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Evaluation of physicochemical, microbiological and cutaneous permeation properties of *Calendula officinalis L.* In different concentrations and dosage forms

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ABSTRACT
C. officinalis is a herbaceous plant that has anti-ulcerative, healing, restorative, anti-edematous and refreshing properties. In this article, the permeation of the glycolic extract of *C. officinalis* in cream and gel at different concentrations was evaluated. Accelerated stability tests were also carried out, as well as organoleptic, physical-chemical and microbiological tests.

Keywords: *Calendula officinalis L.*, Physical-chemical, Microbiological, Skin permeation.

1 INTRODUCTION

The incidence of malignant neoplasms has increased in recent years, with a prevalence of diagnoses of head and neck tumors, with radiotherapy being the main form of treatment. Radiotherapy uses ionizing radiation, promoting the death of malignant cells, in addition to causing lesions that are most often irreversible. Tissue damage induced by radiation emitted by radiation therapy is called radiodermatitis.[1]

In an analysis of publications, the most commonly used allopathic drugs in the prevention and treatment of radiodermatitis are topical corticosteroids, Tetra-Cream® and Bepantol®. However, these drugs have numerous adverse reactions, such as contact dermatitis (due to the preservatives used), skin infections, perioral dermatitis, skin atrophy, and systemic adverse effects (they can also occur due to topical corticosteroid therapy).[2]

The use of plants is an ancient practice that stands out as one of the oldest forms of medicinal practice in humanity. The use of medicinal plants has increased in recent years due to the absence of more economically viable therapies. *C. officinalis*, also known as mal-me-quer, 23 golden daisy and

wonder-of-the-gardens, stands out for its numerous indications, used for pruritus, solar erythema, burns, dry dermatoses, wounds, ulcers, acne, skin toner, skin fungi, jaundice, eye inflammation and prevention of radiodermatitis, among others. Its use is recommended topically for anti-inflammatory and healing therapeutic actions, stimulating tissue regeneration by increasing the metabolism of glycoproteins, nucleoproteins and collagen proteins.[3]

C. officinalis is a plant that has already been used in semi-solid formulations for the treatment of patients who develop radiodermatitis. In a randomized, double-blind clinical study in patients with head and neck cancer, *C. officinalis* cream was effective in the prevention and treatment of radiodermatitis.[4]

The permeation of active ingredients through the skin occurs in two ways: transepidermal (intercellular and intracellular) and transadnexal (transfollicular and transudoridoparous). In vitro skin permeation tests are used using the Franz method. The cells are made up of two compartments: the donor and the recipient, and the skin sample is placed between the two compartments. Due to the physiological similarities with human skin, pigskin is the most suitable animal model for performing this method.[5]

The physicochemical evaluation of formulations consists of a set of tests performed in the laboratory, aiming to evaluate the physical and chemical properties and characteristics of the product. Among the types of tests are pH, density, spreadability and centrifugation.[6] Microbiological tests, on the other hand, identify microbial contamination of a product, analyzing changes with physical and chemical properties, seeking to find signs of risk of infection for the user.[7]

2 OBJECTIVE

To analyze the physicochemical, microbiological and cutaneous permeation properties of two semi-solid dosage forms at different concentrations of the glycolic extract of the *C. officinalis.*

3 MATERIAL AND METHODS

For the development of the formulations, a literature search was carried out, which supported the main active ingredients to be used in cream (Table 1) and gel (Table 2), such as emulsifiers (cream), neutralizing and gelling agents (gel), surfactants, humectants, antioxidants and preservatives.

Table 1. Formulation of anionic cream used to incorporate the glycolic extract of *C. officinalis* at concentrations 7% and 10%

Input	**Usage Percentage**
Oil Phase (Phase B)	
Lanette N	16%
Octyl Stearate	16%
Glycerol	6,35%
Aqueous Phase (Phase A)	
Parabens Solution	3,3%
Water	qsp 800mL
Phase C	
Imide preservative solution	0,6%

Source: From the author (2023).

Table 2. Formulation of carbopol gel used to incorporate C. *officinalis glycolic* extract at 7% and 10% concentrations

Input	**Usage Percentage**
Carbopol 940	1%
Disodium EDTA	0,05%
Propylene Glycol	5,2%
Imide preservative solution	0,5%
AMP-95 Solution 50%	2%
Purified water	qsp 800mL

Source: From the author (2023).

During the production of the formulations, some aspects were analyzed to verify the compatibility of the extract in the vehicles, such as the solubility of *C. officinalis* in the cream and gel, pre-stability, homogeneity and organoleptic characteristics.

3.1 STUDY SAMPLE

For comparative analysis of the cutaneous permeation *of C. officinalis*, the glycolic extract was used at concentrations of 7% and 10% in two vehicles: cream and gel produced in the laboratories of the Pharmacy-School of the University of Vale do Taquari - Univates. The concentrations mentioned were previously taken from the report of the glycolic extract of *C. officinalis,* which recommends a

concentration between 5-10% of the active ingredient in lotions, creams, aftershave and post-waxing products, shampoos, conditioners and soaps. The determination of permeability was analyzed in porcine skin. The skin in question was obtained soon after the slaughter of the pig and there was no scalding process, so that there was no loss of favorable conditions for permeability. After performing the skin permeation tests by the Franz cell method, physicochemical and microbiological tests were performed on the formulations that permeated.

3.2 SKIN PREPARATION

The pig's skin was removed from the cartilage with a scalpel and sanitized with 70% alcohol and water, being intact and with minimal blood vessels and fats. After the skin was removed, it was cut into circles and measured with a caliper to obtain similar sizes. The skin was stored in aluminum foil and kept at -4 °C until the moment of use, so that it did not exceed 30 days, so that there would be no loss of any permeability condition. To perform the test, the skin was thawed and heated in an ultrathermostatic bath at 32 °C, simulating body temperature.

3.3 FRANZ CELLS

The method used to analyze permeability is Franz's, as it is the most widely used method to determine skin permeation in medicines and cosmetics. Franz cells are known to be static, finite-dose diffusion cells where the skin is arranged in a Vertical Diffusion Cell (VDC). Being the best method of quality control in topical formulations.[8]

Franz's method is the most widely used method for verifying the *in vitro* absorption of topical formulations such as gels, creams, ointments, lotions, and transdermal patches. Franz diffusion cells study the cutaneous permeation of semi-solid dosage forms in the skin.

