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Original Research Article

Effect of biosurfactants on the stability of anti-burn ointment of aqueous *Morus alba* leaf extract

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Abstract

Purpose: *To evaluate the anti-burn activity of ointment formulation from Morus alba leaves aqueous extract (MAE) stabilized by a biomolecule product of Marinobacter hydrocarbonoclasticus.*
Methods: *Phytochemical study conducted on MAE includes moisture content, ash content, and pH. Prepared Morus alba leaf ointment was evaluated for organoleptic, consistency, pH, spreadability, and skin irritation study. The effect of temperature variations and physiological and environmental factors on stability was also determined. Anti-burn effectiveness of MAE ointment (250 mg) was tested on the burn skin model of male Albino rabbits.*
Results: *Morus alba leaves contain polyphenols, flavonoids, glycosides and tannins as active chemical components that are useful in infection. Flavonoids exhibit antioxidant activity with indirect inhibition of inflammatory markers. The MAE ointment showed good scores in all test parameters that were evaluated including appearance, texture, pH, spreading ability and non-irritant study. The ointment was stable at different temperatures, and physiological and environmental conditions, and was free from any bacterial contaminations that have the capacity to cause allergies, especially after the addition of stabilizers which kept the ointment stable for longer period.*
Conclusion: *Ointment formulated from MAE is physically, chemically and microbiologically stable and is effective in the treatment of burns in rabbit models. The MAE thus has promising therapeutic potential in the management of skin burns.*

Keywords: *Mulberry leaves, Biomolecule, Marinobacter hydrocarbonoclasticus, Morus alba leaves*

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INTRODUCTION

Plants are useful in medicine, pharmacy, cosmetology and traditional medicine [1]. Since the 20th century, scientists have been interested in the biological properties of plants. Some of these properties are due to essential oil fractions and phenolic compounds contained in plants [1]. White mulberry (*Morus alba*) has interesting medicinal properties. This plant is used for several therapeutic purposes such as for its tonic, depurative, febrifuge, analgesic, and sudorific effects. White mulberry also has a natural anti-anxiety activity [2]. Leaves of white mulberry are parts of the plant containing the most phenolic compounds and flavonoids. These molecules have antioxidant, anti-inflammatory, anticancer, antibacterial, and anti-aging properties [2].

Today, skin burns are a major public health problem due to their frequency [3]. It is defined as the destruction of the skin covering by a causative agent, generally thermal but also electrical, chemical or radiation. Anti-burn activity of plants has been the subject of numerous scientific studies. The mechanism of action of the bioactive molecules of one of these plants, *Morus alba,* is now scientifically recognized and adopted by contemporary medicine as well as the cosmetics industry [3].

This study focuses on the formulation of an ointment from *Morus alba* leaves aqueous extract (MAE) stabilized by a biosurfactant FS. The study includes the determination of physicochemical, microbiological characteristics and anti-burn properties of the formulated ointment and comparison of its therapeutic efficacy with a reference ointment (Biafine ointment).

EXPERIMENTAL

Plant

Plant part used for this study is the leaves from mulberry tree (*Morus alba*). The leaves were harvested from the region of Oued Sly Chlef (Algeria) in March 2024. The harvested plant material was cleaned dried under shade away from light and heat then crushed and sieved to obtain a fine powder. Authentication of the sample was done by Dr Kouidri Mohamed, Maitre De Conference, Chef Department of Nutrition Science Department, Hassiba Ben Bouali University, Chlef. A voucher specimen of the plant was deposited in the herbarium of the University (voucher no. CH 1959).

Physicochemical characterization of the leaves

Determination of moisture

Moisture content was analyzed in homogenized fresh samples using the Association of Official Analytical Chemists (AOAC) procedures. The AOAC 984.25 procedure was employed to determine moisture content by desiccation of the sample at 105 ± 2 °C for 4 – 6 h until a constant weight was achieved.

