be homogeneous, free of lumps, and with adequate consistency because the presence of lumps may indicate incomplete mixing or destabilization of the product, thereby affecting its efficacy and safety. Figure 7 shows that formulations 1, 2, and 5 presented lumps in more than 50% of the tests performed at 40 °C and were therefore discarded.

The undesirable effect in formulations 1 and 2 is probably due to petroleum jelly, which is a mixture of semisolid or solid hydrocarbons that is not soluble in water due to its nonpolar nature. The chemical structure of Vaseline consists of saturated hydrocarbons, whereas water, a polar solvent, contains hydroxyl (-OH) functional groups that dissolve polar substances but not apolar compounds such as Vaseline (Dreher, F et al., 2022, Brown et al., 2009).

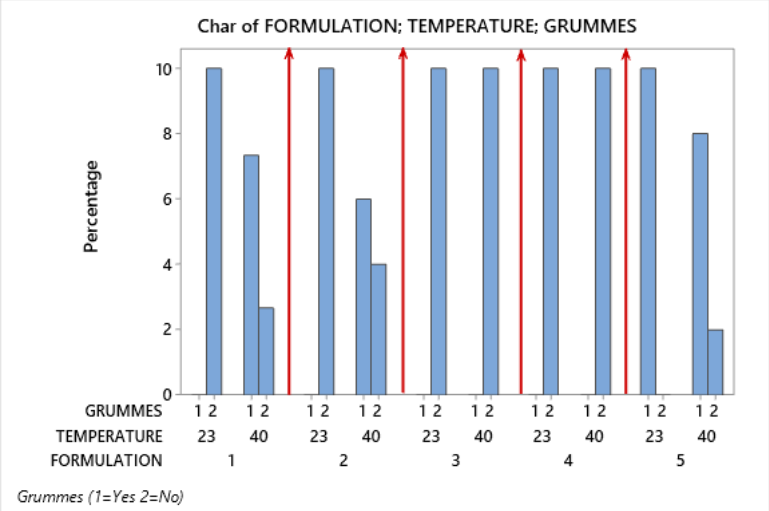


**Figure 7.** Graphical representation of the lumpiness for the 5 formulations at the test temperatures.

*Exudation analysis*

The United States Pharmacopeia (USP) establishes that ointments should be smooth and homogeneous, without exudation, although a small amount is permitted in specific situations. The results of the formulations showed exudation in formulations 1, 2, 4 and 5 at different temperatures (Figure 8). Formulation 1 was exuded at 23 °C and 40 °C, formulation 2 at 40 °C only, formulation 4 at both temperatures, and formulation 5 at 23 °C only. Formulation 3 did

not show exudation, which is consistent with the study of Isabel, M.S. (2017), who achieved a stable ointment with beeswax.

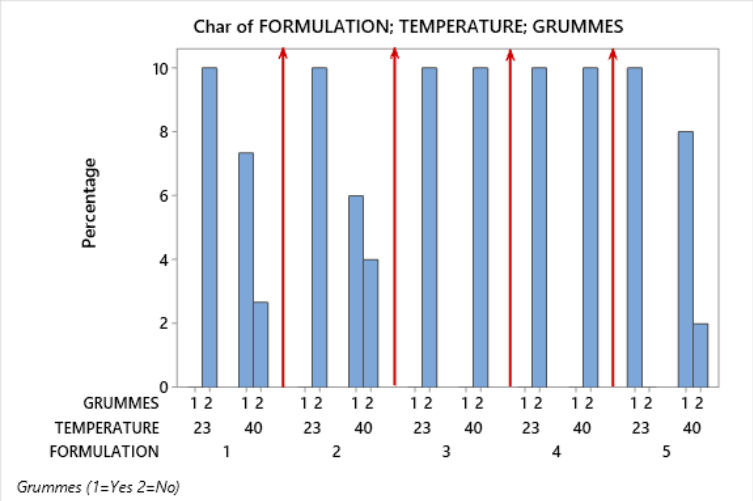


**Figure 8.** Graphical representation of the exudation for the 5 formulations at test temperatures.

According to the 2019 US Pharmacopeia, the ideal ointment texture should be of soft consistency, facilitating its application and uniform spreading on the skin without causing irritation or excessive friction. This texture also allows adequate absorption of active ingredients. Formulation 3 meets this consistency criterion.