In this method, a vertical diffusion cell is used, containing a donor compartment and a recipient compartment, separated by the membrane sample. The formula was applied to pig skin, which had the epidermis and dermis. The method is characterized by the use of diffusion cells, where the active ingredient passes through the membrane by diffusion, to the recipient solution, in which the analytical delimitation of the content of the permeated active ingredient was performed through time.[9]

The analyses were carried out in triplicate at the Chemistry laboratories of the University of Vale do Taquari - Univates. The recipient solution was made with a 1:1 ratio of water and alcohol: 250ml of purified water + 250ml of alcohol. The receptor solution used in the study had a maximum of 17 mL inside the Franz cell. In the donor's compartment, 0.2g of the vehicle containing the glycolic extract was applied. During the experiment in the laboratories, the luminosity was also controlled so that there was no interference. The standardized time was 15 minutes and 30 minutes.

3.4 IN VITRO SKIN PERMEATION

After removing the recipient solution at 15 minutes and 30 minutes, it was placed in an amber bottle so that the light would not interfere with the result. The samples were analyzed in triplicates in a UV spectrophotometer, at a wavelength of 517 nm.[10]

3.5 ORGANOLEPTIC TESTS

Organoleptic assays were the methods used to verify the characteristics of formulations through the sense organs, such as appearance, color, odor and touch. This method analyzes the state of the product in a quick and easy-to-perceive way in cases of alterations such as phase separation, precipitation and turbidity.[6]

3.6 PHYSICOCHEMICAL TESTS

3.6.1 Centrifuge Test

The centrifuge test caused stress on the product, reproducing an increase in the force of gravity, increasing the mobility of the particles and accelerating probable instabilities. The changes were analyzed in the form of precipitation, phase separation, caking and coalescence.[6]

The test was performed with 30g of the analyzed sample, where the sample was submitted to the centrifuge at 3,000 rpm for 30 minutes. The centrifuge test analyzes changes in formulations before stability tests are performed.[11]

3.6.2 Accelerated Stability Tests

Accelerated stability testing is an important quality control to evaluate the efficacy, safety, and quality of the product. This test indicates the degree of stability of the formulation under different environmental conditions that affect the product from its manufacture to its shelf life. According to RDC No. 318 of 2019, this parameter analyzes the chemical, physical and microbiological changes of formulations in a forced state of storage, helping to determine the shelf life of the product. This test looks at the shelf life and compatibility of the product with its storage material. The formulations were stored at different temperatures, to evaluate their characteristics in different environments that may be exposed. The stability assessment period varies according to the specifications of the formula, the active ingredients and preservatives used.[12th]

Due to the time available for the tests, the stability test lasted 60 days, due to the time available after the preparation of the formulations and the development of the skin permeation test. To carry out this research, the samples were stored at room temperature/bench (20 °C - 25 °C), greenhouse temperature (40 °C) and refrigerator temperature (5 °C). The times used in this stability study will initially be at time zero, days seven, 15, 30 and 60. The parameters analyzed were the organoleptic

characteristics, the physicochemical tests (pH, density, spreadability and centrifugation) and the microbiological test performed at time zero, right after production, all physicochemical and microbiological tests were performed in triplicate.[13]

3.6.3 ph

pH determination is by potentiometry, where the electrode is immersed in the sample to determine the activity of hydrogen ions. The scale of values 1 to 14 determines the acidity or alkalinity of a formulation, in which the closer to 1 the more acidic and the closer to 14 the more alkaline, and the value 7 is considered neutral pH. To determine the pH, a device called pH meter was used. Before using the equipment, it is essential to clean and check the sensitivity of the electrode using reference buffer solutions.[6]

In semi-solid formulations, the measurement is not carried out directly on the product, a 10% aqueous solution (1:10) has been prepared. Values should not vary by more than 0.05 pH units in three consecutive readings. The three consecutive readings with a difference of 0.05 were added and divided by three to obtain the average.[14th]

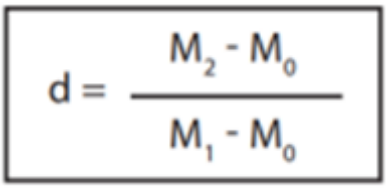
3.7 DENSITY

Density is the ratio of the mass to the volume of the product to be analyzed. The density of the formulations was measured using a metal pycnometer, which is the most suitable for use in semi-solid and viscous formulations.[6]

In this method, the empty pycnometer was weighed and its value (M0) was recorded. Soon after, Milli-Q water was poured until it overflowed and the lid was placed, causing the water to leak through the hole located in the lid, taking care not to generate bubbles. After that, it was dried and weighed again and its value (M1) was noted. Then, the pycnometer was emptied and dried carefully, and then the sample was placed, causing it to leak when the lid was placed. It was dried, weighed and the value (M2) was noted.[6]

Finally, the following formula was applied with the values obtained as shown in Figure 1.

Figure 1. Density Calculation



Source: Brazil (2008, p. 36).

Where: d = density

M0 = mass in grams of empty pycnometer

M1 = mass in grams of the pycnometer with Milli-Q water

M2 = mass in grams of the pycnometer with the sample

Density analysis temperature = 20 °C

3.8 SPREADABILITY

Spreadability is the empirical measure, described by KNORST in 1991, where it determines the ability of the formulation to spread by means of a force that is applied. Aiming to reduce the tangential effort made when applying the formulation to the skin.[15th]

To perform the test, a circular glass mold plate was used, with a hole in the center of the plate containing 1.20 cm in diameter. This circular plate was placed on top of a glass support plate (20 cm x 20 cm). On the top of the square plate there is a sheet of graph paper, with a light source. The sample to be analyzed was placed in the hole of the circular plate and leveled with the aid of a spatula. Then the mold plate was carefully removed and a glass plate was placed on the specimen with the predetermined weight. For each plate added, the measurement of the diameter in two opposite positions and the weight of the plate were recorded. The glass plates were added until it reached a constant value. The interval for the addition of each plate is at least 1 minute.[6]

Spreadability was calculated using the following equation:

Ei= (d2 x π) /4

E = spreadability of weight specimen i

d = average diameter (mm)

3.9 MICROBIOLOGICAL TESTING

The microbiological test identifies microbial contamination of a product, analyzing changes with physical and chemical properties, seeking to find indications of risk of infection for the user. This test is performed in oral and topical formulations, not sterile. For the analysis of microbiological contamination, the acceptable microbial limits for each active ingredient or formulation should be considered.[16]

The microbiological evaluation was performed by the method of counting the total number of mesophilic microorganisms, through the determination of fungi and bacteria, the following culture media was used: casein-soybean conducive to bacterial growth (Table 3) and sabouraud-dextrose agar conducive to fungal growth (Table 4).