Determination of ash

Ash content was determined by calcination at 550 °C, while total mineral content was determined following the stipulated procedure. Dried sample (10 g) was incinerated in an oven (Muffle Furnace MLS 1200 model, Monroe). The temperature was increased gradually to 550 °C. The ashes were removed once discolored to a white powder and were gravimetrically quantified [4].

Determination of pH

A suspension of 10 g of the leaves powder was prepared with 100 mL of distilled water. The pH was measured using a digital pH scale. Once the device was calibrated to homogenize the sample a sufficient volume was entered into the measuring container and the electrodes were immersed in it, the pH was determined [4].

Phytochemical screening

Preparation of Morus alba leaves aqueous extract (MAE)

The extraction of the leaves was carried out using methods described by Dzimitrowicz *et al* [5]. A 50 g sample of dried grounded leaves was extracted with 200 mL of distilled water. The sample was completely submerged in the solvent and then covered with aluminum foil. Maceration was allowed to proceed for 48 h. The extract was decanted and filtered using Watman filter paper No. 1. The filtrates were concentrated in vacuum at 60 °C stored in flasks and refrigerated at 4 °C before use [1]. Phytochemical screening of the leaf extract was carried out separately to identify the presence of active biomolecules like glycosides, tannins, saponins, and flavonoids using a method reported in the literature [4].

Determination of flavonoids

To 1 mL of the extract, 1 mL of 10 % lead acetate solution was added, formation of yellow precipitate indicated the presence of flavonoids [6].

Determination of tannins

To 1 mL of MAE, 2 mL of distilled water and 2 drops of dilute $FeCl_3$ solution was added. The positive test was confirmed by the appearance of a blue-green color indicating the presence of catechins and stanine [6].

Estimation of total phenolic

A drop of 2 % ferric chloride was added to 2 mL of MAE in a test tube. A dark blue or somewhat dark green color indicated the presence of polyphenols [7].

Determination of glycosides

To MAE (1 mL) was mixed with 2 mL of water, followed by 0.2 mL of 0.1 M HCl to hydrolyze the glycosides. Control was maintained in the same manner, except that distilled water was used instead of hydrochloric acid. The mixture was kept boiling for 5 min in a water bath. Finally, equal volumes of Fehling A and Fehling B reagents were added with continuous shaking of the mixture in the water bath for 10 min. A brick-red precipitate formed from the hydrolysis of glycosides indicated the presence of glycosides [6].

Antioxidant activity

The antioxidant activity of the extract was determined by radical DPPH scavenging activity. A 20 µL solution of extracts at different concentrations (100, 500, 1000, 1500, and 2000 ppm) and 180 µL of 0.147 mM DPPH solution were added to each concentration. After 30 min incubation at room temperature in the dark, absorbance was read at 517 nm, with methanol used as blank. The scavenging ability (I) was calculated using Eq 1 [8]. All tests were performed in triplicate. Concentration resulting in 50 % inhibition on DPPH (IC_{50} value) was calculated. Ascorbic acid was used as positive standard.

$$I (\%) = \{(A-A^1)/A\}100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

Where: A: absorbance of standard, A^1: absorbance of crude extract

Preparation of ointment

Simple ointment base was prepared with beef butter (90 g) mixed with *Morus alba* leaves powder (20 g), then kneaded in a mortar until the formation of a homogeneous emulsion. Then the ointment obtained was stabilized with 0.15 % of a bimolecular product from a new bacterial strain, *Marinobacter hydrocarbonoclasticus* isolated from soil contaminated by hydrocarbons in Hassi-Messaoud (Southern Algeria).

Physicochemical characterization of the ointment

Organoleptic evaluation of herbal ointment

The herbal ointment was visually examined for appearance, homogeneity and color. The ointment was tested for characteristic odor, texture, loss of drying, pH value, and topical sensitivity (i.e., signs of allergic reaction).