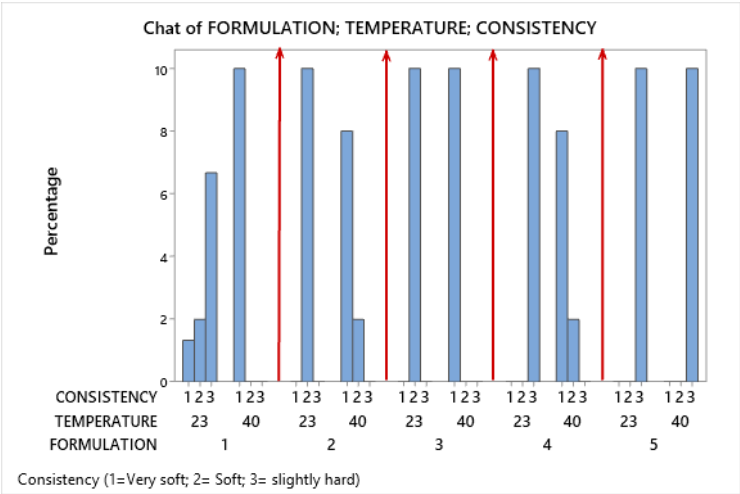


**Figure 9.** Graphic representation of the consistency for the 5 formulations at the test temperatures.

In the analysis of the five formulations, it was found that formulations 1, 2, and 5 did not meet some of the criteria established by the United States Pharmacopeia (USP). While 100% of the tests for formulations 3 at temperatures of 23 °C and 40 °C, (described in Table 1) did not reveal lumps, formulation 3 did not exude and was soft, while formulation 4 did not present lumps but

did exude and was soft. Therefore, the organoleptic characteristics of these two formulations are presented.

*Analysis of the organoleptic characteristics of formulas 3 and 4.*

Formulation 3 showed remarkable organoleptic stability with no sandiness, exudation, or lumps at any working temperature. The sample maintained a stable consistency during 30 days of testing at 23 °C, although it became slightly softer at 40 °C. On the other hand, formulation 4 did not present lumps but did show exudation at both temperatures, and its consistency varied significantly, being slightly hard at 23 °C and very soft at 40 °C, which limits its suitability as the best formulation.

After analyzing various parameters for one month at different temperatures, it was determined that the best formulation was number 3 (Table 1) because it does not present lumps, exudation, or sandiness and has a soft consistency. The beeswax in this formulation contributes to an adequate texture, neither too hard nor too soft, unlike formulations 1 and 2, which only use petroleum jelly as the oil phase. Although formulation 4 also contained beeswax, it showed exudation, especially at 40 °C, suggesting low coconut oil incorporation.

*Characterization of the optimal ointment formulation*

Optimum Formulation (Formulation 3)
- Composition Oil phase (85%): 5% Beeswax. 95% Vaseline.
- Composition Aqueous Phase (15%): 0.25 g of chitosan in 15 mL of acetic acid. 0.2% sodium benzoate.
This is the optimal formulation, as it met all U.S. Pharmacopeia (2019) criteria.
*pH measurement*
- The pH is key to the efficacy of topical products, as the skin has a protective acid mantle with an ideal pH between 4 and 7.
- The pH of formulation 3 is 5.5, which is compatible with human skin, allowing better penetration and favoring topical application.
*Viscosity Test*
- Viscosity is crucial for product stability and texture.
- For petroleum jelly based ointments, a viscosity between 3000-3500 cP is recommended.
- The formulation with petroleum jelly and beeswax showed a viscosity of 2400 cP, which is considered adequate for optimal firmness and application.
*Extensibility Test*
- Extensibility determines the ease with which the ointment is applied and evenly distributed on the skin.
- The extensibility of the ointment was observed to be between 100 and 200 $mm^2$, gradually increasing with applied weights, ensuring optimal application.

Rheological characterization is essential for ointment development because rheological properties affect product stability and texture (Ansari et al., 2022). The study evaluated extensibility and viscosity to ensure that the ointment is evenly applied and distributed on the skin. Adequate viscosity is crucial; 3000-3500 cP is recommended for petroleum jelly-based

ointments (Ansari et al., 2022), although it can range from 40-1500 cP at 37 °C, increasing with thickening agents such as microcrystalline wax (Walters, 2002). The formulation containing petroleum jelly and beeswax has a viscosity of 2400 cP, which is adequate for optimum firmness and application.

Thermal analyses (DSC and TGA) are essential to evaluate the stability, compatibility and ability of the components to withstand manufacturing and storage conditions (Figure 10). In ointment formulation, these analyses provide insight into the thermal properties of the components and the mixture, allowing the evaluation of their stability, compatibility and ability to withstand manufacturing and storage conditions. In addition, this technique is useful to identify any possible interactions between the components of the formulation and to establish the appropriate temperatures for the manufacture of the ointment (Jarnagin et al., 2016).