Table 3. Formulation of casein-soybean culture medium

Casein-soybean culture medium		
Tryptic soja agar		40g
Purified water		1000 mL

Source: Brazilian Pharmacopoeia (2010, digital text).

Table 4. Formulation of sabouraud-dextrose agar culture medium

Sabouraud-dextrose agar culture medium	
Sabouraud dextrose agar	65g
Purified water	1000mL

Source: Brazilian Pharmacopoeia (2010, digital text).

To dilute the sample, a buffer solution of sodium chloride-peptone with a pH of 7.0 was prepared (Table 5).

Table 5. Formulation of Sodium Chloride-Peptone Buffer Solution

Sodium chloride-peptone buffer solution, pH 7.0	
Monobasic potassium phosphate	3.6g
Disodium phosphate dihydrate	7.2g
Sodium chloride	4.3g
Peptona (meat or casein)	1.0g

Purified water	1000mL

Source: Brazilian Pharmacopoeia (2010, digital text).

The microbiological test was performed in a fume hood in triplicate for each dilution. To dilute the 2g of the sample, 20 mL of the buffer solution was used at around 45 °C. The dilutions performed were 1:10, 1:100, 1:1000. The petri dishes were properly identified, where three white dishes were prepared for each sample, one for each culture medium. The buffer and media were sterilized in an autoclave using a validated cycle. In each plate, 1 mL of the diluted sample was transferred and culture medium was placed until the surface was covered (about 20 mL). The temperature of the culture medium was around 45 °C.[16]

The plates were capped when the medium was solidified and then sent to their respective oven:

- Greenhouse at 35 °C - casein-soybean culture medium plates;
- Greenhouse at 25 °C - plates of sabouraud culture medium;

The test shows a visible count of microorganisms within 5 days, in casein-soybean agar at 32.5 °C ± 2.5 °C, and in up to 7 days, in Sabouraud-dextrose agar at 22.5 °C ± 2.5 °C.

3.10 DATA ANALYSIS

To compare skin permeation, the t-test was used for paired samples, for analysis between times at the same concentrations. Between different concentrations, ANOVA followed by Tukey was used.

Organoleptic characteristics were expressed by tables and descriptive analysis was performed. In the centrifugation test, a descriptive analysis of the results was performed.

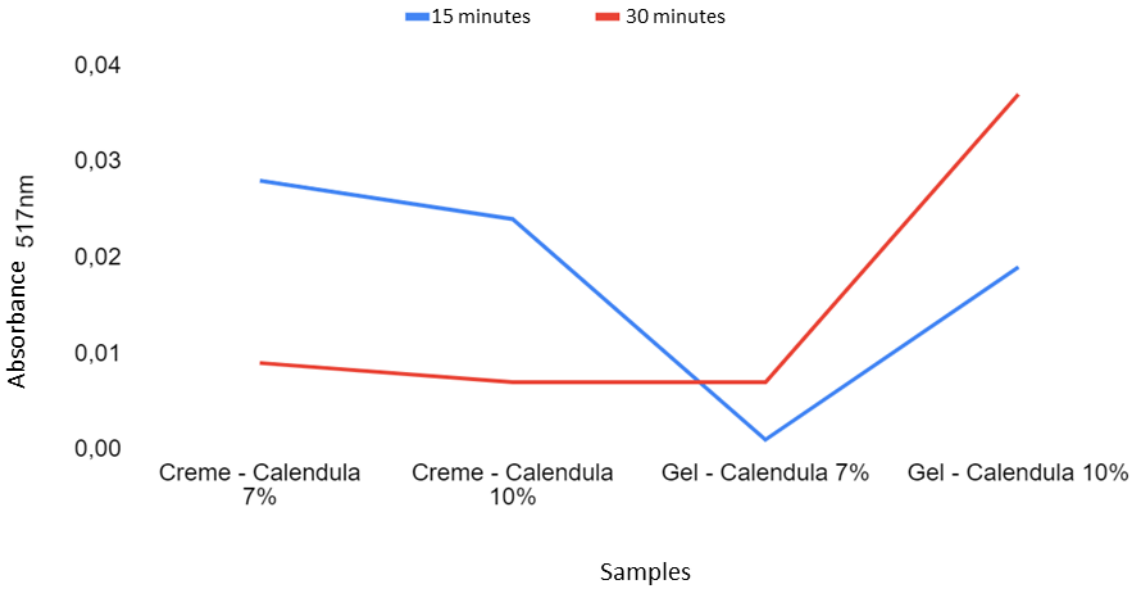
To compare pH and density at different times and concentrations, ANOVA was used, followed by Tukey using the JAMOVI software.[17] Descriptive statistics were used for spreadability. And the microbiological tests were expressed by tables and descriptive analysis was performed.

4 RESULTS AND DISCUSSIONS

4.1 PERMEATION TESTS

With the skin permeation tests performed, it was observed that the 10% *C. officinalis gel sample in 30 minutes has better permeation compared to* the 7% C. officinalis gel and the 7 % and 10% C. *officinalis* cream, *as shown in Graph 1.*

Graph 1. Variation of the absorbance value in the skin permeation test



Source: From the author (2023).

The absorbance results indicate that there was permeability in all concentrations and formulations tested.

The stratum corneum is considered the greatest barrier in the absorption of active ingredients, as it is composed of lipids, which form layers, thus hindering the diffusion of substances and because of this there is little skin permeability. When the active ingredient passes through the stratum corneum, the active ingredient can penetrate layer by layer, the lipophilic to a greater degree and the hydrophilic to a lesser degree.[18th]

With the statistical analysis, it was possible to observe that all samples showed statistically significant difference ($p<0.05$) in the permeation test, considering the times, concentrations and formulations. In gel samples of *C. officinalis*. At 7% and 10% the permeability of the samples was higher in 30 minutes. On the other hand, the cream samples of *C. officinalis* at 7% and 10% had higher permeability in 15 minutes.