Microscopic examination

This examination was performed by placing a small amount of ointment between the slides of a microscope with x40 magnification. Polarization microscopy was performed using a Keyence model VHX-1000 microscope [9]. The emulsion was examined for size, shape and homogeneity. The presence of abnormal particles and air bubbles were also determined by microscopic examination.

Determining the type of emulsion

It consists of an aqueous phase (W) and an oily phase (O). If the dispersed phase is the oily phase, the emulsion is of the oil-in-water type and is referred to as (O/W), while if the continuous phase (external phase) is the opposite, the emulsion is of the water-in-oil type (W/O) [10].

In each of the clean and dry hourglasses, 1 mL of emulsion and 1 mL of each distilled water and olive oil were added to the first and second beakers, respectively. Everything was mixed in each hourglass and the appearance of the product was observed.

Determination of pH

The pH of the ointment was measured using the pH meter after dilution with distilled water. A 1 g of the ointment was mixed with 100 mL of distilled water, and stirred well at 45 °C in a water bath; then the pH was measured [11].

Determination of density

Empty vial (W1) was weighed and the weight of the vial with ointment was also noted and designated as W2. The difference between the weight of the ointment vial and the weight of the empty vial was calculated [12] and designated as ‘W’.

$$W = W2 - W1 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

Then, the vial was weighed with distilled water in it designated as W3. The difference between the weight of the vial with distilled water and the empty vial was determined and noted as W^I.

$$W^I = W3-W2 \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (3)$$

The relative density was calculated using Eq 4.

$$D = W/W^I \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (4)$$

Determination of viscosity

Viscosity was measured with a rheometer (Viscos Tester type. Pin Nº. 4 with digital display, giving a direct reading of viscosity in Pas) at 25 °C at 5, 10, 20, 30 and 50 rpm using 2 mL of sample. Each reading was taken after equilibrating the sample at the end of two minutes. Determination was done in triplicate [13].

Stability tests

Stability at centrifugation

The products to be tested were centrifuged for a fixed time at a speed of 1000, 2000, and 4000 rpm to ensure the presence or absence of creaming, sedimentation or dephasing phenomena [14].

Accelerated stability

Samples were stored at varying temperatures (4, 25 and 40°C) to determine the effect of storage temperatures on the stability of the sample [8].

Microbiological characterization of ointment

Microbiological characterization of the prepared ointment was carried out to determine the presence of viable microorganisms and fungi as was as its potential for anti-microbial activity (yeast and mold) [14]. Biafine (Trolamine, Johnson and Johnson santé beauté France) was used as the control. It is a cream characterized by a whitish color, with a fragrant odour and a non-greasy texture) [15].

Therapeutic evaluation of ointment

The day before the product application, rabbits with healthy skin were shaved on both sides (right and left) and anesthetized using an intramuscular injection of ketamine hydrochloride (15 mg/kg) to anesthetize the rabbits before each burn. The diameter of the burned areas was measured and the product was then applied to the burned area except for control area.

Albino rabbits of New Zealand origin were used for this study. All protocols were approved by the protection service and the use Committee within the Toxicology Laboratory at the Antibiotic Center of Medea (SAIDAL; approval no. M1-20-16). The therapeutic effect and diameter of the area under study were observed at every visit (2 to 5 days), over 20 days.

RESULTS

Physicochemical characteristics of *Morus alba* leaf

The moisture content of *Morus alba* leaves is shown in Table 1. Ash content and pH of the leaves are also shown in Table 1.

Table 1: Physicochemical characteristics of *Morus alba* leaf extract

Setting	Content (%)
Moisture	10
Ash	19.42
pH	7.05

Phytochemical characteristics of MAE

The results, shown in Table 2, indicate that the extract contains flavonoids, polyphenols, tannins and glycosides, as well as tannins.

Table 2: Phytochemical characteristics of aqueous *Morus alba* extract

Compound	Results obtained
Flavonoïdes	Yellow precipitate
Tannins	Blue-green color (+)
Polyphénols	Blue-blackish color (+)
Glycoside	Brick red precipitate (+)

Antioxidant activity.