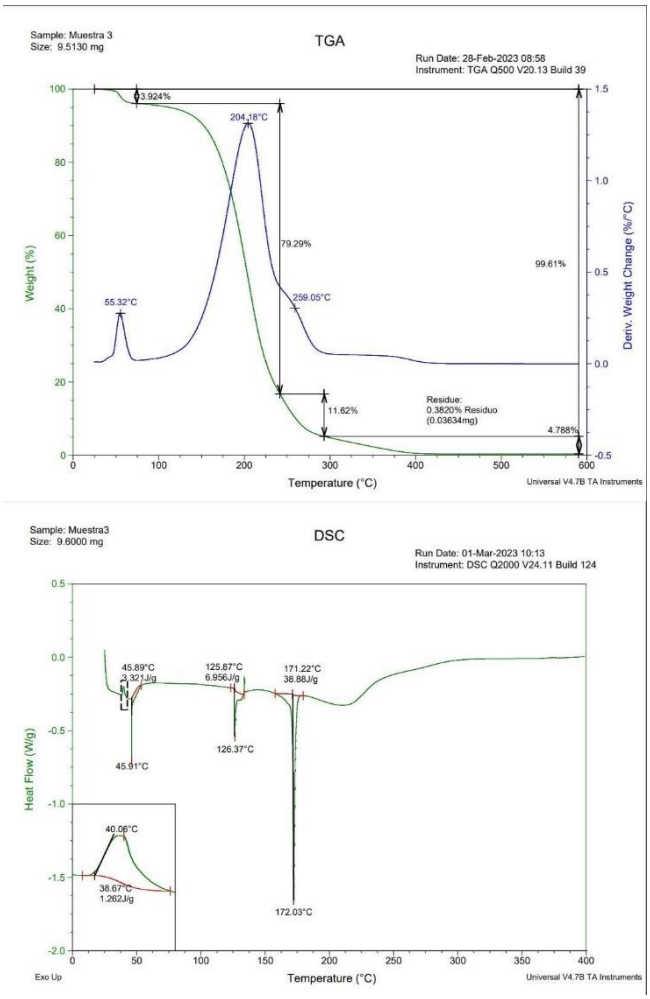


**Figure 10.** Thermal analysis for formulation 3 (ointment).

## 4. DISCUSSION

This band is known as the stretching band of the C=O bond in amide I; the signal 1550-1590 cm-1 due to the bending vibration of the NH-R amine group; at 1417 cm1 corresponds to the vibration of the OH groups in the ring; the signal at 1377 cm1 is due to the CH3 methyl groups; the signal at 1260 cm1 is due to the vibration of the C-O group and at 1150-1070 cm-1 due to the -C-O-C glycosidic bond (Ramirez Barragan et al., 2018).

Thus, medium- and low-molecular-weight Sigma-Aldrich chitosan showed DD values of 76.2% and 80.1%, respectively. In addition, the chitosan Quitoscience has a DD of 82.3% (Ramirez Barragan et al., 2018, Guadalupe, 2016). In addition, chitosan also extracted from Pleurotus Ostreatus has a DD of 80.7%, indicating that the chitosan extracted in this research has 18.6% more deacetylation (Lopez M. et al., 2017).

Thus, DD indicates the total amount of acetamide groups converted to amine, which directly determines properties such as solubility, basicity, and adsorption, functionality, and applications of chitosan (Canepa, 2018). For example, (Cheng et al., 2016) reported that chitosan with a DD >75% favored its antimicrobial activity and tumor cell inhibition. In addition, for (Civantos, 2014) reported that a DD between 77% and 88% favors tissue regeneration, indicating that the obtained chitosan has healing activity.

Characterization of the active principle of chitosan by TGA and DSC is important in the pharmaceutical industry as an ointment because it provides insight into its thermal properties and thermal stability (Ahmad et al., 2018). The obtained information is useful for determining the stability of the active principle in different production and storage processes, as well as for designing stable pharmaceutical formulations. The main peaks observed in TGA and DSC for chitosan (Figure 2), the following can be identified (Cruz et al., 2010):

Moisture loss peak: This peak refers to the moisture loss of chitosan due to its hygroscopic nature. The temperature range of this peak is about 30-120 °C.

Melting peak: This peak refers to the melting of chitosan in its crystalline form. This peak is important for determining the temperature at which a drug melts and can be used to design pharmaceutical formulations. The temperature range of this peak is approximately 130-200 °C; for this sample, the melting peak was recorded at 134.77 °C.