The use of topical products has better patient adherence, being a non-invasive and painless method most of the time, in addition, the product will not be subjected to first-pass metabolism and there are fewer side effects compared to other routes of administration. However, a small amount of active ingredient will reach the target tissue, most of which is wasted due to the partial impermeability of the skin.[19] Thus, the product must have a hydrophilic-lipophilic balance for efficient permeation through the cutaneous route.[20th]

The transdermal route is a route that has been widely used due to the development of drugs and cosmetics with systemic action, where the active ingredient is absorbed into the bloodstream. To achieve this permeation, some allies have been used in the formulations, such as surfactants, ionic pairs

and liposomes, or even alcohol and fatty acids. The physicochemical characteristics of formulations and active ingredients are determining factors for cutaneous absorption.[21]

Zampieri, in his studies with genistein, using gel and emulsion as a vehicle, observed significant greater cutaneous permeation of the gel in relation to the emulsion. The method used in the study was also Franz cells, using porcine skin, but the standardized time for collection of the recipient solution and analysis was 24 hours.[22]

With the result obtained in the analyses, it is possible to observe that the vehicle used in the formulations can alter the characteristics of the stratum corneum, thus influencing the permeation of the active ingredients in the skin. In a study, it is possible to observe that the vehicle used in the formulations alters the permeation of the active ingredients in the skin, altering the skin hydration and the permeability profile.[23rd]

At the end of the study, it was found that there was greater permeation of the gel if associated with the other manipulated bases, due to its formulation being more aqueous. Thus, it is observed that pharmaceutical bases directly influence the permeation of active ingredients in the skin.

4.2 ORGANOLEPTIC TESTS

The samples were placed in a stability study, which presented the organoleptic characteristics contained in Charts 1, 2, 3 and 4.

Table 1. Cream - *C. officinalis* 7%

Condition	**Time (days)**	**Characteristics Organoleptic**
Standard Sample	0	Homogeneous cream smooth and shiny, with light brown coloration and characteristic odor
Environment	7	Remained the same as the standard sample
Environment	15	Remained the same as the standard sample
Environment	30	Remained the same as the standard sample
Environment	60	Remained the same as the standard sample
Refrigerator	7	Remained the same as the standard sample
Refrigerator	15	Remained the same as the standard sample
Refrigerator	30	Remained the same as the standard sample
Refrigerator	60	Homogeneous cream smooth and slightly opaque, with light brown coloration and characteristic odor

Greenhouse	7	Remained the same as the standard sample
Greenhouse	15	Cream with a darker color on the surface and the rest with a lighter color and characteristic odor
Greenhouse	30	Cream containing air bubbles, water droplets, lighter surface than the rest of the cream, and slightly altered odor
Greenhouse	60	Cream containing air bubbles, water droplets, lighter surface than the rest of the cream, and slightly altered odor

Source: From the author (2023).

Table 2. Cream - *C. officinalis* 10%

Condition	**Time (days)**	**Characteristics Organoleptic**
Standard Sample	0	Homogeneous cream smooth and shiny, with light brown coloration and characteristic odor
Environment	7	Remained the same as the standard sample
Environment	15	Remained the same as the standard sample
Environment	30	Remained the same as the standard sample
Environment	60	Remained the same as the standard sample
Refrigerator	7	Remained the same as the standard sample
Refrigerator	15	Remained the same as the standard sample
Refrigerator	30	Remained the same as the standard sample
Refrigerator	60	Homogeneous cream smooth and slightly opaque, with light brown coloration and characteristic odor
Greenhouse	7	Remained the same as the standard sample
Greenhouse	15	Cream with a darker color on the surface and the rest with a lighter color and characteristic odor
Greenhouse	30	Cream containing air bubbles, water droplets, lighter surface than the rest of the cream, and slightly altered odor
Greenhouse	60	Cream containing air bubbles, water droplets, lighter surface than the rest of the cream, and slightly altered odor

Source: From the author (2023).

In both formulations of creams, it was possible to observe that the organoleptic characteristics remained stable at room temperature (20 °C - 25 °C) throughout the study. The samples submitted to the refrigerator cycle remained stable on day 7, 15 and 30, but on day 60 they were slightly opaque.

The cream samples submitted to the oven (40 °C) began to show changes from day 15 onwards, with a darker color on the surface and the rest with a lighter color and characteristic odor. On day 30,

the samples showed air bubbles, water droplets, a lighter surface than the rest of the cream and a slightly altered odor, maintaining these same characteristics on day 60.

Panel 3. Gel - *C. officinalis* 7%

Condition	Time (days)	Characteristics Organoleptic
Standard Sample	0	Homogeneous and shiny gel, with brown coloration and characteristic odor
Environment	7	Remained the same as the standard sample
Environment	15	Remained the same as the standard sample
Environment	30	Remained the same as the standard sample
Environment	60	Remained the same as the standard sample
Refrigerator	7	Remained the same as the standard sample
Refrigerator	15	Remained the same as the standard sample
Refrigerator	30	Remained the same as the standard sample
Refrigerator	60	Remained the same as the standard sample
Greenhouse	7	Remained the same as the standard sample
Greenhouse	15	Gel containing water droplets on the surface, brown coloration, and slightly altered odor
Greenhouse	30	Gel containing air bubbles, with water droplets on the surface, brown coloration, and slightly altered odor
Greenhouse	60	Gel containing air bubbles, with water droplets on the surface, brown coloration, and slightly altered odor

Source: From the author (2023).

Panel 4. Gel - *C. officinalis* 10%

Condition	Time (days)	Characteristics Organoleptic
Standard Sample	0	Homogeneous and shiny gel, with brown coloration and characteristic odor
Environment	7	Remained the same as the standard sample
Environment	15	Remained the same as the standard sample
Environment	30	Remained the same as the standard sample
Environment	60	Remained the same as the standard sample
Refrigerator	7	Remained the same as the standard sample
Refrigerator	15	Remained the same as the standard sample
Refrigerator	30	Remained the same as the standard sample

Refrigerator	60	Remained the same as the standard sample
Greenhouse	7	Remained the same as the standard sample
Greenhouse	15	Gel containing water droplets on the surface, brown coloration, and slightly altered odor
Greenhouse	30	Gel containing air bubbles, with water droplets on the surface, brown coloration, and slightly altered odor
Greenhouse	60	Gel containing air bubbles, with water droplets on the surface, brown coloration, and slightly altered odor

Source: From the author (2023).

In both gel formulations, it was possible to observe that the organoleptic characteristics remained stable at room temperature (20 °C - 25 °C) and in the refrigerator (5 °C) throughout the study period.