The leaf showed a high DPPH scavenging activity (72.505 µg/mL) with a lower IC_{50} value but lower antioxidant capacity compared to ascorbic acid (2.14 µg/mL). The phenolic content of the leaves may contribute to its antioxidant effect through its ability to donate hydrogen.

Formulation of *Morus alba* ointment

The ointment with humidity (10 %), Organic matter (75.38 %), and Mineral matter (24.62 %) also had an anti-burn activity equivalent in characteristics to control ointment.

Physicochemical characterization and organoleptic tests of herbal ointment

The results of physicochemical characterization and the organoleptic tests of the herbal ointments are summarized in Table 3.

Microscopy

Microscopy of the ointment shows that the dispersed particles are medium in size and the dispersion appears homogeneous.

Table 3: Physicochemical characterization of prepared ointment

Parameter	Perception
Colour	Green
Odour	The smell of henna (Characteristic)
pH	6.75
Texture	Downy
Washability	Good
Homogeneity	Homogenous
Application	Refreshing
Hydrations	Refreshing hydrations
Irritation	Non-Irritation
Emulsion type	W/O
Stability at centrifugation (1000 – 4000 rpm)	Stable
Density	0.9767

Emulsion type and pH

After formulating the ointment, it was found that when distilled water was added, a heterogeneous mixture was formed, while when oil was added, a homogeneous mixture was obtained. The ointment formulated/referred to in this work (Table 3) has a pH value of 6.75/7.05 at room temperature.

Density at 25 °C

The density obtained for the prepared ointment as shown in Table 3, is 0.9767. This value is close to the density of 0.9904 for the reference Biafine ointment.

Viscosity

A sharp decrease in viscosity was observed, which indicates non-Newtonian behavior as shown in Figure 1, and the flow curve corresponds to twice application of the ointment, where the ointment is ejected from the container the first time and the second time to spread the latter. This indicates that the repulsive forces are high compared to the attractive forces, so there will be destruction of the emulsion and a decrease in viscosity.

Stability study at different temperatures

Stability is strongly affected by temperatures. Boundary conditions are mainly chosen to investigate the effect of climate change on ointment homogeneity. Phase reversal phenomenon was observed at 4 and 25 °C but no change in lipid structure was observed except at 40 °C as shown in Figure 2.

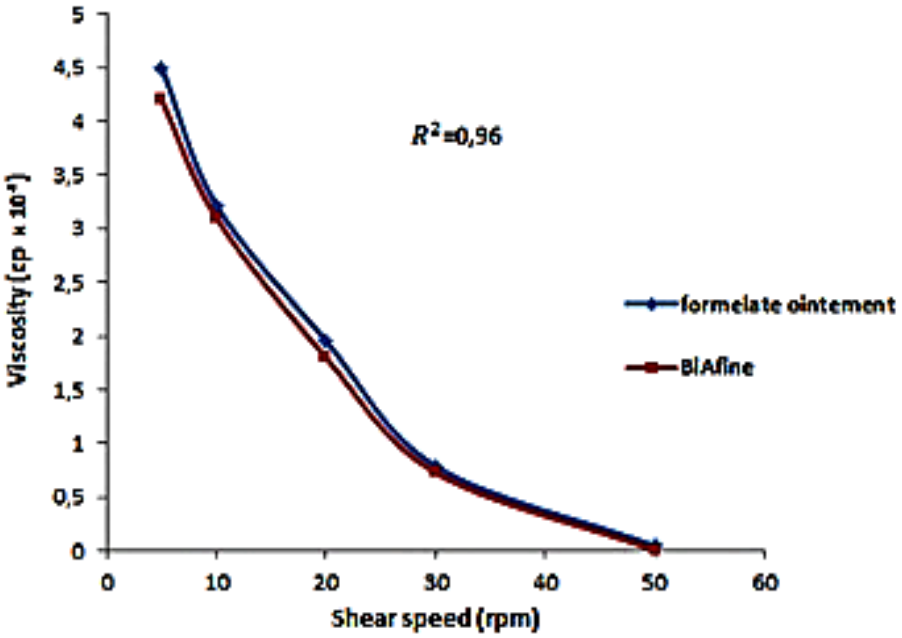

Figure 1: Apparent viscosity variation of two ointments as a function of shear speed