Thermal decomposition peak: This peak refers to the thermal decomposition of chitosan and its degradation. This peak is important for determining the thermal stability of the drug. The temperature range of this peak is approximately 230-350°C; in this case, it was recorded at 291.8°C.

The most representative thermogravimetric and differential scanning calorimetric peaks of chitosan are the thermal decomposition peaks (291.8 °C), which allow determination of the thermal stability of the active ingredient in different production and storage processes.

Regarding the evaporation point, there is no specific evaporation point for coconut oil because it is a complex mixture of lipids. Instead, TGA can be used to determine the temperature at which the thermal decomposition of coconut oil begins. Thus, it has been reported that the thermal stability of coconut oil is high and that significant decomposition does not occur until temperatures above 200 °C (Srivastava et al., 2017). In this study, mass loss can be seen from 300 °C onwards. In the DSC thermogram, two endothermic peaks can be observed above 300 °C, which may correspond to the thermal decomposition of triglycerides present in coconut oil (Elodio-Policarpo et al., 2019).

Characterization by thermal analysis can help identify the presence of impurities or adulterants in beeswax, which is important from the viewpoint of the quality and safety of pharmaceutical products (Buchwald et al., 2008).

This peak refers to the melting of solid components of beeswax, such as cetyl palmitate. This peak is important for determining the temperature at which beeswax melts and can be used to design pharmaceutical formulations. The temperature range of this peak was approximately 60-80 °C (Al-Shehri et al., 2022).

In the case of sodium benzoate, the observed dehydration and thermal decomposition peaks coincide with the results reported by Alaa (2021), which reinforces its predictable thermal behavior and stability under extreme conditions.

On the other hand, palm wax (or vegetable wax) is a mixture of fatty acid esters and long-chain alcohols, so the presence of ester groups may limit their interaction with chitosan and palm wax in oily phases, deriving insolubility (Dos Santos et al., 2017). Additionally, similar results have been reported for soybean oil (Ruyuan et al., 2020).

In terms of future applications, the results indicate that the solubility of chitosan in different components may depend on factors such as the presence of functional groups (hydroxyls, esters), the length of the hydrocarbon chains and the nature of the intermolecular interactions. These findings may be useful for the formulation of new products that require the integration of chitosan in natural oil and wax matrices, with potential applications in the food, pharmaceutical and personal care industries.

The results obtained demonstrate that the 0.25% concentration of chitosan is optimal for the formulation of healing ointments, as it guarantees efficacy without compromising user safety. Previous studies support this concentration as effective in promoting skin healing, while higher concentrations may generate risks of adverse reactions. The exclusion of parabens is also consistent with current trends in the development of safer and more environmentally friendly products, responding to concerns about their impact on health and the environment (Basketter et al., 2012).

The use of preservatives such as paraben was ruled out because they have negative effects on health, ecology, and biodiversity (Basketter et al., 2012, Fang et al., 2019).

Thus, in the design of the formulations, chitosan was taken as a reference, which has been studied and used in formulations with high healing power, (Baltodano et al., 2019), indicating that the formulations should have penetrating power through the skin, so a semi-solid

pharmaceutical form such as a gel or ointment was thought of. Thus, given the physicochemical properties of the chitosan obtained and its solubility, it was decided to design an ointment with a hydrocarbon base (white petroleum jelly) that allows the incorporation of a minimum amount of the aqueous component. Thus, chitosan can be kept in prolonged contact with the skin and acts as an occlusive bandage.

Beeswax is a complex mixture of esters, hydrocarbons, alcohols, and fatty acids produced by bees. Its amphiphilic structure contains polar and apolar functional groups, allowing interactions with water through hydrogen bonds and van der Waals forces, which facilitate the formation of emulsions. Therefore, it is commonly used in cosmetic and pharmaceutical formulations (Brown et al., 2004; Bogdanov, 2016). In formulations 3, 4, and 5, 10% beeswax caused lumps, suggesting that 5% is the optimal amount to avoid this problem.

On the other hand, formulation 3 stood out as the only one that did not present exudation at any of the temperatures tested (23 °C and 40 °C), which is in line with the results reported by Isabel, M.S. (2017), who also achieved a stable ointment using beeswax. This suggests that formulation 3 has a more stable composition, possibly due to a better interaction between its ingredients, which prevents phase separation and exudation of the product at different temperatures.

In contrast, formulations 1, 2 and 5 do not meet several of the criteria established by the USP, since they presented exudation at least one of the temperatures tested. This problem compromises their stability and, therefore, their effectiveness in topical application. Although formulation 4 also exuded at both temperatures, it is important to note that it did not present lumps and maintained a smooth texture, which is a positive aspect in terms of organoleptic evaluation.