The gel samples submitted to the oven (40 °C) began to show changes from day 15, containing water droplets on the surface, brown color and slightly altered odor. On day 30, the samples showed air bubbles, with water droplets on the surface, brown color and slightly altered odor, maintaining these same characteristics on day 60.

The odor changes of the formulations of creams and gels, slightly altered, may be the result of the packaging where the samples were stored, since they were made of plastic.

When products are subjected to extreme temperatures, it is acceptable to have small changes in the appearance of the product, but extreme changes, such as the cream changing its appearance to liquid, are not acceptable. For color and odor variation, it is recommended that they remain in accordance with the standard sample. The sensory of the product is a determining factor in semi-solid formulations, determining whether the product will be accepted or not by the consumer, being analyzed through the organoleptic characteristics.[24th]

The organoleptic characteristics of the product may change depending on where it is stored, such as plastic or glass containers. The plastic container can undergo alterations, due to heat fixation, thermal resistance and oxygen permeation. When this incompatibility between the product and the packaging occurs, phase separation and precipitation of the product can occur, even leading to a change in pH.[25th]

It is possible to observe that both creams and both gels, regardless of the concentration of the glycolic extract of *C. officinalis*, presented the same organoleptic characteristics.

In a study carried out with creams and gels containing grape seed extract, for 60 days, changes in the organoleptic characteristics were also observed from day 15 of analysis in the greenhouse sample, intensifying on day 30 and remaining stable until day 60. The study subjected the product to

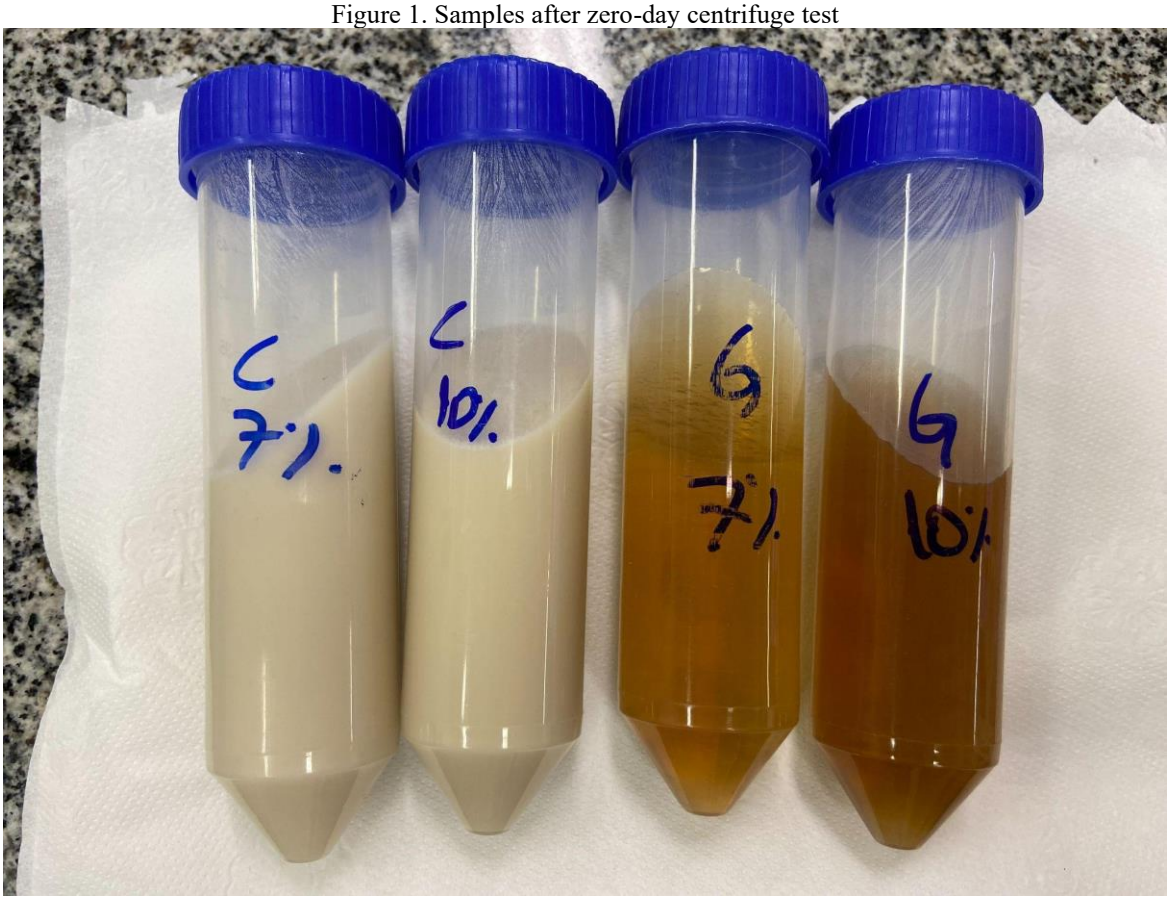
instability tests at room temperature, refrigerator and oven. The study was also carried out with four different formulations, two cream and two gel, in two different concentrations of the extract.[26th]

4.3 PHYSICOCHEMICAL TESTS

4.3.1 Centrifuge Test

After centrifugation test, the products remained stable and without phase separation (Image 1).

Figure 1. Samples after zero-day centrifuge test



Source: From the author (2023).

According to Anvisa's Guide for Quality Control of Cosmetic Products, the centrifugation test is responsible for causing stress in the sample, increasing the circulation of particles, thus anticipating some instabilities, consisting of the force of gravity on the formulation. Changes can be observed through precipitation, coalescence, phase separation, and caking, among others.[6]

The spin test is responsible for identifying possible chemical and physical instabilities that could harm the products. In a study carried out with gel and cream with a product of natural origin, both were stable, and were subsequently submitted to stability studies.[27]

4.3.2 ph

The reference samples/day 0 of the creams presented pH values within the variations allowed according to the Manual of Magistral Pharmacotechnical Management, while the reference samples of the gels did not present values within the standard allowed according to the Manual of Magistral Pharmacotechnical Management, requiring adjustment in the pH, according to the values contained in Table 6, 7 and 8.[28]

The sample that showed the lowest statistical variation of pH values under different storage conditions was the 7% gel. At room temperature and in the oven, the 7% gel showed greater stability, however, when stored in the refrigerator, all formulations showed statistically significant variations.