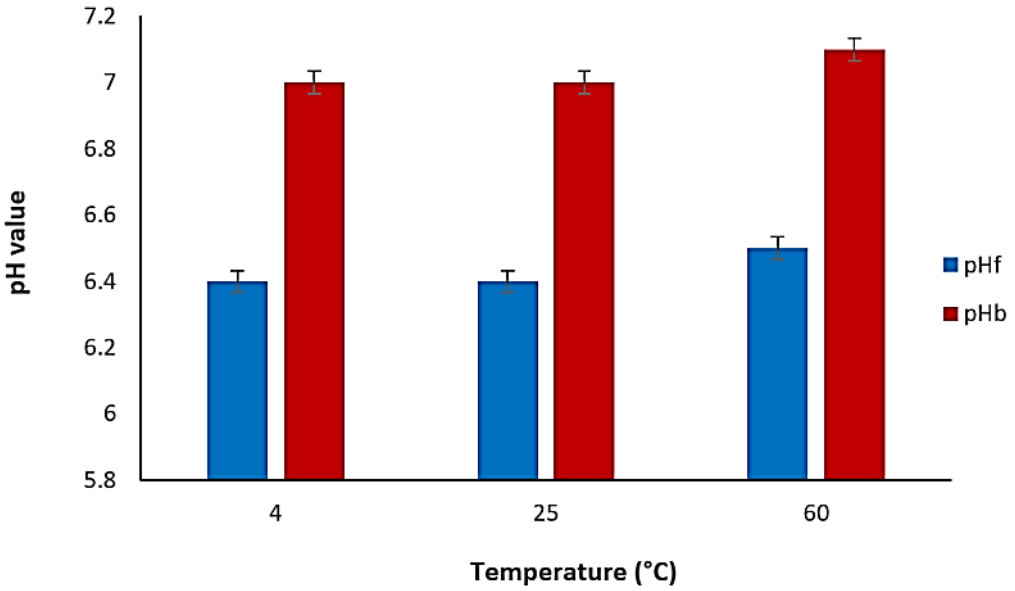

Figure 2: pH values at different temperatures (pHf of formulated and pHb of Biafine (control))

Microbiological characterization of prepared ointment

The presence of a non-pigmented colony in the agar soybean medium was noted which means that there are absence of *Pseudomonas aeruginosa* because this method is selective for this microorganism and this also means the absence of *Staphylococcus aureus*.

Dermophytes effect of MAE ointment

Results of burn wound diameter of rats treated with MAE and Biafine ointments are shown in Figure 3 and Figure 4. Morus alba ointment treated group had a smaller layer of burns compared to Biafine ointment, which had no layer of fur. These results are shown in Figure 3. On the first day, the burn size was 6.1 cm for the prepared ointment and 5.9 cm for Biafine, while on the twentieth day, the burn size was 1 cm for the prepared ointment and 0 cm for Biafine, as shown in Figure 4.

DISCUSSION

In the present study, it was found that treating thermal burns in rabbits with an ointment made from MAE resulted in faster healing and less tissue damage compared to untreated rabbits and was very similar to the results of control ointment.

Moisture content of *Morus alba* leaves was consistent with the specified value of 10%, which is the maximum recommended moisture content for herbal medicines and close to the results of an earlier study by Pereira *et al* [16]. The presence of water in the leaves and raw materials provides an enabling environment for the growth of bacteria and fungi. To ensure the quality of raw materials, the ash content was determined and this was aimed at verifying the absence of inorganic and non-volatile impurities that cause contamination of herbal medicines [16]. The hydrogen potential of the raw material is also suitable for the preparation of skin ointment as the ointment had a neutral pH (7.05) [17].