Organoleptic analysis of the formulations showed that while formulations 1, 2 and 5 have exudation problems, formulations 3 and 4 were smooth to the touch and did not present lumps. This indicates that, from an organoleptic point of view, both formulations offer a pleasant sensory experience. However, the exudation observed in formulation 4 puts it at a disadvantage in terms of stability compared to formulation 3, which is the only one that meets all USP criteria.

Formulation 3, which passed all US Pharmacopeia 2019 criteria, contains in the 85% oil phase: 5% Beeswax and 95% Vaseline; and in the 15% aqueous phase: 0.25 g Chitosan in 15 mL acetic acid and 0.2% sodium benzoate in the 15% aqueous phase, which is the optimal formulation to be characterized.

pH regulation in ointment manufacturing is crucial, as the skin's effectiveness in fighting infections and environmental stress depends on the pH balance. The skin has a protective acid mantle, with a pH ideally between 4 and 7, which protects it from bacteria, fungi, viruses, and pollutants, keeping it soft and supple. It is recommended that skin products have a pH between 4.5 and 6.0 to maintain homeostasis. Formulation 3, with a pH of 5.5, is compatible with the skin and allows greater penetration, thereby favoring topical application.

Extensibility is crucial in the formulation of ointments because it affects the ease of application and coverage of the skin with a minimum amount of product, improving user

satisfaction and product efficacy. An ointment should spread easily without excessively increasing friction (Walters, 2002; Orlandi, 2004). In this study, the extensibility of the ointment was between 100 and 200 mm², gradually increasing with the weights applied, ensuring optimal application on the skin (Pongo & Herrera, 2015).

In ointment formulation, thermal analyses allow evaluation of the stability, compatibility, and ability of components to withstand manufacturing and storage conditions. They also help identify interactions between components and establish suitable manufacturing temperatures (Jarnagin et al., 2016). Figure 10 shows that the formulation exhibits adequate thermal stability with no significant changes in thermal properties. Thermogravimetric and differential scanning calorimetry analyses identified thermal decomposition and representative peaks for Vaseline and chitosan.

Using thermogravimetry and differential scanning calorimetry, the results indicated that the ointment exhibited good thermal stability and no significant changes in composition. By comparing the individual thermogram of each component of the formulation with the thermogram of the complete formulation, it is possible to identify that the mass loss of the formulation, which occurred around 100 °C, corresponds to the mass loss of the beeswax, and the peaks obtained in the DSC of the formulation correspond to those obtained in the thermogram of each component. The formulation exhibited adequate thermal stability with no significant decomposition observed during the study. In addition, the results showed that the ointment did not significantly change the chemical composition or physical appearance during the study period.

## 5. CONCLUSIONS

- The techniques employed allowed us to obtain an optimum chitosan yield of 10.8% and a deacetylation degree of 82.6%, in accordance with previous studies on other fungal species.
- Pleurotus Ostreatus is a good source of chitosan and has established itself as an optimal raw material for its multiple industrial applications, avoiding the use of crustaceans.
- Thermal analysis of the raw materials made it possible to establish their quality, composition, and thermal stability.
- Thermal tests and monitoring of organoleptic characteristics made it possible to obtain an optimum formulation for topical application on the skin.
- The incorporation of chitosan into the formulation offers additional benefits due to its antimicrobial and healing properties, making the ointment a promising alternative for skin care.
- Further studies are required to test the effect of chitosan on different skin conditions, optimize formulations or compare its effectiveness with existing commercial products.
- The formulation containing 5% Beeswax and 95% Vaseline is the most promising due to its thermal stability, absence of exudation, compatibility with skin pH and optimal organoleptic and extensibility characteristics. This positions it as an effective and safe option for use in topical applications.

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## Study limitations

- It is important to perform in-vitro and in-vivo tests to determine the degree of healing of the ointment.
- Long-term stability studies should be performed to evaluate the microbiological characteristics of the product.
- In-vitro and in-vivo tests are necessary to evaluate the efficacy of the ointment prior to its use in humans, reducing health risks and ensuring product quality.
- These tests can also optimize the formulation of the ointment and adjust the concentrations of components, such as coconut oil, to avoid phenomena such as exudation.

## Ethical Approval

Ethical Approval is not applicable for this article.

## Statement of Human and Animal Rights

This article does not contain any studies with human or animal subjects.

## Statement of Informed Consent

There are no human in this article and informed consent is not applicable.

## Declaration of Competing Interest

The authors declare that they have no financial interests or personal relationships that could have influenced this work.

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## Data Availability

Data will be made available on request.

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