Table 6. Mean and standard deviation values of pH at room temperature of C. *officinalis* cream and gel samples at 7% and 10% on days zero, 7, 15, 30 and 60

Samples - ENVIRONMENT	**pH day 0**	**pH day 7**	**pH day 15**	**pH day 30**	**pH day 60**
Creams - Calendula 7%	6.99± 0.03a	6.88± 0.03b	7.04± 0.01c	7.13± 0.02d	6.84± 0.03e
Creams - Calendula 10%	6.66± 0.01a	6.74± 0.02b	6.95± 0.02c	7.04± 0.01d	6.66± 0.02e
Ice - Calendula 7%	7.62± 0.02A	8.00± 0.01B	7.99± 0.03B	7.97± 0.02b	7.59± 0.02C
Ice - Kalendula 10%	7.71± 0.02a	7.53± 0.02b	7.97± 0.02C	7.64± 0.00D	7.34± 0.02e

Source: From the author (2023).

*Different lowercase letters on the same line indicate a statistical difference ($p<0.05$) between the sample means.

Table 7. Mean and standard deviation pH values in refrigerator of C. *officinalis* cream and gel samples at 7% and 10% on days zero, 7, 15, 30 and 60

Samples - REFRIGERATOR	**P.S. is 0.**	**P.S. is 7.**	**P.S. is 15.**	**P.S. is 30.**	**P.S. is 60.**
Creams - Calendula 7%	6.99± 0.03A	7.11± 0.03B	7.26± 0.02C	7.11± 0.02d	6.94± 0.00e
Creams - Calendula 10%	6.66± 0.01a	6.83± 0.03B	6.92± 0.00C	6.93± 0.03D	6.80± 0.00E
Ice - Calendula 7%	7.62± 0.02A	7.87± 0.03B	7.99± 0.03C	8.25± 0.01d	7.50± 0.03E
Ice - Kalendula 10%	7.71± 0.02a	7.58± 0.00b	7.71± 0.02C	7.96± 0.01d	7.33± 0.02e

Source: From the author (2023).

*Different lowercase letters on the same line indicate a statistical difference ($p<0.05$) between the sample means.

Table 8. Values of the mean and standard deviation of pH in an oven of *C. officinalis* cream and gel samples at 7% and 10% on days zero, 7, 15, 30 and 60

Samples - GREENHOUSE	**P.S. is 0.**	**P.S. is 7.**	**P.S. is 15.**	**P.S. is 30.**	**P.S. is 60.**
Creams - Calendula 7%	6.99± 0.03A	7.15± 0.02B	6.56± 0.03C	6.78± 0.03d	6.78± 0.02d
Creams - Calendula 10%	6.66± 0.01a	6.56± 0.00b	6.35± 0.01c	6.61± 0.02d	6.51± 0.03e
Ice - Calendula 7%	7.62± 0.02a	7.91± 0.02b	7.93± 0.01b	7.80± 0.03c	7.84± 0.02c
Ice - Kalendula 10%	7.71± 0.02a	7.55± 0.02b	7.51± 0.01b	7.66± 0.02c	7.46± 0.02d

Source: From the author (2023).
*Different lowercase letters on the same line indicate a statistical difference ($p<0.05$) between the sample means.

According to the Manual of Pharmacotechnical Management of Magistral Creams, the pH range indicated is from 6.0 to 7.0. For formulations using carbopol gel-based, the recommendation is a pH range between 6.0 to 7.0. [28]

The samples stored at room temperature showed a statistically significant variation in most formulations and times, with the exception of the 7% C. *officinalis* gel, which did not change significantly between day 7 and day 30. On the other hand, all samples stored in the refrigerator showed statistically significant variation. The samples stored in the oven varied in the statistical results, with the 7% cream showing stability only from day 30 to day 60, the 10% cream showed statistical variation at all times, and the 7% gel showed stability between day 7 to day 15 and between day 30 to day 60. and the 10% gel showed stability between day 7 and day 15.

Physiological skin has a slightly acidic pH (4.0 - 6.5), which changes according to gender, location, and age. The pH of a semi-solid formulation is an essential factor for its efficacy and safety, contributing to bactericidal and fungicidal protection on the surface of the skin, and formulations with acidic pH have better skin permeation, as they are less ionized, causing less irritation to the skin. Formulations with a pH between 4.0 and 8.0 are less harmless to the skin.[29th]

The Practical Guide of Farmácia Magistral recommends that semi-solid formulations with pH values outside the expected standard should be corrected with a pH correcting agent when necessary.[30] In the case of the formulations developed in the present study, their correction with a citric acid solution would be recommended to adjust the pH and favor skin permeation.

When high pH changes occur in formulations, the indication is of degradation of compounds in the product, thus compromising its efficacy and safety over time. Therefore, stability tests in semi-solid formulations are indispensable to analyze physicochemical parameters and stability of active ingredients in different vehicles. For a product to remain stable, the pH changes must be small in the

storage time, not fluctuating too much from the pH at time 0, because the stability of products is related to high pH variations.[31]

In a stability study, carried out with glycolic extract of *C. officinalis* for 60 days, using an emulsion developed with non-ionic self-emulsifying wax (Polawax), it showed instability, not supporting large temperature variations, presenting pH changes. The use of wax in the development of emulsions is difficult, because during all the tests the pH temperature varied, not supporting temperature changes, so the way of storage and transport influence the stability and physicochemical tests of the products containing glycolic extract of *C. officinalis* in non-ionic emulsions.[32th]

The cream samples showed statistically significant variation in practically all storage conditions, but the variation of these values is not significant when referring to the established pattern that delays changes in the parameters of the product. On the other hand, the gels showed less statistically significant variation when compared to the cream samples, not altering the stability of the product, but requiring correction of pH values, thus favoring even more the permeation of the product.[13]

4.4 DENSITY

The reference formulations/day 0 of the creams and gels presented density analysis values within the standard allowed according to the Cosmetic Products Quality Control Guide, according to the values contained in Tables 9, 10 and 11.[6]

The analyzed samples did not show statistically significant changes in most formulations and times in the density analyses. Since the 10% gel formulation of *C. officinalis* is the only formulation with no statistical change when stored at room temperature and in the refrigerator, showing alteration only between days 7 and 15 in the oven.