The study conducted on phytochemical screening of MAE found that the leaves contain flavonoids, polyphenols, tannins and glycosides. These phytochemical compounds are useful as medicine sources, as flavonoids contain biological response modifiers that have the ability to modify the body's response to infections and microbes. Flavanoids are also used in herbal medicine and cosmetics [18]. Due to the tannins present in them, they possess antibacterial and antiviral properties. They are also used as antioxidants, anti-inflammatory and antifungal agents. The presence of these phytochemical compounds in the raw material of *Morus alba* plant will enhance the quality of the ointment [18].

Microscopic examination of the ointment sample found to be of W/O type, showed that the granules were of medium size and the dispersion appeared homogeneous. Thus, the good dispersion of water globules in the emulsion confirms the stability of the ointment which is in accordance with the results of the study of Inoui *et al* [6].

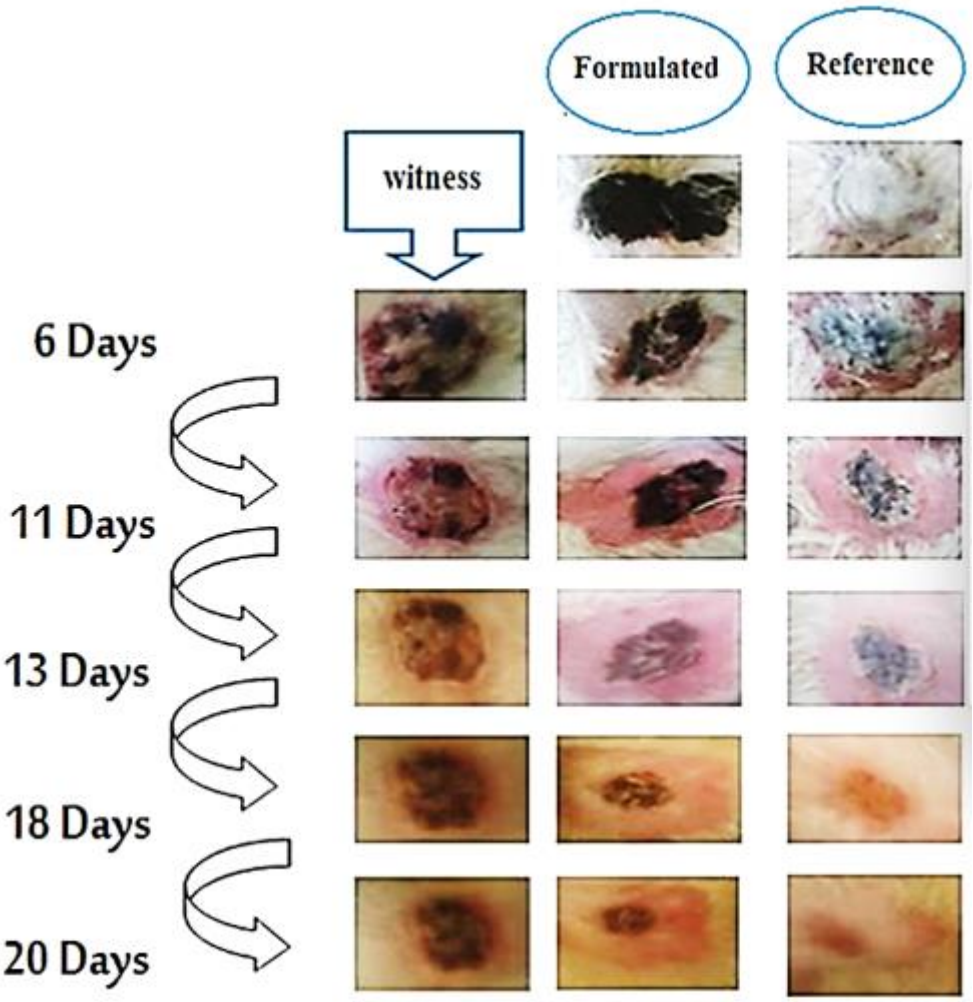

Figure 3: The evolution of the treatment of burns using the formulated and reference ointments