Table 9. Mean and standard deviation values of density at room temperature of *C. officinalis* cream and gel samples at 7% and 10% on days zero, 7, 15, 30 and 60

Samples - ENVIRONMENT	Day 0 Density	Density Day 7	Density Day 15	Density Day 30	Density Day 60
Creams - Calendula 7%	0.876± 0.00A	0.861± 0.00A	0.862± 0.00A	0.912± 0.00b	0.901± 0.01b
Creams - Calendula 10%	0.903± 0.00A	0.853± 0.00b	0.860± 0.01b	0.908± 0.01c	0.915± 0.00c
Ice - Calendula 7%	0.998± 0.00A	0.972± 0.00b	0.996± 0.01c	0.979± 0.02c	0.980± 0.00c
Ice - Kalendula 10%	0.975± 0.00A	0.970± 0.00A	0.976± 0.00A	0.975± 0.01a	0.984± 0.01a

Source: From the author (2023).

*Different lowercase letters on the same line indicate a statistical difference ($p<0.05$) between the sample means.

Table 10. Values of the mean and standard deviation of the refrigerator density of C. *officinalis* cream and gel samples at 7% and 10% on days zero, 7, 15, 30 and 60

Samples - REFRIGERATOR	Day 0 Density	Density Day 7	Density Day 15	Density Day 30	Density Day 60
Creams - Calendula 7%	0.876± 0.00A	0.865± 0.01a	0.853± 0.00A	0.879± 0.00b	0.920± 0.05B
Creams - Calendula 10%	0.903± 0.00A	0.940± 0.01B	0.894± 0.01c	0.926± 0.01d	0.843± 0.01e
Ice - Calendula 7%	0.998± 0.00A	1.02± 0.03A	0.970± 0.01b	0.986± 0.01c	0.957± 0.01c
Ice - Kalendula 10%	0.975± 0.00A	0.977± 0.00A	0.977± 0.00A	0.991± 0.01a	0.993± 0.01a

Source: From the author (2023).

*Different lowercase letters on the same line indicate a statistical difference ($p<0.05$) between the sample means.

Table 11. Values of the mean and standard deviation of the greenhouse density of *C. officinalis* cream and gel samples at 7% and 10% on days zero, 7, 15, 30 and 60

Samples - GREENHOUSE	Day 0 Density	Density Day 7	Density Day 15	Density Day 30	Density Day 60
Creams - Calendula 7%	0.876± 0.00A	0.876± 0.00A	0.871± 0.00A	0.878± 0.01a	0.917± 0.00b
Creams - Calendula 10%	0.903± 0.00A	0.903± 0.00A	0.883± 0.00b	0.938± 0.01c	0.916± 0.01c
Ice - Calendula 7%	0.998± 0.00A	0.998± 0.00A	0.983± 0.01b	0.990± 0.00b	0.986± 0.00b
Ice - Kalendula	0.975± 0.00A	0.975± 0.00A	0.959± 0.00b	0.975± 0.01b	0.969± 0.01b

10%					

Source: From the author (2023).
*Different lowercase letters on the same line indicate a statistical difference ($p<0.05$) between the sample means.

At room temperature, the 7% cream showed statistical changes only between days 15 and 30, remaining unchanged at the other times, while the 10% cream showed changes between days 0 and 7 and days 15 and 30. The 7% gel showed changes between days 0 and 7, 7 and 15, stabilizing in the rest of the other times, while the 10% gel remained without statistical changes in all times.

When stored in the refrigerator, the 7% cream sample showed statistically significant changes between days 15 and 30, remaining stable in the other times, and the 10% cream showed statistical variation in all times. The 7% gel showed statistical variation between days 7 and 15 and between days 15 and 30, and the 10% gel did not show statistically significant variation at any of the times.

In the greenhouse, the 7% cream showed statistically significant variation only between days 30 and 60, while the 10% cream showed changes between days 7 and 15 and days 15 and 30. The 7% and 10% gels showed statistical variation only between days 7 and 15, remaining stable in the rest of the times.

According to the Quality Control Guide for Cosmetic Products, formulations based on anionic cream, the density value range is 0.85-0.95 g/mL. For formulations based on carbopol gel, the permissible range is 0.9-1.0 g/mL.[6]

In a study carried out with a vegan O/A emulsion, containing oily extract of *C. officinalis* and glycolic extract of *Chamomilla recutita*, the formulation was submitted to organoleptic and physicochemical characterization tests. The density tests in the study were performed only on day 0, presenting density values within the standard established by the Cosmetic Products Quality Control Guide, density of 0.933± 0.00.[33rd]

Density is a quality control that indicates whether there has been air incorporation or loss of volatile inputs. The samples, in most storage times and conditions, did not show statistically significant variation, however, the few changes they presented It is not significant when referring to the established standard that delays changes in product parameters.

4.5 SPREADABILITY

In all samples of creams and gels, it was possible to observe statistically significant changes in the results of the spreadability tests when compared to the different times. If we compare all times, temperatures and samples, it was observed that the *C. officinalis* at 10% in the greenhouse showed greater spreadability. However, due to changes that the product presented when stored in an oven, this form of storage is not considered adequate, therefore, the sample that had the greatest spreadability at room temperature and in the refrigerator was the formulation of gel of *C. officinalis* at 7%.

The samples showed satisfactory results in relation to spreadability, as the values increased as the weights/glass plates were added.

Each formulation showed different spreadability results in each storage condition. The 7% cream showed greater spreadability when stored in the refrigerator on day 60. On the other hand, the 10% cream showed greater spreadability in the oven on day 7. The 7% gel showed greater spreadability when stored at room temperature and in the refrigerator on day 30. On the other hand, the 10% gel showed greater spreadability when stored at room temperature on day 30.

In the different storage conditions, the samples showed different spreadability values when compared in the oven, refrigerator and room temperature. At room temperature and in the refrigerator, the 7% gel showed greater spreadability, whereas when the samples are stored in the oven, the 10% cream showed greater spreadability.

It is observed that all formulations did not show continuity in relation to spreadability in their storage conditions and times, because at some times the values increased and others decreased, this is due to the temperature variation of the central air of the laboratory that was stored and also to the temperature variation of the minibar that was made available, no matter how much the temperatures are controlled and programmed.

Spreadability is an essential quality control in semi-solid formulations, indicating the relationship between the formulation and the site of action on the skin. This test indicates whether the formulation should be applied to the skin in greater or lesser amounts, taking into account the presence or expansion of the lesion. Cold-brewed formulations have better spreadability compared to hot-brewed formulations.[34th]

In a study carried out with cream and gel formulations containing hydroethanolic extract of calendula, spreadability was evaluated according to the area obtained in relation to the weight applied, and the gel samples showed greater spreadability in relation to the cream. During the period of analysis, the samples remained stable and the physicochemical characteristics were preserved. In the study, it was observed that the samples kept in the refrigerator preserved their initial characteristics better.[31]

The spreadability of a product is a determining factor, as it is related to the level of user acceptance and adherence to treatment. Spreadability is the ability of the product to spread over the place where it will be applied and the action is expected. A product with low spreadability presents a change in the application dose and a change in the permeation of the active ingredient in the skin, leading to a low level of adherence by users.[35th]

4.6 MICROBIOLOGICAL TESTING

Considering that in this study, four semi-solid formulations were analyzed in triplicate, verifying the microbial contamination soon after the manipulation of the formulations, at time 0.

Table 5 shows the results of microbial contamination of bacteria and fungi in samples of creams and gels containing *Calendula officinalis L* in different concentrations. It was observed that all samples showed contamination by bacteria and fungi in the Casein culture medium, however, in the Sabouraud culture medium, only the formulations of the creams showed fungal growth, with no bacterial growth.

Table 5. Results of bacterial and fungal contamination analyses in casein-soybean and sabouraud-dextrose agar culture medium.

SAMPLE (time 0)	CASEIN	SABOURAUD
CREAM 7% Bacteria	3.2 x 10^2 CFU/mL	< 1 X 10^1 CFU/mL (Est.)
CREAM 7% Fungi	9.0 x10^1 CFU/mL	1.0 x10^1 CFU/mL
CREAM 10% Bacteria	2.1 x 10^2 CFU/mL	< 1 X 10^1 CFU/mL (Est.)
CREAM 10% Fungi	4.0 x10^1 CFU/mL	1.0 x10^1 CFU/mL
GEL 7% Bacteria	9.0 x10^1 CFU/mL	< 1 X 10^1 CFU/mL (Est.)
GEL 7% Fungi	3.0 x10^1 CFU/mL	< 1 X 10^1 CFU/mL (Est.)
GEL 10% Bacteria	3.8 x 10^2 CFU/mL	< 1 X 10^1 CFU/mL (Est.)
GEL 10% Fungi	5.0 x10^1 CFU/mL	< 1 X 10^1 CFU/mL (Est.)

Source: From the author (2023).

The samples had a bacterial count within the limits allowed by the Brazilian Pharmacopoeia for non-sterile products of a maximum of 1.0 x 10^3 CFU of bacteria/g.[14]

The samples also showed fungal counts within the microbial limits acceptable by the Brazilian Pharmacopoeia, which establishes a maximum of 1.0 x 10^2 CFU of fungi/g for non-sterile products.[14]

In light of this, According to the results of the microbiological tests, both specific plates for the growth of bacteria and fungi, respectively, containing the formulation, analyzed at time 0, after production, did not show growth of microorganisms outside the specified standards.

Microbiological testing of non-sterile formulations determines the limited microbial presence due to the use of the product. To minimize the risks of microbial contamination, it would be recommended that the product be accompanied by a spatula for minimal contact of the product with the hands, thus preserving the original characteristics of the product for longer, since contamination by microorganisms is identified in most cosmetic products.[36th]

According to the Brazilian Pharmacopoeia, quality control and microbial control of non-sterile products must be within pre-established safe limits for the consumer. The limits within the acceptable standard are 1.0 x 10^3 CFU of bacteria/g and 1.0 x 10^2 CFU of fungi/g.[14]

In the study by Soares and his collaborators, the presence of bacteria in cosmetic samples was verified, in which six cosmetic products were analyzed, with bacterial and fungal growth in 75% of the manipulated formulations, while the industrialized ones did not show such microbial growth.

Proper quality control is essential to ensure that the product is used by the consumer effectively and safely. [37]

In the study carried out by Borella, with carbopol gel and anionic emulsifier base, containing an input of natural origin, microbial growth occurred in both formulations, however, within the limits established by the legislation, ensuring the microbiological safety of the formulations. In the study, two different bases were standardized, carbopol gel and anionic cream, containing papain in different concentrations, used for wound healing and debridement. The following analyses were performed: pH, organoleptic analysis, spreadability, microbiological analysis, centrifugation test and enzymatic activity, to evaluate stability, contamination by microorganisms and proteolytic action.[38]

As the main raw material used for the production of galenic bases was water, which is the main contributor to microbial growth. Thus, as the formulations were manipulated following Good Manufacturing Practices, Rosa and his collaborators believe that formulations that use water as their main raw material, thus contributing to microbial growth in semi-solid formulations. Another factor that contributes to microbial growth is the use of glycolic extract, which is of natural origin, conducive to the growth of microorganisms.[39]

5 CONCLUSION

With the study it was possible to verify the dosage form and the concentration of the glycolic extract of calendula that permeate more efficiently in the skin, identifying that the gel of calendula *C. officinalis* 10% obtained superior permeation performance in 30 minutes compared to other formulations and times.

Regarding the organoleptic characteristics, the gel samples remained stable when stored at room temperature and in the refrigerator, only those stored in the oven showed alterations.

In the pH test, the sample that showed the least statistically significant variation was the 7% gel sample of *C. officinalis.* In the density test, the sample that did not show statistically significant changes when stored at room temperature and in the refrigerator was the 10% C. *officinalis* gel , showing changes only between days 7 and 15 in the oven.

In the spreadability test, statistical changes were observed in all times, storage conditions and formulations analyzed, however, the results are satisfactory, because the spreadability increased as the glass weights/plates were added to the samples. The 10% cream sample showed greater spreadability when stored in the oven, but when stored at room temperature and in the refrigerator, the 7% gel showed greater spreadability.

All samples had bacteria and fungi counts within the microbial limits established by the Brazilian Pharmacopoeia.

In view of the results, it can be concluded that the gel sample of *C. officinalis* 10% is the most adequate, as it presented better skin permeation and density, organoleptic characteristics and adequate microbiological count and good spreadability, however, the only alteration presented was related to pH values, requiring correction to further favor the permeability of the product, since more acidic products have better skin permeation.

Stability studies are considered an important safety standard for the manufacturer and consumer who use these products, but studies on semi-solid formulations containing active ingredients of natural origin are still scarce. Thus, the need for further studies on permeation, physicochemical and microbiological tests of topical formulations containing active ingredients of natural origin is ratified.

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