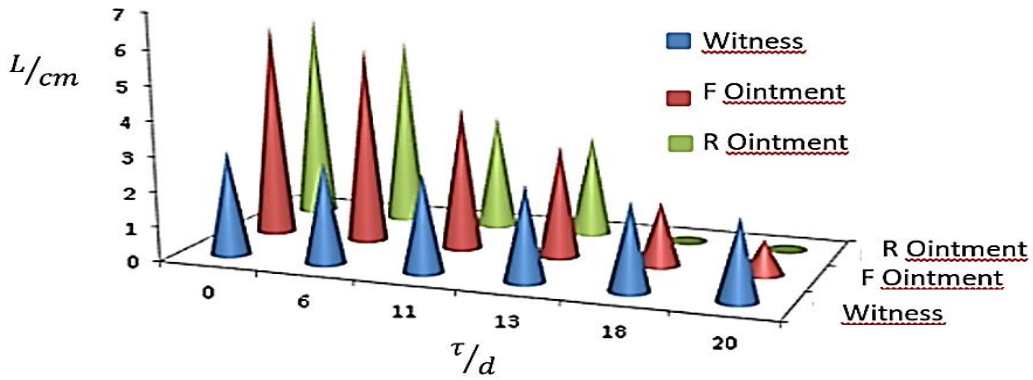

Figure 4: The evolution of burn treatment witness, R ointment (reference ointment), F Ointment (Formulation ointment)

Also, the pH value of the ointment (6.75/7.05) is close to the pH value of the skin (4 and 7) and that of the reference ointment (Biafin) as shown in a similar study conducted by Ijaz and Badia [8,10].

The density of the extract is also a very important criterion for evaluating its quality in many fields, namely pharmacy, cosmetics, etc. It easily gives an overview of the product quality as well as fraud and counterfeiting attempts [7]. The density

value obtained from prepared ointment is close to the density of the reference Biafine ointment. This reflects that the prepared ointment is of good quality.

It should be noted that the rheological behavior of the prepared ointment is similar to that of the reference ointment. The viscosity measurement of the ointments corresponds to the primary and secondary skin feel. The primary skin feel describes the sensations at the beginning of application, while the secondary feel describes the sensations at the end of application when the product is almost completely rubbed into the skin. An emulsion for topical use should have a low viscosity so that it spreads easily on the skin [11]. The viscosity of the prepared ointment and the reference ointment showed similar consistencies of the prepared ointment with Biafine ointment.

For any development of a new formulation, it is important to have as much information as possible about this product in order to anticipate any stability problems that may occur on a larger scale. However, the visual tests do not provide sufficient information on the instabilities of the ointments Therefore it is necessary to use other analytical techniques that are not always suitable for highly complex systems such as cosmetic products.

The results obtained also showed that the ointment is resistant to mechanical action, temperature, oxidation and mold. The prepared ointment has a physical, chemical and microbiological stability close to that of the reference ointment. The same applies to appearance, consistency, texture and penetration.

CONCLUSION

The ointment formulated from *MAE* is physically, chemically and microbiologically stable and is effective in the treatment of skin burns. The burns treated with the prepared ointment show significant improvement compared to the reference product, and thus has therapeutic potential in the management of burns.

DECLARATIONS

Acknowledgement/Funding

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Ethical approval

All protocols were approved by the protection service and the use Committee within the Toxicology Laboratory at the Antibiotic Center of Medea (SAIDAL; approval no. M1-20-16).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflict of interest

No conflict of interest associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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